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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OPP OFFICIAL RECORD
HEALTH EFFECTS DIVISION
SCIENTIFIC DATA REVIEWS
EPA SERIES 361

APR 16 1997

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

Subject: **IPRODIONE** Mechanistic Data / Testes and Liver

From: Linda L. Taylor, Ph.D. 4/11/97
Toxicology II Branch, HED (7509C)

K. Clark Swentzel, Head, Section 4/11/97
Toxicology II Branch, HED (7509C)

Yiannakis Ioannou, Ph.D., Acting Chief
Toxicology II Branch, HED (7509C) 4/11/97

To: Christina Scheltema
RCAB
Health Effects Division [7509C]

Registrant: Rhone-Poulenc Ag Company
Chemical: **Iprodione** 3-(3,5-dichlorophenyl)-N-isopropyl-2,4-dioxoimidazo-lidine-1-carboxamide; 3-(3,5-dichlorophenyl)-N-(1-methylethyl)-2,4-dioxo-1-imidazolidinecarboxamide
Synonym: Iprodione; RP26019; Rovral®; Glycophene
P.C. Code: 109801
Caswell No.: 470A
Submission No.: S481268
DP Barcode: D232064/225159

Action Requested: None specified.

Comment: The Registrant submitted four mechanistic studies on Iprodione in support of their premise that the liver and testicular tumors observed in the mouse and rat studies, respectively, are both threshold phenomena. These four studies have been reviewed, and the DERs are appended.

MRID 44171901 Effects of Iprodione and its Metabolites RP36112 and RP36115 on Testosterone Secretion in Cultured Leydig Cells: Sites of Action.

EXECUTIVE SUMMARY: In an in vitro study [MRID 44171901] using immature porcine cultured Leydig cells, Iprodione [99.7%] and two of its metabolites [RP36112 (99.2%) and RP36115 (96.7%)] inhibited

testosterone secretion when Leydig cells were stimulated with (1) the gonadotropin hCG, (2) with drugs that enhance cAMP production [(a) cholera toxin, which stimulates Gs protein; (b) forskolin, which stimulates adenylate cyclase catalytic unit, and (3) with a cAMP analog [8-bromo-cAMP]. Because there were no effects observed on gonadotropin-stimulated cAMP production with Iprodione, it is hypothesized that the inhibition of testosterone secretion by Iprodione is downstream from cAMP production. At the next step in testosterone biosynthesis, inhibition of testosterone secretion by Iprodione was not observed when the substrate 22ROHCT was added to the culture medium, which indicates that the step that is inhibited is located between the cAMP production and the movement/penetration of cholesterol into the mitochondria. Since 22ROHCT is a cholesterol substrate that passes through the mitochondrial membrane without the need of an active transport system, it is postulated by the Registrant that the sensitive site of inhibition of testosterone synthesis by Iprodione [or RP 36115] is the transport/availability of cholesterol substrate for the cholesterol side chain cleavage enzyme. The RP 36112 metabolite appears to act downstream from the cholesterol step; i.e., at the level of steroidogenic enzyme 17 αhydroxylase/17, 20 lyase. This latter step is inhibited by Ketoconazole also. Iprodione and its metabolites appear to modulate Leydig cell steroidogenesis by interfering at the level of cholesterol transport and/or steroidogenic enzyme activity.

This nonguideline study is classified Acceptable.

MRID 44171902 Iprodione Exploratory 14-Day Toxicity Study in the Mouse by Dietary Administration.

EXECUTIVE SUMMARY: In this 3-day and 14-day oral exposure study [MRID # 44171902], groups of CD1 male mice [15/dose/group/chemical; 7 weeks old on arrival] were administered (1) **IPRODIONE** via the diet at dose levels of 4000 ppm [696 mg/kg/day] or 12000 ppm [2138 mg/kg/day]; (2) **KETOCONAZOLE** via the diet at a dose of 2000 ppm [341 mg/kg/day]; (3) **PHENOBARBITAL** via gavage at a dose level of 75 mg/kg/day; and (4) **CYPROTERONE ACETATE** via gavage at a dose level of 40 mg/kg/day. The control for the dietary studies was basal diet, and 0.5% methylcellulose was the control of the gavage studies. The objective of the study was to examine the potential liver effects of Iprodione in mice and to compare these effects with those produced by well characterized liver enzyme inducers and/or rodent liver carcinogens. Ketoconazole was selected as a positive control for its potential to inhibit testosterone secretion; Phenobarbital and Cyproterone acetate were selected for their potential to induce early liver changes and subsequent liver tumor formation in rodents. All of the liver effects produced by Ketoconazole, Phenobarbital, and/or Cyproterone acetate [increases in liver weight, alanine aminotransferase, aspartate aminotransferase, # hepatocytic mitoses, total cytochrome P-450 content, staining for isoforms CYP 2B and CYP 3A, benzoxyresorufin

[BROD], ethoxyresorufin [EROD], pentoxyresorufin [PROD] enzyme activities, and hepatocyte proliferation, in addition to increases in the incidence of liver enlargement, centrilobular hypertrophy, diffuse hypertrophy, centrilobular/midzonal fine vacuolation] were exhibited by Iprodione at 12000 ppm. An effect observed following Iprodione exposure that was not observed following any of the other test material exposures was an increase in lauric acid hydroxylation. Although several of the effects observed in the liver following Iprodione exposure are analogous to those observed following the positive controls, especially Phenobarbital [centrilobular hypertrophy, liver weight, increased BROD, PROD, and EROD activities, cell proliferation after 3 days], in several cases the liver effect observed was most pronounced in the Iprodione mice compared to the positive controls [centrilobular/midzonal fine vacuolation, increased number of mitoses, cell proliferation at day 15].

This study demonstrates that Iprodione, at dose levels that are 5- and 15- fold greater than the LOEL for liver effects observed in the mouse carcinogenicity study, induces (1) liver cell proliferation, (2) increased microsomal enzyme activities, (3) an increase in total cytochrome P-450 content, and (4) centrilobular hypertrophy. These observations most closely resemble the pattern of liver effects observed following Phenobarbital exposure. Hepatocytic hypertrophy was observed at the high-dose level of Iprodione following both the 3- and 14-day exposure periods but only following the 14-day exposure period at the low dose. Liver cell proliferation was observed after both the 3-day and 14-day exposure periods at both dose levels of Iprodione. Increased cytochrome P-450 content and increased microsomal enzyme activities were observed at both dose levels of Iprodione following the 14-day exposure period, but neither analysis was performed following the 3-day exposure period. The dose level where liver tumors were observed in the mouse carcinogenicity study [604 mg/kg/day] is comparable to the low dose used in the current study. The findings in this study support the Registrant's arguments that the liver tumors observed in the Iprodione mouse carcinogenicity study may be secondary to liver toxicity. However, several pieces of data are lacking. The current study does not address whether cytochrome P-450 content and the microsomal enzyme activities are increased initially [after the 3-day exposure period]; therefore, one cannot determine whether the cell proliferation and hepatocytic hypertrophy observed after 3-days exposure to Iprodione is due to a direct effect of Iprodione on the liver or the result of adaptive processes. Additionally, the current study does not identify a NOEL for the liver effects monitored over a 14-day exposure period or address the question of whether these liver effects occur initially at the lower doses utilized in the mouse carcinogenicity study. Another outstanding question is whether the liver effects [hepatocytic hypertrophy, increased total cytochrome P-450 content, increased microsomal activities, cell proliferation] observed in the current study persist throughout a long-term exposure. It is to

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be noted that Phenobarbital produces a short-term increase in hepatocyte proliferation that is not sustained [Jirtle, et al. 1991, Standeven and Goldsworthy, 1993]. In a paper on proliferation and liver tumor development [CIIT Activities, vol. 15 (8), August, 1995], it is stated that the proliferative response seen after acute exposure does not always reflect the proliferative response observed after chronic exposure.

This study is classified Acceptable [non-guideline].

MRID 44171903 Toxicity Testing of a Fungicide, Iprodione: Endocrine Toxicology Studies of Testes from Adult Male CD® Sprague-Dawley Rats Exposed to Iprodione In Vitro.

The objective of this in vitro study [MRID 44171903] was to determine the effect of in vitro Iprodione [99.7%] exposure on basal testosterone secretion and stimulated release from testicular sections in culture media [in vitro Endocrine Challenge Test (ECT) using human chorionic gonadotropin (hCG)]. The effects of prior in vivo exposure of the male rats via the diet [3000 ppm Iprodione for 14 days] was also evaluated. Testicular sections obtained from 12 male CD® Sprague-Dawley rats administered Iprodione via the diet for 14 days at dose levels of 0 ppm or 3000 ppm were incubated with 0, 1, 10, or 100 µg/ml Iprodione for one hour. Half of these testicular sections from each in vitro treatment group were challenged with human chorionic gonadotrophin and the other half of the sections were monitored for basal testosterone secretion. Media testosterone concentrations were monitored at hourly intervals for 3 hours after challenge.

There was a dose-related reduction in testosterone secretion from testicular sections incubated in vitro with Iprodione, with and without hCG stimulation. Prior exposure of the rats to Iprodione in vivo for 14 days appeared to have little effect on the secretion of testosterone, with and without hCG stimulation, from testicular sections incubated in vitro other than a slight increase at t=0. At sacrifice following the 14-day exposure period to Iprodione in vivo, plasma LH concentrations were significantly increased compared to the control and, although plasma testosterone was not significantly affected, the levels were somewhat increased compared to the control [132% of control]. The significant increase in plasma LH at necropsy suggests a possible stimulation of the homeostatic mechanism. Under the conditions of this 14-day study, Iprodione was shown to produce a reduction in testosterone secretion from testicular sections following incubation in vitro with Iprodione. Prior exposure of male rats to Iprodione in vivo via the diet for 14 days did not alter the reduction in testosterone secretion observed in their testicular sections exposed to Iprodione in vitro. Although the in vitro inhibition appeared to be dose-related, it appears that a maximum response may have occurred between the 10 and 100 µg/mL dose levels. The data presented provide pieces to the "puzzle" but not a complete picture

of what may be occurring in the testes/rat that ultimately results in testicular tumors. Although it appears that the premise is that Iprodione produces testosterone biosynthesis inhibition, resulting ultimately in the increased incidence of Leydig cell tumors, there are inconsistencies in the in vitro and in vivo data, and the in vitro effects observed in the short-term studies to date have not been demonstrated to occur in long-term studies, nor is it clear that the levels at which the in vitro effects were observed are attained in vivo.

This study is classified Acceptable [non-guideline].

MRID 44171904 Toxicity Testing of a Fungicide, Iprodione: Endocrine Toxicology Studies of Testes from Adult Male CD® Sprague-Dawley Rats Exposed to Iprodione In Vivo.

EXECUTIVE SUMMARY: In an in vivo study [MRID 44171904], no changes in testicular function, as assessed by measuring testosterone levels in plasma and testicular homogenates from 15 male Sprague-Dawley rats administered Iprodione [97.3%] via the diet at doses levels of 0 ppm and 3000 ppm for 2, 7 or 14 days, were observed. Decreased body weight [95% of control after 2 days, 90-91% of control after 7 days, and 87% of control after 14 days], body-weight gain [negative gain after 2 days, 32% of control after 7 days, 44% of control after 14 days], and food consumption were observed following all exposure intervals. Organ-weight effects included decreased absolute liver, kidney, epididymis, and total accessory sex organs [TASO]; increased absolute and relative adrenal; and decreased relative TASO. The objective of this study was to assess the effects of in vivo Iprodione exposure on plasma and testicular homogenate testosterone concentrations in the male rat following a human chorionic gonadotrophin [hCG] Endocrine Challenge Test (ECT). There were no significant differences in either peripheral plasma or testicular homogenate testosterone levels observed in samples collected one hour after human chorionic gonadotrophin [hCG] challenge. Under the conditions of this study, Iprodione did not produce alterations in testicular function following dietary exposure at 3000 ppm for up to 14 days.

This study is classified Acceptable [non-guideline].

CONCLUSION: These data, along with two previous mechanistic studies on Leydig cells, were presented to a "mini peer review" group on April 1, 1997. The "mini peer review" group concluded that the data available do not provide a definitive mode of action with respect to the Leydig cell tumors, and there are inconsistencies between the in vivo and in vitro data and organ weight data that have not been fully addressed by the Registrant. Additionally, the effects observed in these short-term studies have not been assessed in longer-term studies and therefore cannot be extrapolated to long-term findings. Although the Registrant contends that the mechanistic research has determined that tumor formation is linked

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to a prolonged hormonal perturbation, prolonged perturbation has not been demonstrated. A sustained cell replication/turnover in the Leydig cells has not been demonstrated. TB II notes that other possible mechanisms for the Leydig cell tumors [other than the elimination of androgen receptor antagonism] have not been discussed or eliminated as factors; e.g., there has been no discussion about a possible adrenal involvement or an estrogenic component.

With respect to the liver tumors, Iprodione was shown to exhibit similar effects as observed following Phenobarbital exposure. Although the Registrant concludes that the large number of non-genotoxic chemicals that induce mouse liver tumors strongly suggests that cell proliferation plays an important role in mouse liver tumorigenesis, cell proliferation following Iprodione exposure has been demonstrated only after a 14-day exposure period. A definitive mode of action for Iprodione with respect to liver tumorigenesis was not identified by the Registrant. As the Registrant is aware, liver cell tumorigenesis is a widely discussed/argued topic in the scientific community, and the Agency at present has no set policy for regulating such chemicals as threshold carcinogens.

The Registrant has not demonstrated for either tumor type a clear mode of action to justify a change in the classification of Iprodione with respect to carcinogenicity. The specific mechanism involved in the tumorigenesis in either the Leydig cell or the liver has not been identified. As the Registrant is aware, both Leydig cell tumors and liver carcinogenesis are topics widely debated in the scientific community. The registrant should be encouraged to meet with the Agency, not to argue their position, but to discuss/identify gaps in the mechanism data for both tumor types that need to be addressed before the Agency can determine whether a change in classification is justified.

cc: Vivian Prunier, CRM
Special Review Branch
SRRD (7508W)

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[**IPRODIONE**] Mechanistic Study/Testosterone Secretion/Sites of Action

EPA Reviewer: Linda L. Taylor, Ph.D. Linda Lee Taylor 1/16/97
Review Section II, Toxicology Branch II (7509C)
EPA Secondary Reviewer: K. Clark Swentzel K. Clark Swentzel 1/17/97
Review Section II, Toxicology Branch II (7509C)

DATA EVALUATION RECORD

STUDY TYPE: Mechanistic Study [isolated Leydig cells from immature porcine testes] OPPTS/§ none

DP BARCODE: D232064 SUBMISSION CODE: S481268

P.C. CODE: 109801 TOX. CHEM. NO.: 470A

TEST MATERIAL (PURITY): Iprodione [99.7%]; RP36112 [99.2%]; RP36115 [96.7%]

CHEMICAL: 3-(3,5-dichlorophenyl)-N-isopropyl-2,4-dioxoimidazo-lidine-1-carboxamide; (dichloro-3,5 phenyl)carbamoyl-1 hydantoin; 1-(3,5-dichlorophenyl)biuret

SYNONYMS: for Iprodione: RPA095207, RPA590611, RP26019

CITATION: Benahmed, M. (October 21, 1996) Effects of Iprodione and Its Metabolites RP 36112 and RP 36115 on Testosterone Secretion in Cultured Leydig Cells: Sites of Action. INSERM U 407, Communication Cellulaire en Biologie de la Reproduction. Bât. 3B, Centre Hospitalier Lyon-Sud, France. Report INSERM/U 407/96001, [study dates not provided]. MRID 44171901. Unpublished.

SPONSOR: Rhone-Poulenc Agrochimie-Centre de Recherche- 355, rue Dostoïevski- BP153-F-06903 SOPHIA ANTIPOLIS Cedex

EXECUTIVE SUMMARY: In an in vitro study [MRID 44171901] using immature porcine cultured Leydig cells, Iprodione [99.7%] and two of its metabolites [RP36112 (99.2%) and RP36115 (96.7%)] inhibited testosterone secretion when Leydig cells were stimulated with (1) the gonadotropin hCG, (2) with drugs that enhance cAMP production [(a) cholera toxin, which stimulates Gs protein; (b) forskolin, which stimulates adenylate cyclase catalytic unit, and (3) with a cAMP analog [8-bromo-cAMP]. Because there were no effects observed on gonadotropin-stimulated cAMP production with Iprodione, it is hypothesized that the inhibition of testosterone secretion by Iprodione is downstream from cAMP production. At the next step in testosterone biosynthesis, inhibition of testosterone secretion by Iprodione was not observed when the substrate 22ROHCT was added to the culture medium, which indicates that the step that is inhibited is located between the cAMP production and the movement/penetration of cholesterol into the mitochondria. Since 22ROHCT is a cholesterol substrate that passes through the mitochondrial membrane without the need of an active transport system, it is postulated by the Registrant that the sensitive site of inhibition of testosterone synthesis by Iprodione [or RP 36115] is the transport/availability of cholesterol substrate for the cholesterol side chain cleavage enzyme. The RP 36112 metabolite appears to act downstream from the cholesterol step; i.e., at the level of steroidogenic enzyme 17

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αhydroxylase/17, 20 lyase. This latter step is inhibited by Ketoconazole also. Iprodione and its metabolites appear to modulate Leydig cell steroidogenesis by interfering at the level of cholesterol transport and/or steroidogenic enzyme activity.

This nonguideline study is classified Acceptable.

COMPLIANCE: Signed and dated GLP and Data Confidentiality statements were provided. There was no Quality Assurance statement per se. The GLP statement indicated that the mechanistic study was not conducted in compliance with the Good Laboratory Practices Regulations, but the Sponsor's Quality Assurance Unit audited the raw data and final report. No flagging statement was provided.

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[**IPRODIONE**] Mechanistic Study/Testosterone Secretion/Sites of Action

I. **MATERIALS AND METHODS**

A. MATERIALS

1. Test Material: (1) Iprodione, (2) RP36112 (metabolite), (3) RP36115 (metabolite); Description: white powders; Batch #: (1) TV3015C, (2) BES1526, (3) BESS129; Purity: (1) 99.7%, (2) 99.2%, (3) 96.7%; Stability: information not provided; CAS #: (1) 36734-19-7.

Structure: (1)

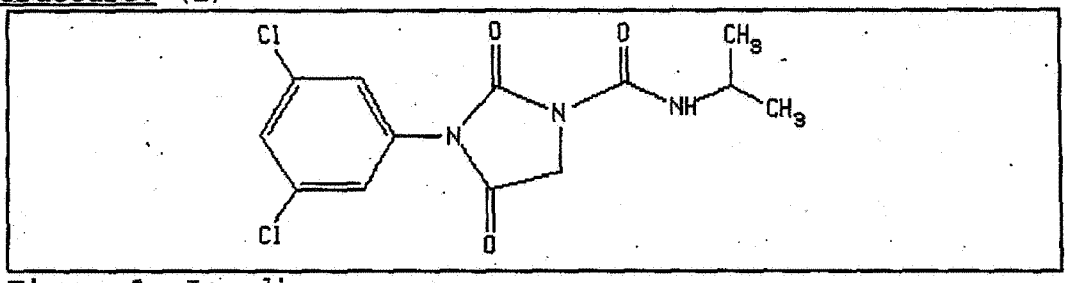

Figure 1 Iprodione

Formula: (1) $C_{13}H_{13}Cl_2N_3O_3$;
Source: Rhone-Poulenc Secteur Agro/France
(2) and (3) are shown in Certificates of Analysis [pages 52 and 53 of report, copies appended to File Copy of DER].

2. Vehicle: absolute ethanol or DMSO or DMF; Batch #: not provided. Positive control: Ketoconazole [dissolved in absolute ethanol]; Batch #: not provided; Source: Biolmol Laboratories distributed through TEBU France. It was stated that Iprodione and RP 36115 were readily dissolved in ethanol but RP 36112 required more time to dissolve under sonication. All three compounds were said to be readily dissolved in DMSO and DMF. All three solvents were tested on basal and hCG-stimulated testosterone secretion in cultured Leydig cells, and at concentrations used in the culture medium [5/1000 v/v], no significant effect on steroid hormone secretion was reported [Table 1].

Table 1. Effects of Solvents on Testosterone Secretion [ng/10^5 cells/3 hours]

Testosterone Secretion	None	Ethanol	DMF	DMSO
basal	0.26±0.17	0.24±0.14	0.28±0.22	0.21±0.16
hCG	6.64±0.80	6.84±1.22	4.86±0.90	6.21±0.71

data from page 13 of report

3. Test animals: Species: immature porcine [isolated Leydig cells from testes]; Strain: not provided; Age: 3 weeks old; Source: not provided.

B. STUDY DESIGN and METHODS

1. In life dates - not provided. It was stated that all experiments were performed within a week's time.

2. Preparation of Cultures - Isolated Leydig cells were prepared according to the methods described by Mather and Phillips [1984], as modified by Benahmed, et al. [1987]. As described in the Methods'

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section of the report, decapsulated testes were minced, washed twice in DME-F12 medium, and after collagenase dissociation, the cells were washed by centrifugation [200 x g for 10 minutes]. The pellets were resuspended and submitted to two successive sedimentations of 5 and 15 minutes. The crude interstitial cells were recovered from the supernatants, and Leydig cells were prepared from this fraction by Percoll density gradient centrifugation. Leydig cells were recovered from this gradient and characterized by their ability to bind LH/hCG and to produce testosterone in response to this hormone. The report stated that the percentage of Leydig cells in this final preparation [as established by staining for 3β-hydroxysteroid dehydrogenase activity] was always greater than 90%.

Leydig cells were plated in 24-multiwell plates and cultured at 32° C in a humidified atmosphere of 5% CO_2-95% air [Heraeus incubator] in DME-F12 medium containing sodium bicarbonate, HEPES, and gentamicin. This medium was supplemented with insulin, transferrin, and vitamin E. It was stated that differentiated [steroidogenic] activity of cultured porcine Leydig cells had been shown previously to remain stable for at least one week.

?a After seeding, the medium was changed from each culture every day for the first 2 days. Iprodione, RP36112, or RP36115 were then [apparently after being dissolved in one of the vehicles and] added to fresh culture medium and incubated with cultured Leydig cells for 72 hours.

3. Leydig Cell Testosterone Secretion - Leydig cells were incubated for 72 hours with Iprodione, RP 36112, RP 36115, and Ketoconazole [10 μg/mL], and the cells were then stimulated for 3 hours with hCG [3 ng/mL], or cholera toxin [5 μg/mL], or forskolin [5 x 10^{-5}M], or 8-bromo-cAMP [1 mM]. Iprodione and Ketoconazole were dissolved in ethanol, and RP 36112 and RP 36115 were dissolved in DMSO. Testosterone secretion was measured in the culture medium.

Leydig cells were incubated in the absence [control] or presence of Iprodione, RP 36112, RP 36115, and Ketoconazole [10 μg/mL] for 72 hours. The cells were then incubated for 3 hours with hCG [3 ng/mL], or the substrates 22-R hydroxycholesterol [22ROHCT; 5 μg/mL] pregnenolone [P5; 500 ng/mL], 17 αhydroxypregnenolone [17OHP5;500 ng/mL], dehydroepiandrosterone [DHEA; 500 ng/mL], and Δ4 androstenedione [Δ4Dione; 500 ng/mL]. Iprodione and Ketoconazole were dissolved in ethanol, and RP 36112 and RP 36115 were tested dissolved in either ethanol, DMSO, or DMF. Testosterone secretion was measured in the culture medium.

Leydig cells from each control and treatment group were cultured in 3 separate wells. Using three samples of different volumes [50, 100, and 200 μL], testosterone concentration in each well was determined by specific Radioimmunoassay [RIA] in triplicate. Testosterone concentration [ng/mL] in each well was then calculated from the concentrations of these 3 samples, and the results were expressed as

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the mean of testosterone concentrations from the 3 different wells of each control and treatment group.

testosterone control values: testosterone concentrations in untreated cultures that have been challenged by an hCG stimulation
testosterone basal values: testosterone concentrations in untreated or treated cultures that have not been challenged by an hCG stimulation

4. c-AMP Production - Leydig cells were cultured in the absence [control] or presence of Iprodione [10 µg/mL] for 72 hours. The cells were then stimulated for 30 minutes with increasing concentrations of hCG [0.01-3 ng/mL], and cAMP was measured in Leydig cell culture medium.

Leydig cells from each control and treatment group were cultured in 3 separate wells. Following 30 minutes of stimulation with the gonadotrophin, the culture medium was collected in order to evaluate the extracellular cAMP levels, which reflect the production of the nucleotide considered as the intracellular second messenger of LH/hCG. The cAMP concentration in each well was determined by specific Radioimmunoassay [RIA], in duplicate [100 µL], and the cAMP concentration [pmol/mL] in each well was then calculated from the concentrations of these 2 samples. The results are expressed as the mean of cAMP concentrations from the 3 different wells of each control and treatment group.

C. EXPRESSION OF FINDINGS

1. Softwares - Excel [version 3.0] was used to input individual results and to calculate concentrations and standard deviations [SD]. Cricket Graph [version 1.3.2] was used to convert x-axis in log scale and to include SD.

2. Calculations with Excel - The results tables contain 9 or 10 columns, and the calculations are made for

column 4 = column 3/column 2 [respective volume] in pg/mL
column 5 = mean of the 3x3=9 values of each test column 4 in pg/mL
column 6 = standard deviation of column 5 in pg/mL
column 8 = dilution factor [concerns only data in Appendix III]
column 9 = column 5/column 7 in ng testosterone/10^6 cells
column 10 = standard deviation of column 8 in ng testosterone/10^6 cells

II. **RESULTS**

A. Effects of Iprodione, RP 36112, RP 36115, or Ketoconazole on hCG, 8-bromo-cAMP, forskolin, and cholera toxin-stimulated testosterone secretion: Leydig cell testosterone production stimulated with (1) gonadotropin [hCG], (2) substances that elevate cAMP production [cholera toxin and forskolin], and (3) a cAMP analog [8-bromo-cAMP] was inhibited by Iprodione, RP 36112, RP 36115, and Ketoconazole [Table 2]. Ketoconazole displayed the greatest inhibition in each case, and Iprodione displayed the smallest.

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[IPRODIONE] Mechanistic Study/Testosterone Secretion/Sites of Action

Table 2. Inhibition of Testosterone Secretion [ng/10^6 cells]				
Compound	hCG	Cholera Toxin	Forskolin	8-Bromo-cAMP
control	6.65±2.32	3.53±0.72	3.23±0.83	4.03±1.70
Iprodione	2.63±0.66 [40]♪	1.09±0.33 [31]	0.99±0.20 [31]	1.77±0.70 [44]
control	7.39±2	3.58±1.04	3.15±0.81	3.53±1.56
Ketoconazole	0.07±0.14 [<1]	0.04±0.08 [1.1]	0.02±0.07 [<1]	0 [0]
control	5.16±1.79	2.69±0.85	2.29±0.47	3.75±1.69
RP 36112	0.07±0.13 [1.4]	0.04±0.10 [1.5]	0.02±0.04 [<1]	0.06±0.12 [1.6]
control	6.01±2.63	3.07±0.75	2.40±0.56	2.83±1.30
RP 36115	0.48±0.35 [8]	0.33±0.20 [11]	0.06±0.08 [2.5]	0.66±0.34 [23]

data from Table 2 [page 18] of the report and Appendix I, pages 26-30; ♪ [% of control]

B. Effects of Iprodione on LH/hCG-Stimulated cAMP production: Iprodione was not shown to have an effect on cAMP production in cultured Leydig cells [Table 3]. The data were presented in Figure 3 of the report [copy appended to File Copy of DER].

Table 3. Effects on hCG-Stimulated cAMP Production		
Group	mean of cAMP from 3 cells	cAMP concentration pmol/10^6cells
basal	0.23±0.06	0.75±0.19
Iprodione	0.20±0.00	0.65±0.00
hCG 0.01 ng	0.23±0.06	0.75±0.19
Iprodione + hCG 0.01 ng	0.30±0.00	0.97±0.00
hCG 0.03 ng	0.40±0.00	1.29±0.00
Iprodione + hCG 0.03 ng	0.30±0.00	0.97±0.00
hCG 0.1 ng	1.33±0.12	4.30±0.37
Iprodione + hCG 0.1 ng	0.90±0.44	2.90±1.41
hCG 0.3 ng	5.53±1.03	17.85±3.31
Iprodione + hCG 0.3 ng	4.60±1.87	14.84±6.04
hCG 1 ng	10.10±1.55	32.58±5.01
Iprodione + hCG 1 ng	11.67±1.00	37.63±3.23
hCG 3 ng	15.17±1.86	48.92±5.99
Iprodione + 3 ng	16.40±7.85	52.90±25.32
basal	0.27±0.06	0.86±0.19
Iprodione	0.25±0.07	0.81±0.23

data from Appendix II, page 32

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[**IPRODIONE**] Mechanistic Study/Testosterone Secretion/Sites of Action

C. Effects of Iprodione, RP 36112, RP 36115, and Ketoconazole on Testosterone Secretion in Leydig Cells Incubated With Different Substrates: The inhibitory effect of Iprodione on testosterone secretion was not observed [Table 4] when Leydig cells were incubated with 22-R hydroxycholesterol [22ROHCT], known to be a cholesterol substrate, which passes readily through the mitochondrial membranes without requiring active transport. This result supports an hypothesis that Iprodione reduces hormone-stimulated testosterone formation in Leydig cells by preventing the active transport of cholesterol substrate to the mitochondria where it is converted to pregnenolone. Additionally, the inhibitory effect of Iprodione on testosterone secretion was also not observed in Leydig cells incubated with other steroid substrates. Although the author contends that RP 36115 shows a similar site of action to that of Iprodione, TB II notes that when DMF was the vehicle, inhibition was observed with 22ROHCT and 17OHP5. With respect to RP 36112, inhibition was evident with 22ROHCT, P5, and 17OHP5 as substrates with all 3 solvents and when DHEA was the substrate and DMF and DMSO were solvents. The author states that these results suggest that this metabolite inhibits testosterone formation by inhibiting the steroidogenic enzyme 17 αhydroxylase/17, 20 lyase, although because of solubility problems, the data for RP 36112 should be viewed with caution as to the exact site(s) of inhibition of testosterone formation. Ketoconazole, used as a positive control, exhibited its well characterized inhibitory action on 17 αhydroxylase/17, 20 lyase [Feldman, D. (1986) Endocrine Reviews 7, 409-420].

Table 4. Effects on Testosterone Secretion in Leydig Cells Incubated With Different Substrates

Compound/Substrate	hCG	22ROHCT	P5	17OHP5	DHEA	Δ4 Dione
Control Iprodione	7.57±1.86 2.26±1.06 [30]♪	70±34 108±23	73±23 87±18	110±51 140±58	132±41 167±80	271±99 314±164
Control Ketoconazole	10.47±7.61 0.43±0.41 [4]	68±21 24±10 [35]	50±7 27±12 [54]	117±29 47±12 [40]	137±50 199±98	470±252 341±118 [73]
Control RP 36112 EtOH	9.99±5.56 1.55±0.36 [16]	48±9 30±5 [63]	51±10 23±4 [45]	111±31 84±15 [76]	137±69 132±31	397±192 388±131
Control RP 36112 DMSO	14.10±7.38 0.56±0.18 [4]	66±12 30±10 [45]	85±37 18±7 [21]	199±56 88±19 [44]	174±86 141±21 [81]	472±226 665±293
Control RP 36112 DMF	19.73±12.80 2.65±0.81 [13]	139±26 51±7 [37]	112±17 34±7 [30]	278±77 203±26 [73]	258±101 173±27 [67]	536±185 635±210
Control RP 36115 EtOH	13.34±6.24 1.60±0.56 [4]	49±14 89±25	57±15 83±25	112±48 96±21 [86]	118±35 108±34	501±336 516±149
Control RP 36115 DMSO	21.06±8.76 3.41±1.34 [16]	77±12 152±23	103±33 122±25	237±66 224±65	276±106 258±154	497±145 530±241
Control RP 36115 DMF	19.84±5.82 1.28±0.46 [6]	75±33 44±8 [59]	63±18 73±22	223±49 130±40 [58]	180±45 195±63	528±168 720±248

data from page 21 of the report and Appendix III, pages 34-49; ♪ [% of control value]; 22-R hydroxycholesterol [22ROHCT]; pregnenolone [P5]; 17 αhydroxypregnenolone [17OHP5]; dehydroepiandrosterone [DHEA]; Δ4 androstenedione [Δ4Dione]

B

[IPRODIONE] Mechanistic Study/Testosterone Secretion/Sites of Action

III. **DISCUSSION**

A. The objective of this study was to identify the possible sites of inhibition of Iprodione and Metabolites RP 36112 and RP 36115 on LH/hCG-stimulated testosterone formation by evaluating several parameters related to the different biochemical steps involved in LH/hCG action on steroid hormone formation.

The parameters evaluated in this <u>in vitro</u> model using porcine cultured Leydig cells were (1) testosterone production after LH/hCG, 8-bromo-cAMP, forskolin, and cholera toxin stimulation; (2) cAMP production, (3) testosterone production in Leydig cells incubated with 22-R hydroxycholesterol, a cholesterol substrate derivative that passes readily through cell membranes and does not require active transport to be available for the P-450ssc enzyme in the inner mitochondria membrane; and (4) steroidogenic enzymes involved in testosterone formation by using different steroid substrates [P5, 17OHP5, DHEA, Δ4 dione. Iprodione and 2 of its metabolites inhibited testosterone secretion when Leydig cells were stimulated with (1) the gonadotropin hCG, (2) with drugs that enhance cAMP production [(a) cholera toxin, which stimulates Gs protein; (b) forskolin, which stimulates adenylate cyclase catalytic unit, and (3) with a cAMP analog [8-bromo-cAMP]. Because there were no effects observed on gonadotropin-stimulated cAMP production with Iprodione, it is stated that the inhibition of testosterone secretion by Iprodione would appear to be downstream from cAMP production. At the next step in testosterone biosynthesis, inhibition of testosterone secretion by Iprodione was not observed when the substrate 22ROHCT was added to the culture medium, which indicates that the step that is inhibited is located between the cAMP production and the movement/penetration of cholesterol into the mitochondria. Since 22ROHCT is a cholesterol substrate that passes through the mitochondrial membrane without the need of an active transport system, it is postulated by the Registrant that the sensitive site of inhibition of testosterone synthesis by Iprodione [or RP 36115] is the transport/availability of cholesterol substrate for the cholesterol side chain cleavage enzyme. The RP 36112 metabolite appears to act downstream from the cholesterol step; i.e., at the level of steroidogenic enzyme 17 αhydroxylase/17, 20 lyase. This latter step is inhibited by Ketoconazole also. It is concluded by the Registrant that Iprodione and its metabolites appear to modulate Leydig cell steroidogenesis by interfering at the level of cholesterol transport and/or steroidogenic enzyme activity [Figure 4 of report; copy appended to File Copy of DER]. TB II notes that there is no discussion regarding the possibility that a decrease in cholesterol resulting from an inhibition of protein kinase A might be a means by which Iprodione and/or its metabolites modulate Leydig cell steroidogenesis.

B. <u>Study deficiencies</u> None that would affect interpretation of the study. The write-up of the report was hard to follow. For example, a description of what was done in each experiment was in the Results section of the report instead of in the Methods section. Additionally, some of the details of the methods were listed only in the legend of the table of results. The cholesterol desmolase P-450 side chain cleavage is referred to as P-450ssc on pages 9, 10, and 12 and as P-450scc on pages 10, 11, and 23.

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[IPRODIONE] 14-Day Oral Study [§ none]

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Section Head, Toxicology Branch II/Section II/HED (7509C)

DATA EVALUATION REPORT

STUDY TYPE: 14-Day Oral - Mouse [§none, suppl. study; OECD none]

DP BARCODE: D232064 Submission: S481268

CASWELL NUMBER: 470A PC Code: 109801

MRID NUMBER: 44171902

TEST MATERIAL: Iprodione [97.1%]

CHEMICAL NAME: 3-(3,5-dichlorophenyl)-N-isopropyl-2,4-dioxoimidazolidine-1-carboxamide

SYNONYMS: Iprodione, Glycophene, Promidione, RP 26019

CITATION: Bigot, D. (1996). Iprodione Exploratory 14-Day Toxicity Study in the Mouse by Dietary Administration. Rhone-Poulenc Agrochimie/France. SA 95131. October 21, 1996. MRID 44171902. Unpublished.

SPONSOR: Rhone-Poulenc Agrochimie

EXECUTIVE SUMMARY: In this 3-day and 14-day oral exposure study [MRID # 44171902], groups of CD1 male mice [15/dose/group/chemical; 7 weeks old on arrival] were administered (1) **IPRODIONE** via the diet at dose levels of 4000 ppm [696 mg/kg/day] or 12000 ppm [2138 mg/kg/day]; (2) **KETOCONAZOLE** via the diet at a dose of 2000 ppm [341 mg/kg/day]; (3) **PHENOBARBITAL** via gavage at a dose level of 75 mg/kg/day; and (4) **CYPROTERONE ACETATE** via gavage at a dose level of 40 mg/kg/day. The control for the dietary studies was basal diet, and 0.5% methylcellulose was the control of the gavage studies. The objective of the study was to examine the potential liver effects of Iprodione in mice and to compare these effects with those produced by well characterized liver enzyme inducers and/or rodent liver carcinogens. Ketoconazole was selected as a positive control for its potential to inhibit testosterone secretion; Phenobarbital and Cyproterone acetate were selected for their potential to induce early liver changes and subsequent liver tumor formation in rodents. All of the liver effects produced by Ketoconazole, Phenobarbital, and/or Cyproterone acetate [increases in liver weight, alanine aminotransferase, aspartate aminotransferase, # hepatocytic mitoses, total cytochrome P-450 content, staining for isoforms CYP 2B and CYP 3A, benzoxyresorufin [BROD], ethoxyresorufin [EROD], pentoxyresorufin [PROD] enzyme activities, and hepatocyte proliferation, in addition to increases in the incidence of liver enlargement, centrilobular hypertrophy, diffuse hypertrophy, centrilobular/midzonal fine vacuolation] were exhibited by Iprodione at 12000 ppm. An effect observed following Iprodione exposure that was not observed following any of the other test material exposures was an increase in lauric acid hydroxylation. Although several of the effects observed in the liver following Iprodione exposure are analogous to those observed following the positive controls, especially

[IPRODIONE] 14-Day Oral Study [§ none]

Phenobarbital [centrilobular hypertrophy, liver weight, increased BROD, PROD, and EROD activities, cell proliferation after 3 days], in several cases the liver effect observed was most pronounced in the Iprodione mice compared to the positive controls [centrilobular/midzonal fine vacuolation, increased number of mitoses, cell proliferation at day 15].

This study demonstrates that Iprodione, at dose levels that are 5- and 15-fold greater than the LOEL for liver effects observed in the mouse carcinogenicity study, induces (1) liver cell proliferation, (2) increased microsomal enzyme activities, (3) an increase in total cytochrome P-450 content, and (4) centrilobular hypertrophy. These observations most closely resemble the pattern of liver effects observed following Phenobarbital exposure. Hepatocytic hypertrophy was observed at the high-dose level of Iprodione following both the 3- and 14-day exposure periods but only following the 14-day exposure period at the low dose. Liver cell proliferation was observed after both the 3-day and 14-day exposure periods at both dose levels of Iprodione. Increased cytochrome P-450 content and increased microsomal enzyme activities were observed at both dose levels of Iprodione following the 14-day exposure period, but neither analysis was performed following the 3-day exposure period. The dose level where liver tumors were observed in the mouse carcinogenicity study [604 mg/kg/day] is comparable to the low dose used in the current study. The findings in this study support the Registrant's arguments that the liver tumors observed in the Iprodione mouse carcinogenicity study may be secondary to liver toxicity. However, several pieces of data are lacking. The current study does not address whether cytochrome P-450 content and the microsomal enzyme activities are increased initially [after the 3-day exposure period]; therefore, one cannot determine whether the cell proliferation and hepatocytic hypertrophy observed after 3-days exposure to Iprodione is due to a direct effect of Iprodione on the liver or the result of adaptive processes. Additionally, the current study does not identify a NOEL for the liver effects monitored over a 14-day exposure period or address the question of whether these liver effects occur initially at the lower doses utilized in the mouse carcinogenicity study. Another outstanding question is whether the liver effects [hepatocytic hypertrophy, increased total cytochrome P-450 content, increased microsomal activities, cell proliferation] observed in the current study persist throughout a long-term exposure. X ?

This study is classified Acceptable [non-guideline].

Compliance: Signed and dated Quality Assurance, GLP Compliance, and Data Confidentiality statements were provided. No Flagging statement was submitted.

[IPRODIONE] 14-Day Oral Study [§ none]

I. **MATERIALS AND METHODS**

A. MATERIALS

1.(a) Test Material: Iprodione
Chemical name: 3-(3,5-dichlorophenyl)-N-isopropyl-2,4-dioxoimidazolidine-1-carboxamide
Description: white granular powder
Batch #: 9426801
Purity: 97.1%
Stability: stable [Appendix N, Table A2, page 455]
CAS #: 36734-19-7
Structural formula: $C_{13}H_{13}Cl_2N_3O_3$
[Structure]

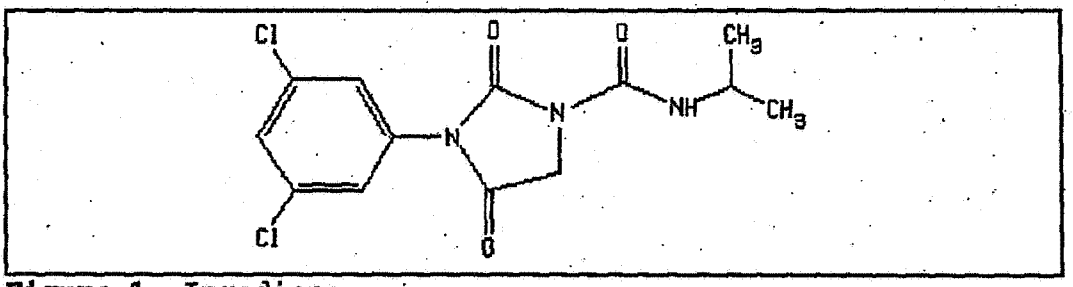

Figure 1 Iprodione

Formula: $C_{13}H_{13}Cl_2N_3O_3$;
Source: Rhone-Poulenc Secteur Agro [Lyon, France]

(b) Test Material: Ketoconazole
Chemical name:
Description: off-white solid
Batch #: P1446
Purity: 98%
Stability: stable [Appendix N, Table A4, page 456]
CAS #:
Structural formula: $C_{26}H_{28}Cl_2N_4O_4$

(c) Test Material: Phenobarbital sodium salt
Chemical name:
Description: white powder
Batch #: Lot # 122H0143
Purity: 99%
Stability: no data provided
CAS #:

(d) Test Material: Cyproterone acetate
Chemical name:
Description: white powder
Batch #: Lot # 054H0362
Purity: 99.5%
Stability: stable [Appendix N, page 453]
CAS #:

2. Vehicle: Certified Rodent Pellet Diet for Iprodione/Ketoconazole
Vehicle: 0.5% methylcellulose for Phenobarbital/Cyproterone Acetate

3. Test animals: Species: mouse
Strain: CD1 [males]

Age on arrival: 7 weeks old
Weight: 31.4-39.5 grams on day of treatment
Source: Charles River France, St. Albin-les-Elbeuf, France.
Housing: individual cages
Diet: Certified Rodent Pellet Diet A04C P1 [Usine d'Alimentation Rationnelle, Villemoisson-sur-Orge/France; Batch #: not provided, ad libitum
Water: filtered and softened municipal water ad libitum
Environmental conditions: standard laboratory conditions
Acclimation period: 13 days

B. STUDY DESIGN

1. In-Life Dates - initiated: 4/11/95; terminated: 7/27/95.

2. Animal Assignment: Fifteen male rats/group were administered the test material [control diet, Iprodione (4000 ppm or 12000 ppm), or Ketoconazole (2000 ppm)] via the diet or by gavage [vehicle (0.5% methylcellulose), Phenobarbital (75 mg/kg/day; dose volume of 5 mL/kg/day) or Cyproterone (40 mg/kg/day; dose volume of 5 mL/kg/day)] for 14 days. A satellite subgroup of 15 male mice was added to each group, and these mice were sacrificed after 3 days of treatment to assess hepatic cellular proliferation and liver histopathology. Four days before study initiation, an automatic procedure was used to select mice from the middle of the weight range of available mice. Each mouse was assigned an identification number within groups using a randomization procedure that "ensured a similar body-weight distribution among group for each sex." TB II notes that only **males** were used in this study.

3. Dose preparation and analysis: Iprodione and Ketoconazole were dry-mixed into the diet [no other details were provided in the Methods' section of the report; in Appendix N (Analytical Report), it states that analysis was performed to assess homogeneity, stability, and concentration of Iprodione and Ketoconazole in ground diet. The feed provided the rats was stated to be a pellet diet]. Both diets were prepared once. The mixed diets were stored at ≈-18°C when not in use. Homogeneity of Iprodione and Ketoconazole in the diet was verified prior to study start and during the study at all concentrations. The stability of each in the diet was demonstrated in a pre-study [SA 95131-A0, Appendix N]. Samples of Iprodione [4000 ppm and 12000 ppm] and Ketoconazole [2000 ppm] were kept frozen for 10 and 43 days, thawed, placed in the animal room for one week, and then analyzed. Daily, appropriate amounts of Phenobarbital and Cyproterone Acetate were suspended in 0.5% methylcellulose in distilled water. No analyses were performed on these latter 2 test materials. [Iprodione dose levels were chosen based on the dose levels used in previous subchronic [rat: 1000, 2000, 3000, and 5000 ppm] and oncogenicity [old mouse study: 200, 500, 1250 ppm; new mouse study: 160, 800, 4000 ppm; new rat: 150, 300, 1600 ppm; old rat: 125, 250, 1000 ppm] studies.

RESULTS

Results of analyses indicate that the homogeneity and concentrations attained were in the acceptable ranges for both test materials in rodent chow [Tables A1-A4, Appendix N, pages 454-456 of the report]. Concentrations attained were within an acceptable range for both test materials also [Tables B1 and B2, Appendix N, pages 459 and 460 of the report].

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4. Statistics: **body weight, body-weight change, food consumption, liver weight, clinical biochemistry data, hepatic microsomal enzyme activities, P-450 content:** Iprodione vs control Bartlett's test for homogeneity of variances; if homogeneous, group means compared using the analysis of variances [ANOVA] followed by Dunnett's test. If heterogeneous variances, group means compared using non-parametric Kruskall-Wallis analysis of variance by rank; if significant, the mean of each Iprodione group was compared to the mean of the control using Mann-Whitney test. Ketoconazole vs control [diet], Phenobarbital vs control [gavage], Cyproterone vs control [gavage] F' test performed to test for homogeneity of variances; if homogeneous, Student's t-test was performed for comparison of means; if heterogeneous, a modified t test was computed. **cell proliferation using PCNA evaluated at days 4 and 15:** Iprodione vs control group means compared using non-parametric Kruskal-Wallis analysis of variance by ranks; if significant, the means were compared using Mann-Whitney test. Ketoconazole vs control [diet], Phenobarbital vs control [gavage], Cyproterone vs control [gavage] means compared using Mann-Whitney. Statistical analysis was performed using SAS programs.

C. METHODS

1. Survival and clinical observations: All mice were observed for mortality, moribundity, and signs of toxicity twice a day [once on weekends], and the cages and cage trays were inspected for blood and loose feces.

2. Body weight and food consumption: Each mouse was weighed once during acclimation, on the first day of dosing, weekly thereafter, and at sacrifice. Food consumption was recorded weekly by calculating the difference between the amount of food given and that remaining at the end of the food consumption period. On day 4, food consumption was evaluated for the mice in the satellite groups.

3. Test material intake: The amount of Iprodione and Ketoconazole ingested was calculated.

4. Clinical pathology: Blood samples were collected from all surviving mice on day 15 [all groups] via puncture of the retro-orbital venous plexus [anesthetized via ether inhalation([not fasted)]. The CHECKED (X) parameters were evaluated. No hematology parameters were monitored.

Blood Chemistry

	Electrolytes:		Other:
	Calcium	X	Albumin
	Chloride		Blood creatinine
	Magnesium	X	Blood urea nitrogen
	Phosphorous	X	Cholesterol
	Potassium		Globulin
	Sodium		Glucose
	Iron		Phospholipids
	Enzymes	X	Total bilirubin
X	Alkaline phosphatase (ALK)	X	Total Protein (TP)
	Cholinesterase (ChE)		Triglycerides
	Creatine kinase (CK)		A/G ratio
	Lactate dehydrogenase (LAD)		Triiodothyronine [T3]
X	Serum alanine aminotransferase		Thyroxine [T4]
X	Serum aspartate aminotransferase		
	Gamma glutamyl transferase (GGT)		
	Glutamate dehydrogenase (GLDH)		
	Ornithine carbamyltransferase (OCT)		
	Electrophoretic protein fractions		

5. Urinalysis: No urine samples were collected.

6. Ophthalmoscopy: Eye examinations were not performed.

7. Gross Pathology: The satellite mice were sacrificed [not fasted] on day 4, and all survivors from the main study were sacrificed [not fasted] on day 15 under deep anesthesia [pentobarbital; i.p. injection (50-60 mg/kg)]. All mice were subjected to a complete post-mortem examination of all major organs, tissues, and body cavities, and all abnormalities were recorded. Only the liver was weighed. With the exception of the testes and epididymes, which were preserved in Davidson's fixative, all tissues were fixed in 10% buffered formalin.

8. Histopathology: In the satellite mice, only the liver and duodenum were preserved for histological and immunohistochemical assessment. The liver, testes, epididymes, seminal vesicles, prostate, duodenum, and adrenals were preserved from the main study mice. Histological examination was performed on hematoxylin and eosin stained liver sections from the control and treated groups.

9. Hepatotoxicity Testing: At the day 15 necropsy only, the remaining portions of the liver from all surviving mice were pooled into 3 groups within each group for microsomal preparations. Cytochrome P-450 specific isoenzyme content and enzymatic activities were assessed using a two-step methodology: (1) Western blotting for qualitative investigation and (2) enzymology for quantitative determination of P-450 activities. **There was no explanation as to why these analyses were not performed following the 3-day exposure period.**

a. Total Cytochrome P-450 Content: Total cytochrome P-450 content in microsomal preparations was determined by spectrophotometry using a reduced CO differential spectrum between 400 nm and 500 nm. Two determinations were performed for each microsomal preparation.

b. Western Blotting With Anti P-450 Antibodies: The potential changes of cytochrome P-450 isoenzymes were examined by western blotting

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after electrophoresis of microsomal preparations [from page 21 of report]. The different antibodies used were respectively specific of the following rat isoenzymes: CYP 1A1, CYP 2B1/2, CYP 2E1, CYP 3A.

To run microsomal preparations from all of the groups on the same gel, it was necessary to pool the 5 microsomal preparations of each group and to load the same amount of proteins of each of these pooled preparations onto the polyacrylamide gel. Then it was possible to compare directly the different immunostained bands obtained on the blot, as the staining procedure was rigorously identical for each sample. On each electrophoresis, protein molecular weight standards and well characterized reference compound-induced rat microsomes were run along samples to be examined. Following the electrophoretic separation and transfer to membrane, the blots were immunostained with an antibody raised against a specific rat P 450 isoenzyme listed above, a biotinylated secondary antibody and a streptavidin horseradish peroxidase conjugate. The detection was performed using ECL detection reagents [Amersham] utilizing the bound horseradish peroxidase to catalyze the oxidation of luminol. Following oxidation, the luminol was in an excited state, which decayed to the ground state via a light-emitting pathway. This light was captured on radiographic film.

Table 1. Cytochromes P-450 Isoenzymes, Activities, and Inducers

Family	Enzymatic Activity	Typical Inducing Agents
CYP 1A1 & 1A2	EROD	β-naphtoflavone
CYP 2B CYP 2E	PROD (BROD)	phenobarbital steroids isoniazid
CYP 3A	PROD (BROD)	steroids phenobarbital
CYP 4A	LAURIC ACID HYDROXYLATION	peroxisome proliferators

EROD [ethoxyresorufin O-deethylation];
BROD [benzoxyresorufin O-debenzylation];
PROD [pentoxyresorufin O-depentylation]; from page 22 of report

c. P-450 Enzymatic Activities: Specific cytochrome P-450 enzymatic activities were evaluated by spectrofluorimetry using (1) benzoxyresorufin, (2) ethoxyresorufin, and (3) pentoxyresorufin, and by HPLC with fluorimetric detection following derivatization by 4-(bromomethyl)-7-methoxycoumarin of 12-hydroxy-lauric acid (lauric acid used as substrate). Ethoxyresorufin is a highly selective substrate for the isoform 1A; the isoform 2B induces preferentially the O-dealkylation of pentoxyresorufin; and the benzoxyresorufin O-debenzylation is mainly induced by the isoform 3A. Cytochrome P-450 dependent dealkylation of resorufin derivatives was followed over a period of 1, 5, or 7 minutes at 37°C according to the type of substrate. Three determinations were performed for each microsomal preparation.

The hydroxylation of lauric acid by the isoform 4A was followed over a period of 10 minutes at 37°C. 12-Hydroxylauric acid was quantified in the incubation mixtures using the method N° ANL/046-94E.

Rat liver microsomes induced by well characterized reference compounds β-naphtoflavone, phenobarbital, pregnenolone 16α-carbonitrile, and clofibric acid were measured at the same time as the study samples to have positive controls for each assay.

d. Liver Cell Proliferation: An immunohistochemical staining was used to detect the proliferating cell nuclear antigen [PCNA] in order to assess liver cell proliferation. A monoclonal antibody raised against PCNA was applied to formalin fixed paraffin-embedded, deparaffinized liver sections. The immunological reaction was amplified by a secondary antibody biothynile. After submitting the liver sections to a complex streptavidine-peroxylase, the reaction was revealed using a chromogen, amino-ethyl-carbazol [AEC]. Microscopic examination was performed at a magnification of 40, using an eye-piece reticule of 1 square millimeter of surface divided into 100 squares. Five centrilobular and five periportal areas were randomly selected for assessment. All the liver PCNA-positive hepatocytes present in 10 reticular surfaces were counted and classified according to their staining characteristics, and the mean group values were calculated. Cell cycling assessment was performed on all but 4 mice. [from page 23 of report]

II. **RESULTS**

A. Survival and clinical observations: There was one death, which occurred in the 12000 ppm Iprodione group. This mouse was found dead on day 7. On days 4 and 7 this mouse was cold to touch, displayed tremors, reduced motor activity, prostration, palpebral ptosis, and general pallor. At necropsy, dark red fluid was reported in the small intestine, and moderate centrilobular hypertrophy and moderate centrilobular hydropic degeneration were observed in the liver. There were no other treatment-related signs reported for any of the groups.

B. Body weight and body-weight gain: **IPRODIONE** Both treated groups displayed body weights that were comparable to the control values on days 1 and 14, but the 12000 ppm group displayed a significant decrease [91% of control value] from the control value on day 8 [Table 2]. A negative body-weight gain [Table 3] was observed in the 12000 ppm mice during the first week of dosing, but both Iprodione groups displayed increased gains compared to the control during the second week. **KETOCONAZOLE** Ketoconazole mice displayed decreased body weight on days 8 and 14 compared to the control values [Table 2] and decreased body-weight gains during both weeks of the study [Table 3]. **CYPROTERONE ACETATE** Cyproterone acetate mice also displayed decreased body weight on days 8 and 14 compared to the control values, but body-weight gains were decreased during the first week only [Tables 2 & 3]. **PENTOBARBITAL** Phenobarbital mice displayed comparable body weights to the control during the study [Table 2].

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Table 2. Body Weight Data [grams]			
Group/Dose/Day	1	8	14
Iprodione			
control	36.00	37.17	37.90
4000 ppm	35.63	36.70	37.92
12000 ppm	34.75(97)	33.88**(91)	36.74(97)
Ketoconazole			
control	36.00	37.17	37.90
2000 ppm	35.43	34.92**(94)	35.32**(93)
Phenobarbital			
control	34.98	34.96	35.47
75 mg/kg/day	34.94	34.82	35.04
Cyproterone			
control	34.98	34.96	35.47
40 mg/kg/day	35.36	32.82**(94)	33.33**(94)

data from Table 2 of report [pages 63-66]/Appendix B [pages 161-167];
NOTE: #s differ slightly between these 2 sources; ** p<0.01;

Table 3. Body-Weight Change Data [g/day]		
Group/Dose/Interval	days 1-8	days 8-14
Iprodione		
control	0.166	0.122
4000 ppm	0.153(92)	0.202*(166)
12000 ppm	-0.158**	0.476**(390)
Ketoconazole		
control	0.166	0.122
2000 ppm	-0.072**	0.066(54)
Phenobarbital		
control	-0.002	0.085
75 mg/kg/day	-0.018	0.036(42)
Cyproterone		
control	-0.002	0.085
40 mg/kg/day	-0.362**	0.084

data from Table 3 of report [pages 68-71]; ** p<0.01;
#s vary slightly from those in Appendix C, pages 168-172

C. Food consumption and test material intake: Overall food consumption [Table 4] was comparable among the groups, with the exception of the high Iprodione group, which displayed a significant decrease [88% of control] during the first week and a significant increase [118% of control] during the second week compared to the control. Food efficiency was not calculated.

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Table 4. Food Consumption Data [g/day]		
Group/Dose/Interval	days 1-8	days 8-14
Iprodione		
control	5.97	5.89
4000 ppm	6.14	6.38
12000 ppm	5.28*(88)	6.96**(118)
Ketoconazole		
control	5.97	5.89
2000 ppm	5.91	6.09
Phenobarbital		
control	5.85	6.00
75 mg/kg/day	5.51	5.83
Cyproterone		
control	5.85	6.00
40 mg/kg/day	5.61	5.88

data from Table 4 of report [pages 73-76]; ** p<0.01

The mean intake of test material is shown in Table 5.

Table 5. Achieved Dose [mg/kg/day]			
Group/Week	week 1	week 2	mean [week 1-2]
Iprodione			
4000 ppm	696	696	696
12000 ppm	1812	2464	2138
Ketoconazole			
2000 ppm	322	349	341

data from Table 5, page 78 of report

D. Clinical pathology: **Clinical chemistry** - There were several differences noted among the groups compared to their respective control groups [Tables 6-9]. **IPRODIONE** Dose-related increases were observed in total protein, albumin, total cholesterol, ASAT, and ALAT [Table 6] following the 14-day exposure period. The apparent increase in alkaline phosphatase at the high dose is attributed to the high levels observed in 3 mice; excluding these 3 values gives a value for this parameter that is comparable to the control [54.8].

Table 6. Clinical Chemistry Data - IPRODIONE			
Parameter/Dose	control	4000 ppm	12000 ppm
albumin [g/L]	28.800	29.333	34.615**
cholesterol [mmol/L]	2.692	2.953	3.878**
total protein [g/L]	51.666	54.133**	66.076**
urea [mmol/L]	13.756	10.572	10.970
alanine aminotransferase [IU/L]	41.866	74.666*	128.923**
alkaline phosphatase [IU/L]	52.666	51.533	85.923♪
aspartate aminotransferase [IU/L]	55.666	61.000	95.692**
total bilirubin [µmol/L]	0.686	0.000**	0.038**

data from Table 6 of report [pages 80-81]; ♪ 3 of the 13 mice examined displayed values of 110, 128, & 331 [Appendix E]; excluding these, the mean is 54.8; ** p<0.01

KETOCONAZOLE There were treatment-related increases in ASAT and ALAT compared to the control values [Table 7].

Table 7. Clinical Chemistry Data - KETOCONAZOLE

Parameter/Dose	control	2000 ppm
albumin [g/L]	28.800	28.857
cholesterol [mmol/L]	2.692	2.617
total protein [g/L]	51.666	51.000
urea [mmol/L]	13.756	14.382
alanine aminotransferase [IU/L]	41.866	74.642**
alkaline phosphatase [IU/L]	52.666	57.785
aspartate aminotransferase [IU/L]	55.666	115.428**
total bilirubin [µmol/L]	0.686	0.171*

data from Table 6 of report [pages 82-83]; * p<0.05; ** p<0.01

PHENOBARBITAL There were small increases observed in total protein and ALAT compared to the control values [Table 8].

Table 8. Clinical Chemistry Data - PHENOBARBITAL

Parameter/Dose	control	2000 ppm
albumin [g/L]	28.666	28.133
cholesterol [mmol/L]	2.537	2.544
total protein [g/L]	52.066	54.666*
urea [mmol/L]	12.440	12.552
alanine aminotransferase [IU/L]	48.933	93.733*
alkaline phosphatase [IU/L]	54.800	55.533
aspartate aminotransferase [IU/L]	66.466	75.266
total bilirubin [µmol/L]	0.753	0.060*

data from Table 6 of report [pages 84-85]; * p<0.05; ** p<0.01

CYPROTERONE ACETATE Increases were observed in total protein, albumin, total cholesterol, ALAT, and ASAT, and a decrease was observed in alkaline phosphatase compared to the control [Table 9].

Table 9. Clinical Chemistry Data - CYPROTERONE ACETATE		
Parameter/Dose	control	2000 ppm
albumin [g/L]	28.666	34.066**
cholesterol [mmol/L]	2.537	6.529**
total protein [g/L]	52.066	66.666**
urea [mmol/L]	12.440	12.499
alanine aminotransferase [IU/L]	48.933	115.333*
alkaline phosphatase [IU/L]	54.800	37.733**
aspartate aminotransferase [IU/L]	66.466	152.533
total bilirubin [µmol/L]	0.753	0.613

data from Table 6 of report [pages 86-87]; * p<0.05; ** p<0.01

F. Sacrifice and pathology

1. Gross pathology: **IPRODIONE** In the 12000 ppm Iprodione mouse that was found dead on day 7, a dark red fluid was observed at necropsy in the small intestine, and moderate centrilobular hypertrophy and moderate centrilobular hydropic degeneration were observed in the liver. At interim sacrifice [day 4], diffuse enlargement of the liver was observed in 3 of the 15 mice at the high-dose level compared to none in the low and control groups. Hepatic enlargement, which was associated with accentuated lobular pattern and friability in several mice, was observed at both dose levels [dose-related] at final sacrifice [Table 10] and considered treatment-related. **KETOCONAZOLE** At the final sacrifice only, enlargement of the liver and adrenals was observed in the Ketoconazole mice [Table 10], along with several other liver lesions [abnormal dark color, accentuated lobular pattern, friable]. **PHENOBARBITAL** One of the 15 Phenobarbital mice displayed an enlarged liver at the interim sacrifice, and 2 displayed enlarged adrenals at the final sacrifice [Table 10]. **CYPROTERONE ACETATE** With the exception of one enlarged liver, there were no gross lesions observed at either sacrifice.

Table 10. Gross Pathology - Terminal Sacrifice							
Group/Dose/ Tissue/ Lesion	Iprodione			Ketoconazole		Phenobarbital	
	0 ppm	4000 ppm	12000 ppm	0 ppm	2000 ppm	0 mg/kg	75 mg/kg
LIVER							
enlargement	0/15	10/15	14/14	0/15	2/15	nr	nr
accentuated lobular pattern	0/15	3/15	10/14	0/15	1/15	nr	nr
friable	0/15	0/15	2/14	0/15	2/15	nr	nr
dark abnormal color	0/15	0/15	1/14	0/15	14/15	0/15	2/15
ADRENAL							
enlargement	0/15	2/15	7/14	0/15	3/15	0/15	2/15

nr not reported; data from Table 9, pages 107-115 of the report

2. Organ weights: Liver weight was increased in all test material groups compared to their respective controls [Table 11].

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[IPRODIONE] 14-Day Oral Study [§ none]

Table 11. Liver Weight

Group/Dose/Sacrifice	interim	final
Iprodione		
absolute		
control	2.04	2.01
4000 ppm	2.43** [119]	2.70** [134]
12000 ppm	2.86** [140]	4.51** [224]
relative		
control	5.68	5.25
4000 ppm	6.80** [120]	7.04** [134]
12000 ppm	8.43** [148]	12.11** [231]
BODY WEIGHT		
control	35.9	38.2
4000 ppm	35.6	38.3
12000 ppm	33.8** [94]	37.0 [97]
Ketoconazole		
absolute		
control	2.04	2.01
2000 ppm	2.18	2.41** [120]
relative		
control	5.68	5.25
2000 ppm	6.15** [108]	6.77** [129]
BODY WEIGHT		
control	35.9	38.2
2000 ppm	35.4	35.5** [93]
Phenobarbital		
absolute		
control	1.90	1.91
75 mg/kg/day	2.20** [116]	2.30** [120]
relative		
control	5.35	5.35
75 mg/kg/day	6.26** [117]	6.43** [120]
BODY WEIGHT		
control	35.5	35.8
75 mg/kg/day	35.0	35.6
Cyproterone		
absolute		
control	1.90	1.91
40 mg/kg/day	1.95	2.23** [117]
relative		
control	5.35	5.35
40 mg/kg/day	5.93** [111]	6.57** [123]
BODY WEIGHT		
control	35.5	35.8
40 mg/kg/day	32.8** [92]	34.0* [95]

data from Tables 7 & 8 of report (pages 89-96 & 98-105)
* $p<0.05$; ** $p<0.01$

3. Microscopic findings: **IPRODIONE** Moderate centrilobular hypertrophy and moderate centrilobular hydropic degeneration was observed in the liver of the mouse found dead on day 7. Slight to moderate centrilobular hypertrophy was observed in the liver of the high-dose mice at the interim sacrifice [Table 12], and the number of hepatocytic mitoses was increased also. One high-dose mouse displayed slight centrilobular/midzonal fine vacuolation. There were no liver lesions observed in the 4000 ppm mice at the interim sacrifice [3 days of treatment]. Treatment-related effects observed in the liver at the terminal sacrifice [14 days of treatment] are listed in Table 12. All of the Iprodione mice displayed hepatocytic hypertrophy.

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[IPRODIONE] 14-Day Oral Study [§ none]

Table 12. Microscopic Findings - IPRODIONE			
Lesion/Dose	0 ppm	4000 ppm	12000 ppm
LIVER - interim sacrifice			
hepatocytic hypertrophy - centrilobular	0/15	0/15	15/15
increased number of mitoses	0/15	0/15	9/15
centrilobular/midzonal fine vacuolation	0/15	0/15	1/15
midzonal microvesicular fatty change	0/15	0/15	2/15
LIVER - final sacrifice			
hepatocytic hypertrophy - centrilobular	0/15	15/15	6/14
hepatocytic hypertrophy - diffuse	0/15	0/15	8/14
increased number of mitoses	0/15	0/15	2/14
centrilobular/midzonal fine vacuolation	0/15	3/15	10/14

data from Table 10, pages 118-119 of the report

KETOCONAZOLE At the interim sacrifice, centrilobular hypertrophy and increased number of mitoses were observed in the treated mice [Table 13]. At the terminal sacrifice, slight to moderate hepatocytic hypertrophy was observed in all treated mice, which in a few cases was associated with slight to mild increased number of mitoses and with slight to mild midzonal microvesicular fatty change or slight to moderate centrilobular/midzonal fine vacuolation.

Table 13. Microscopic Findings - KETOCONAZOLE		
Lesion/Dose	0 ppm	2000 ppm
LIVER - interim sacrifice		
centrilobular hypertrophy	0/15	3/15
increased number of mitoses	0/15	1/15
LIVER - final sacrifice		
centrilobular hypertrophy	0/15	15/15
increased number of mitoses	0/15	2/15
midzonal microvesicular fatty change	0/15	3/15
centrilobular/midzonal fine vacuolation	0/15	2/15

data from Table 10, pages 120-121 of the report

PHENOBARBITAL Slight to moderate centrilobular hypertrophy and slight to mild increased number of mitoses were observed in the livers of the treated mice compared to no incidence in the control at the interim sacrifice [Table 14]. All of the treated mice displayed centrilobular hypertrophy at the terminal sacrifice.

Table 14. Microscopic Findings - PHENOBARBITAL		
Lesion/Dose	0 ppm	75 mg/kg/day
LIVER - interim sacrifice		
centrilobular hypertrophy	0/15	14/15
increased number of mitoses	0/15	3/15
centrilobular/midzonal fine vacuolation	0/15	1/15
LIVER - final sacrifice		
centrilobular hypertrophy	0/15	15/15

data from Table 10, pages 122-123 of the report

CYPROTERONE ACETATE The observed changes in the liver were comparable between the treated and control mice and were considered as representative of the usual background pathology found in mice of the age and strain used in the study.

G. Hepatotoxicity Testing

1. Total Cytochrome P-450 Content: There was a dose-related increase in total cytochrome P-450 content in mice fed Iprodione for 14 days compared to the control mice [Table 15], and Ketoconazole, Phenobarbital, and Cyproterone acetate each increased total cytochrome P-450 content compared to their respective control values also [Table 15].

Table 15. Individual Cytochrome P-450 Content

Group	microsomal proteins [mg/mL]	P-450 content [nmol/mg protein]
diet control	8.688	1.19
4000 ppm Iprodione	14.152	1.61** [135]♪
12000 ppm Iprodione	15.322	1.76** [148]
2000 ppm Ketoconazole	15.028	1.47** [124]
methyl cellulose control	8.400	1.21
75 mg/kg/day Phenobarbital	15.51 [185]	1.87** [155]
40 mg/kg/day Cyproterone acetate	12.44 [148]	1.88** [155]

♪ [% of control]; p <0.01; data from Table 11, pages 127-130, Appendix J, pages 425-427 of the report;

2. Western Blotting with Anti P-450 Antibodies: **IPRODIONE** The staining of the isoforms CYP 2B and CYP 3A were increased in a dose-related manner following Iprodione treatment, and the dose-related effect was said to be more marked for the isoform 2B. The staining of CYP 2E was comparable to that in the control microsomes, and no staining was obtained for the isoform 1A in either the control or Iprodione group. **KETOCONAZOLE** The staining of the isoform CYP 3A was markedly increased after Ketoconazole treatment and of similar intensity to the staining obtained after Cyproterone acetate treatment but at a dose that was 8 times larger. No changes were observed in the staining for CYP 2B, 2E, and 1A compared to the controls. **PHENOBARBITAL** There was increased staining of the isoforms CYP 2B and CYP 3A, but no change in the staining of isoform 2E. No positive staining was found for CYP 1A in either the treated or control mice. **CYPROTERONE ACETATE** Both CYP 2B and CYP 3A were induced by Cyproterone acetate, but Pentobarbital was more potent in inducing the isoform 2B and less potent than Cyproterone acetate in inducing isoform 3A. Compared to the control, Cyproterone acetate decreased slightly the staining of the isoform 2E. No positive staining was obtained for the isoform 1A in either the control or treated mice. [as described on pages 30, 36, 40, and 44 of the report].

3. P-450 Enzymatic Activities: **IPRODIONE** There was a dose-related increase in pentoxyresorufin [PROD] and benzoxyresorufin [BROD]

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activities, and 12000 ppm Iprodione increased ethoxyresorufin [EROD] activity also. A marginal dose-related increase in lauric acid hydroxylation was induced by Iprodione administration compared to the control [Table 16]. **KETOCONAZOLE** There was a decrease in BROD, EROD, and PROD activity following Ketoconazole administration. Additionally, Ketoconazole did not have any effect on lauric acid hydroxylation. **PHENOBARBITAL** Significant increases in BROD, EROD, and PROD activities were induced by Pentobarbital administration at 75 mg/kg/day. There was no effect on the hydroxylation of lauric acid. **CYPROTERONE ACETATE** There was a significant increase in BROD and PROD activities following administration of Cyproterone acetate at 40 mg/kg/day, but there was no effect on EROD activity or the hydroxylation of lauric acid.

Table 16. Hepatic Microsomal Activities - Iprodione			
Substrate/Dose [ppm]	0	4000	12000
pentoxyresorufin	3.86	10.33** [268] ♪	26.17** [678]
ethyoxyresorufin	32.63	47.23 [145]	79.31** [243]
benzoxyresorufin	6.00	47.73** [796]	103.90** [1732]
lauric acid	0.74	0.99* [134]	1.25** [169]

* p<0.05; ** p<0.01; ♪ [% of control]; data from Table 12, page 132 of the report

Table 17. Hepatic Microsomal Activities - Ketoconazole		
Substrate/Dose [ppm]	0	2000
pentoxyresorufin	3.86	2.15** [56] ♪
ethyoxyresorufin	32.63	14.98** [46]
benzoxyresorufin	6.00	0.52* [9]
lauric acid	0.74	0.77

* p<0.05; ** p<0.01; ♪ [% of control]; data from Table 12, page 133 of the report

Table 18. Hepatic Microsomal Activities - Phenobarbital		
Substrate/Dose	0 [mg/kg/day]	2000 [mg/kg/day]
pentoxyresorufin	6.24	110.69** [1774] ♪
ethyoxyresorufin	25.42	70.99** [279]
benzoxyresorufin	0.00	522.18
lauric acid	0.88	0.93

** p<0.01; ♪ [% of control]; data from Table 12, page 134 of the report

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[IPRODIONE] Mechanistic Study/Testosterone Secretion/Rats [§ none]

EPA Reviewer: Linda L. Taylor, Ph.D.
Review Section II, Toxicology Branch II (7509C)
EPA Secondary Reviewer: K. Clark Swentzel
Review Section II, Toxicology Branch II (7509C)

4/8/97

9/9/97

DATA EVALUATION RECORD

STUDY TYPE: Mechanistic Study [rat testicular sections]; OPPTS/§ none

DP BARCODE: D232064 SUBMISSION CODE: S481268

P.C. CODE: 109801 TOX. CHEM. NO.: 470A

TEST MATERIAL (PURITY): Iprodione [97.3%]

CHEMICAL: 3-(3,5-dichlorophenyl)-N-isopropyl-2,4-dioxoimidazolidine-1-carboxamide

SYNONYMS: Iprodione, Glycophene, Promidione, RP 26019

CITATION: Fail, P.A., Anderson, S.A., and Pearce, S.W. (1996). Toxicity Testing of a Fungicide, Iprodione: Endocrine Toxicology Studies of Testes From Adult Male CD®Sprague-Dawley Rats Exposed to Iprodione In Vitro. Laboratory of Reproductive and Endocrine Toxicology/Center for Life Sciences and Toxicology/Chemistry and Life Sciences/Research Triangle Institute. RTI ID No. 65C-6169; Rt95-IPDB; RTI-532. November 26, 1996. MRID 44171903. Unpublished.

SPONSOR: Rhone-Poulenc Ag Company/Research Triangle Park, NC

EXECUTIVE SUMMARY: The objective of this in vitro study [MRID 44171903] was to determine the effect of in vitro Iprodione [99.7%] exposure on basal testosterone secretion and stimulated release from testicular sections in culture media [in vitro Endocrine Challenge Test (ECT) using human chorionic gonadotropin (hCG)]. The effects of prior in vivo exposure of the male rats via the diet [3000 ppm Iprodione for 14 days] was also evaluated. Testicular sections obtained from 12 male CD® Sprague-Dawley rats administered Iprodione via the diet for 14 days at dose levels of 0 ppm or 3000 ppm were incubated with 0, 1, 10, or 100 µg/ml Iprodione for one hour. Half of these testicular sections from each in vitro treatment group were challenged with human chorionic gonadotrophin and the other half of the sections were monitored for basal testosterone secretion. Media testosterone concentrations were monitored at hourly intervals for 3 hours after challenge.

There was a dose-related reduction in testosterone secretion from testicular sections incubated in vitro with Iprodione, with and without hCG stimulation. Prior exposure of the rats to Iprodione in vivo for 14 days appeared to have little effect on the secretion of testosterone, with and without hCG stimulation, from testicular sections incubated in vitro other than a slight increase at t=0. At sacrifice following the 14-day exposure period to Iprodione in vivo, plasma LH concentrations were significantly increased compared to the control and, although plasma testosterone was not significantly affected, the levels were somewhat increased compared to the control [132% of control]. The significant increase in plasma LH at necropsy suggests a possible stimulation of the homeostatic mechanism.

[**IPRODIONE**] Mechanistic Study/Testosterone Secretion/Rats [§ none]

Under the conditions of this 14-day study, Iprodione was shown to produce a reduction in testosterone secretion from testicular sections following incubation in vitro with Iprodione. Prior exposure of male rats to Iprodione in vivo via the diet for 14 days did not alter the reduction in testosterone secretion observed in their testicular sections exposed to Iprodione in vitro. Although the in vitro inhibition appeared to be dose-related, it appears that a maximum response may have occurred between the 10 and 100 µg/mL dose levels. The data presented provide pieces to the "puzzle" but not a complete picture of what may be occurring in the testes/rat that ultimately results in testicular tumors. Although it appears that the premise is that Iprodione produces testosterone biosynthesis inhibition, resulting ultimately in the increased incidence of Leydig cell tumors, there are inconsistencies in the in vitro and in vivo data, and the in vitro effects observed in the short-term studies to date have not been demonstrated to occur in long-term studies, nor is it clear that the levels at which the in vitro effects were observed are attained in vivo.

This study is classified Acceptable [non-guideline].

COMPLIANCE: Signed and dated Data Confidentiality, Quality Assurance, and GLP statements were provided. No flagging statement was submitted.

[**IPRODIONE**] Mechanistic Study/Testosterone Secretion/Rats [§ none]

I. **MATERIALS AND METHODS**

A. MATERIALS

1. Test Material: Iprodione
Chemical name: 3-(3,5-dichlorophenyl)-N-isopropyl-2,4-dioxoimidazolidine-1-carboxamide
Description: off-white powder consisting of large spherical granules
Lot #: 8906201;
Purity: 97.3%
Stability: stable [from Material Safety Data Sheet]
CAS #: 36734-19-7
Structural formula: $C_{13}H_{13}Cl_2N_3O_3$
[Structure]

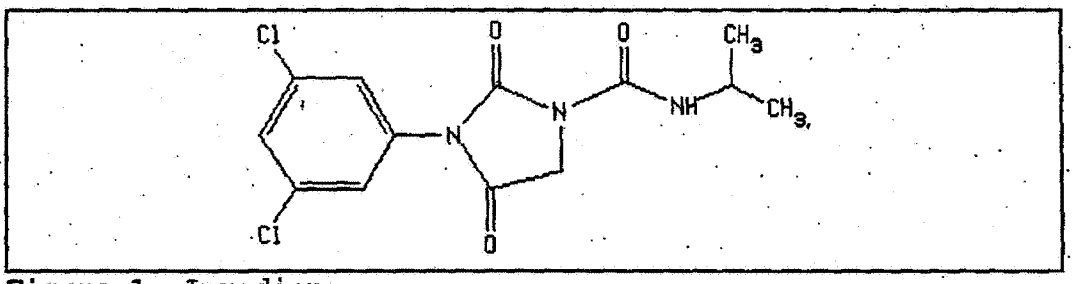

Figure 1 Iprodione

Formula: (1) $C_{13}H_{13}Cl_2N_3O_3$;
Source: Rhone-Poulenc [Research Triangle Park, NC]

2. Vehicle: #5002 Purina Certified Ground Rodent Chow; Batch #: not provided.

3. Test animals: Species: rat
Strain: virus antibody free [VAF/Plus®], Cesarean-derived (CD®), Sprague-Dawley (SD), barrier reared (BR), outbred albino [Crl:CD® (SD) BR]
Age on arrival: ~8 weeks old
Weight: 282.02-317.16 grams at randomization
Source: Charles River Laboratories, Inc. (Crl), Raleigh, NC.
Housing: individual cages
Diet: #5002 Purina Certified Ground Rodent Chow ad libitum
Water: deionized/filtered tap ad libitum
Environmental conditions: standard laboratory conditions, except rooms were maintained on a 14:10 hour light:dark cycle
Acclimation period: 7 days

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[**IPRODIONE**] Mechanistic Study/Testosterone Secretion/Rats [§ none]

B. STUDY DESIGN and METHODS

1. In life dates - initiated: July 26, 1995; completed: August 9, 1995

2. Animal assignment: Twelve adult virgin male rats were assigned to one of two experimental groups [either 0 and 3000 ppm Iprodione] by a randomization process and stratified by body weight; at study initiation, the body weights of both groups were homogeneous by statistical analysis.

3. Dose preparation and analysis - Iprodione was ground into a fine powder, and a known amount of this was added to a known amount of feed and mixed thoroughly. Test diets were analyzed for adequacy of mixing, stability under both freezer [-20°C] and study conditions [room temperature], and attained concentration. The test diets were prepared once at study initiation and stored under freezer conditions. Fresh diet was provided on days 1 and 7 of the 14-day feeding study.

RESULTS

The results of the storage stability study of diets containing 3000 ppm Iprodione indicate that the test diets were stable for a period of 125 days when stored under freezer conditions and for 9 days at room temperature [results shown on pages 118 and 119 of report (APPENDIX II)]. TB II notes that on page 120 of the report [APPENDIX II], it states that test diets can be stored for periods of time up to 35 days at freezer temperature [0 to -20°C]. The mixing procedure was shown to be adequate [page 111 of report, APPENDIX II]. The test diets were found to contain the appropriate amount of test material [106% of nominal concentration; results shown on page 135 of the report].

4. Preliminary experiment to determine hCG challenge parameters - This experiment was performed to establish an effective dose and time response regimen to detect a response in testicular testosterone following addition of human chorionic gonadotropin [hCG] to media. Fifteen untreated male rats [same supplier/strain/age of those used in definitive study] were sacrificed by rapid decapitation without anesthesia, and the right testis was removed and cut into 4 sections. Each section was weighed and incubated in 5 mL of modified medium 199 with 0.1% bovine serum albumin, sodium bicarbonate, HEPES, and soybean trypsin at ~34°C on a shaker in 5% CO_2/95% air. After an initial incubation of 60 minutes, samples of media were collected just prior to hCG challenge and at one, two, and three hours after hCG challenge for analysis of testosterone concentration. The results are presented in Table 1. The media testosterone concentration increased with increasing hCG concentration until a maximal response was attained at 0.5 IU hCG/0.5 mL of media. Doses greater than this dose did not elicit a greater response. The approximate effective dose 50 [ED50] of hCG, i.e., the dose of hCG that results in 50% of the maximal testosterone release, was determined to be 0.005 IU/0.5 mL of media.

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[**IPRODIONE**] Mechanistic Study/Testosterone Secretion/Rats [§ none]

Based on these data, the dose and sampling times for the definitive study were established at 0.005 IU hCG/0.5 mL media and 0, 1, 2, and 3 hours.

Table 1. Results of Preliminary Study - In Vitro Testosterone after hCG Challenge

hCG [IU/0.5 mL]	Testosterone [ng/mL media]♪			
	baseline	1 hour	2 hour	3 hour
0	6.58±1.59	7.62±1.47	9.54±1.24	10.00±1.07
0.005	6.26±1.77	10.74±2.34	23.75±6.34*	32.02±9.068*
0.05	4.71±1.01	18.95±2.15*	37.15±5.17*	47.68±8.27*
0.25	5.07±1.14	20.98±3.18*	38.49±5.86*	46.56±5.98*
0.5	5.84±1.15	22.53±2.60*	42.48±4.19*	52.97±5.05*
2.5	6.88±1.40	25.98±5.13*	47.83±10.18*	58.86±9.83*
5	6.15±0.88	23.47±1.70*	41.60±2.25*	54.76±3.92*
25	6.31±0.96	23.81±3.31*	39.46±5.26*	50.96±6.27*
50	8.29±1.09	28.35±4.45*	45.96±7.14*	53.05±8.30*
250	8.26±1.54	26.87±2.45*	46.98±3.84*	53.33±3.13*

♪ mean ± standard error of mean; * p <0.05; data from Table MB-1, page 18 of the report

5. Endocrine measurements - Hormone concentrations were determined in the plasma using radioimmunoassays [RIA] validated [using male CD rat plasma and/or media, as appropriate, and cross-reactivity of Iprodione was checked at 28 µg/mL (maximum possible blood level in rats treated with 3000 ppm Iprodione)] for male rats at RTI. **Rat LH Mini RIA** - A double antibody ^{125}I-RIA was used [details listed on pages 21 & 22 of the report], which utilized rat LH antibody, ^{125}I-labeled rat LH, rat LH antigen for use as a reference standard, and a second antibody [goat anti-rabbit gamma globulin]. The standard curve and iodinated rat LH were prepared in RIA Buffer [0.01 M sodium phosphate + 0.86% (w/v) sodium chloride with 0.05 M EDTA, 0.3% (v/v) normal rabbit serum, and 0.1% (w/v) sodium azide, pH 7.6]. The second antibody was prepared in PBS/EDTA buffer [0.01 M sodium phosphate + 0.85% (w/v) sodium chloride with 0.05 M EDTA and 0.1% (w/v) sodium azide, pH 7.6]. Rat LH controls were prepared by adding known concentrations of rat LH to plasma [added mass]. For the RIA procedure, the sample was pipetted directly to the bottom of a glass tube, LH antiserum was added, and the tubes were vortexed and incubated overnight [≈4°C]. On the second day, the second antibody was added, and the tubes were vortexed and incubated for ≈ 48 hours at ≈4°C. Following the 2-day incubation with the second antibody, the tubes were centrifuged, the supernatant was aspired, and the tubes containing pellets were counted in a gamma counter [ICN 10/600 Plus]. **Testosterone RIA** - A no-extraction, solid-phase ^{125}I-RIA was used, which utilized testosterone-specific, antibody-coated tubes and ^{125}I-testosterone [details from pages 22-23 of the report]. The standard curve was prepared in a PBS-Gel buffer [0.1 M sodium phosphate + 0.85% (w/v) sodium chloride with 0.1% (w/v) sodium azide and 0.1% (w/v) gelatin, pH 7.4]. Testosterone controls were prepared in the same matrix as unknown samples by adding known concentrations of testosterone to plasma or media [added mass], as appropriate. For the RIA procedure, the sample [plasma or media] was pipetted directly to the bottom of the tube, ^{125}I-testosterone was added, and the tubes were vortexed and then incubated in a ≈37°C water bath for 3 hours.

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After incubation, the supernatant was aspirated, and the tubes were counted in a gamma counter [same as above].

Assay characteristics are presented in Table 2. **LH**: Recovery of rat LH from plasma was ≈64%; intra-assay variations for blank and spiked samples were ≈ 7% or less for plasma; inter-assay variation was not applicable since only one assay was run. The parallelism for the LH assay in plasma was excellent [≈99%]. There was no evidence of cross-reactivity. From the control values, the intra- and inter-assay coefficient of variation, % recovery, and index of parallelism for the assays were determined. The sensitivity of this assay was 4 pg. **Testosterone**: Intra-assay variations for blank and spiked samples were ≈12% or less for plasma and ≈14% or less for media. From the control values, the intra- and inter-assay coefficient of variation, % recovery, and the index of parallelism for the assays were determined. The sensitivity of this assay was 3.5 pg.

Table 2. Characteristics of RIAs Validated for Determination of LH and Testosterone in Male Rats

Parameter	Hormone Assay		
	plasma testosterone	media testosterone	plasma LH
sensitivity [pg]	3.5	3.5	4.0
intra-assay variation♪ blank mass added	0/8.7% 2/11.8% 8/5.2%	NA 0.5/14.2% 2/8.5% 8/5.8%	0/3.6% 1/3.4% 5/7.3% 10/6.3%
interassay variation♪ # of assays blank mass added	1 NA NA	3 0.5/5.7% 2/5.9% 8/3.1%	1 NA NA
% recovery of added mass♫	2/92.5% 8/94.3%	0.5/158.7% 2/141.5% 8/124.3%	1/54.0% 5/68.8% 10/69.6%
index of parallelism (%)♦	127.1%	113.8%	99.4%
cross reactivity of Iprodione (%)	0%	NT	0%

♪ #s are mass added [ng/mL]/% variation [for intra-assay variation, # of samples assayed was 10 in each case; NT = not tested; NA = not applicable; ♫ #s are mass added [ng/mL]/% recovered; ♦ concentration of low volume ÷ concentration of high volume x 100; data from Table MB-3, page 24, of the report

6. Definitive study design - **In Vivo Phase** Two groups of 12 male rats each were administered Iprodione via the diet at dose levels of 0 ppm and 3000 ppm for 14 consecutive days, during which time daily clinical signs, body weight [days 1, 7, and 14], feed and water consumption [days 1, 7, and 14], and mortality were monitored. Following the 14-day exposure period, the rats were sacrificed via rapid decapitation on day 15 and trunk blood was collected for the determination of plasma testosterone and luteinizing hormone [LH]. A complete necropsy

was performed on each rat, and the liver, paired adrenals, paired kidneys, each epididymis, each testis, and total accessory sex organs [TASOs] weighed as a unit with fluids [seminal vesicles, prostate, coagulating glands; bladder removed] were weighed. The left and right testicles were removed, trimmed of fat, weighed, and sectioned into 4 sections each [8 sections per rat]. The sections were randomly assigned to one of eight in vitro treatment groups within the 2 in vivo treatment groups [Table 3].

Table 3. Experimental Design - Endocrine Toxicity of Iprodione on Rat Testes [In Vitro Challenge]

In Vitro Treatment			In Vivo Iprodione Treatment	In Vitro Challenge hCG [IU]/0.5 mL	In Vitro Exposure Iprodione [µg/mL]
group #	# testicular sections	# sections/rat			
1	12	1	0	0	0
2	12	1	0	0	1
3	12	1	0	0	10
4	12	1	0	0	100
5	12	1	0	0.005	0
6	12	1	0	0.005	1
7	12	1	0	0.005	10
8	12	1	0	0.005	100
9	12	1	3000 ppm	0	0
10	12	1	3000 ppm	0	1
11	12	1	3000 ppm	0	10
12	12	1	3000 ppm	0	100
13	12	1	3000 ppm	0.005	0
14	12	1	3000 ppm	0.005	1
15	12	1	3000 ppm	0.005	10
16	12	1	3000 ppm	0.005	100

data from Table MB-2, page 19 of the report

In Vitro Phase Endocrine challenge test - Testicular sections within each in vitro treatment group were exposed either to Iprodione [dissolved in ethanol] in media at dose levels of 0, 1, 10, and 100 µg/mL or to equal volumes of ethanol-spiked media. The Iprodione solutions used were said to have been prepared immediately prior to use. After one hour of incubation, a 0.5 mL sample was taken [baseline release], and then all sections were challenged with either 0.5 mL plain media or 0.005 IU hCG in 0.5 mL media. Testosterone release was monitored by taking a 0.5 mL sample at 1, 2, and 3 hours after the challenge. Of the 8 in vitro treatment groups obtained from each of the in vivo pretreated groups, half were monitored for basal testosterone secretion and half were challenged with 0.005 hCG/0.5 mL of media. The sample media volume taken at each time point was not replaced, and testosterone values were corrected for this difference.

4. Statistics: Parametric statistical procedures were applied to parametric data. Appropriate General Linear Models [GLM] procedures (SAS Institute Inc.) for the Analyses of Variance [ANOVA] or Student's T-test were employed using SAS/STAT®. software [SAS® Version 6.08]. Prior to GLM analysis, which was used to determine whether significant dose effects had occurred for selected measures, a $\log_{10}$ transformation was performed on all testosterone data [Snedecor and Cochran, 1967] to

allow use of parametric methods. An F' [folded] statistic was used to test for equality of variances, and a univariate procedure was used to test for normality of distribution. A two-tailed test was used for all pairwise comparisons, body and organ weight parameters, and feed consumption. Because the dose-response data represent repeated measurements from multiple pieces of each testis, a linear dose-response model was used to test hypothesis of interest using SUDAAN software [Versions 6.4 and 7.0] and the Generalized Estimating Equations [GEE] model-fitting method of longitudinal continuous outcomes. Analysis of the preliminary study utilized GLM procedures for the ANOVA and Dunnett's test for pairwise comparisons of treatment groups back to the control. The purpose of the investigation was to determine whether exposure to Iprodione and/or hCG challenge have an effect on testosterone levels. The response variables used in the analysis were (1) testosterone levels at 0, 1, 2, and 3 hours post hCG challenge; (2) time to maximum testosterone level over the 3-hours period; and (3) area under the testosterone-time curve, using the trapezoid rule based on linear interpolation [Crowder and Hand, 1990]. Six broad sets of hypotheses were tested in the analysis. **Hypothesis Set #1** [relates to the main effects and interactions among in vivo Iprodione, hCG challenge, in vitro Iprodione dose, and time since administration]: Models included (1) a main-effects-only model, (2) a main-effects + 2-way interaction [in vitro Iprodione by hCG challenge], and (3) a main-effects + 3-way interaction [in vitro Iprodione by hCG challenge by in vivo Iprodione] model. Because the 2-way interaction effect was significant, a main effects model was fitted testing the effects of time, in vivo Iprodione, and in vitro Iprodione dose within levels of hCG challenge. Additionally, because the 3-way interaction effect was significant, a main effects model was fitted testing the effects of time and in vitro Iprodione dose within cross-classified levels of hCG challenge by in vivo Iprodione. **Hypothesis Set # 2** [tests the effect of in vivo Iprodione on testosterone levels at time = 0, when there is no exposure to either hCG challenge or in vitro Iprodione dose]. **Hypothesis Set # 3** [test the effect of in vitro Iprodione dose on testosterone levels at time = 0]. Seven models were tested: first, within each of the 4 cross-classified levels of hCG challenge by in vivo Iprodione, then within each level of in vivo Iprodione but adjusted for hCG challenge, and finally adjusted for both in vivo Iprodione and hCG challenge. Each of the 7 models contained effects of in vitro Iprodione, with pairwise comparisons of each dose group to control. When adjusting for the effects of hCG challenge and in vivo Iprodione, these effects were included in the model also. **Hypothesis # 4** [tests the effects of hCG challenge and in vitro Iprodione dose on AUC testosterone (within levels of in vivo Iprodione)]. **Hypothesis # 5** [tests effects of hCG challenge and in vivo Iprodione within each level of in vitro Iprodione dose]. **Hypothesis # 6** [tests the effects of in vivo Iprodione on AUC testosterone when all other effects are set to their control levels (hCG challenge = 0 IU and in vitro Iprodione dose = 0 μg)].

[**IPRODIONE**] Mechanistic Study/Testosterone Secretion/Rats [§ none]

II. **RESULTS**

A. In Vivo Phase

1. Survival/observations/body weight/food consumption - All rats survived until study termination, and there were no apparent treatment-related signs. The Iprodione rats displayed decreased body weights by day 7 [93% of control value] of the study and by day 14, their body weight was 10% less than the controls. Body-weight change overall for the Iprodione rats was 38% of the control value [Table 4]. The Iprodione rats also ate less food [77%-82% of control value] than the controls during both weeks of the study. On a mg/kg/day basis, the amount of Iprodione ingested was ≈175.

Table 4. Body-Weight Data

Parameter/Group	0 ppm	3000 ppm
Body Weight [g]/Day		
1	341.5	345.2
7	382.9	355.1*[93]♪
14	420.7	379.8*[90]
Body-Weight Change [g]	74.1	28.1*[38]
Body-Weight Change [%]	21.5	8.2*

* p<0.05; ♪ [% of control]; data from Table B-1, page 35 of the report

2. Organ weights - Compared to the control rats, the Iprodione rats displayed decreased kidney, total accessory sex organs [TASO], and total epididymides weights and increased adrenal weights. The relative adrenal and testes weights were significantly increased and the relative TASO weight was decreased in the Iprodione rats compared to the controls [Table 5]. Brain weight was not provided and consequently, endocrine-sensitive organ weight relative to brain weight could not be calculated. This may have provided a more relevant assessment of the organ-weight data.

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[IPRODIONE] Mechanistic Study/Testosterone Secretion/Rats [§ none]

Table 5. Organ-Weight Data

Organ/Group	0 ppm	3000 ppm
liver [g]	16.34	15.92 [97]♪
kidneys [g]	2.97	2.63* [89]
adrenals [mg]	62.06	78.65* [127]
right testis [g]	1.61	1.62
left testis [g]	1.61	1.61
total testes [g]	3.22	3.23
right epididymis [mg]	494.97	466.30 [94]
left epididymis [mg]	491.33	455.69* [93]
total epididymes [mg	986.30	921.99* [93]
TASO [g]	2.23	1.72* [77]
relative♦		
liver [g]	38.79	41.92 [108]
kidneys [g]	7.09	6.89
adrenals [mg]	0.15	0.21* [140]
right testis [g]	3.83	4.28* [112]
left testis [g]	3.82	4.24* [111]
total testes [g]	7.65	8.52* [111]
right epididymis [mg]	1.21	1.25
left epididymis [mg]	1.19	1.21
total epididymes [mg	2.40	2.45
TASO [g]	5.49	4.41* [80]
Body Weight [g]	420.7	379.8* [90]

♪ [% of control]; ♦ mean of organ weight [mg]/g body weight; data from Table B-3, page 37 of report

3. Endocrine values at necropsy - There were no statistically significant differences in peripheral plasma testosterone values at necropsy between the groups, although the mean value for the Iprodione group was 32% greater than the control value [Table 6]. LH concentrations were significantly greater [double] in the Iprodione rats than in the control rats [Table 6].

Table 6. Plasma Testosterone and LH Values

Hormone/Group	0 ppm	3000 ppm
testosterone [ng/mL]	1.16±0.16	1.53±0.38 [132]♪
LH [ng/mL]	0.20±0.04	0.42±0.09* [210]

* $p<0.05$; ♪ [% of control]; data from Table B-5, page 39 of the report

B. In Vitro Phase

1. Endocrine Measures: (a) Testosterone synthesis and release from testicular sections incubated in vitro: The release of testosterone into the media increased over time under all conditions tested. In testicular sections from male rats not exposed to Iprodione in vivo and not exposed in vitro, the ED_{50} of hCG produced a statistically-significant difference in testosterone release into the media, as evidenced by the 4-fold increase [64.43 vs 255.47] in AUC compared to non-stimulated controls [Tables 7&10]. In vitro exposure to Iprodione

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reduced both the baseline secretion [no hCG] and the stimulated release [with hCG] of testosterone [Table 11]. (b) Effects of Iprodione on testosterone synthesis and release from testicular sections incubated in vitro - The release of testosterone into the media increased over time under all conditions tested, but the addition of Iprodione to the media resulted in a smaller release of testosterone into the media than that of the control [Table 7, left top quadrant]. Iprodione exposure in vitro produced a reduction [appears dose-related] in testosterone release into culture media, with and without hCG stimulus [Table 7 & Table 11]. The total testosterone produced over the 4-hour period was 2.2-fold higher in control slices than for the high-dose, Iprodione-treated slices [AUC, Table 7, top left quadrant]. After hCG stimulation, this difference was 6.2-fold higher [AUC, Table 7, top right quadrant]. (c) Effects of in vivo Iprodione on testosterone synthesis and release from testicular sections incubated in vitro - With respect to the effect of in vivo pretreatment with Iprodione, neither the basal secretion nor the hCG-stimulated release of testosterone was significantly affected by the 14-day exposure to 3000 ppm Iprodione in the diet [Tables 8 and 13]. The total testosterone produced over the 4-hour period was 1.5-fold higher in control slices than for the high-dose, Iprodione-treated slices [AUC, Table 7, bottom left quadrant]. After hCG stimulation, this difference was 3.7-fold higher [AUC, Table 7, bottom right quadrant]. It appears that exposure to Iprodione in vivo results in a higher basal concentrations of testosterone [Table 7]; in all cases, the testosterone value at 0 hour is greater for the 3000 ppm Iprodione in vivo exposure than for the 0 ppm Iprodione in vivo exposure. Comparing Tables 11 and 12, which display the effects of in vitro Iprodione exposure at 10 µg/mL to 0 µg mL, in vivo Iprodione exposure does not appear to have any effect on testosterone release other than the increase in basal testosterone levels.

Table 7. Testicular Testosterone [ng/g testis] of Male Rats Following 14 Days of Iprodione Administration in Diet - In Vitro Incubation w/ Iprodione in Media

I in vivo	0 ppm Iprodione							
hCG in vitro	0 IU hCG				0.005 IU hCG			
I in vitro	0 µg/mL	1 µg/mL	10 µg/mL	100 µg/mL	0 µg/mL	1 µg/mL	10 µg/mL	100 µg/mL
testosterone♪								
0 hr.	58.06±5.45	51.61±6.25	46.98±5.26	44.11±6.27	61.49±5.10	47.93±7.39	41.42±4.56	46.94±5.71
1 hr.	75.57±7.01	62.50±7.52	57.82±4.93	53.30±6.36	94.48±6.67	70.95±9.13	53.01±4.58	58.06±6.27
2 hr.	85.76±7.65	69.10±7.83	60.94±5.70	54.73±5.34	173.48±10.72	129.05±13.07	56.26±4.16	64.75±6.42
3 hr.	96.50±8.19	72.18±7.28	67.25±5.79	61.75±6.13	282.44±17.79	223.17±18.04	69.44±4.53	71.04±6.73
max. T [hr]♦	2.67±0.19	2.50±0.26	2.92±0.08	2.75±0.18	3.0±0	3.0±0	3.0±0	2.83±0.11
AUC♫	64.43±6.19	38.68±8.66	34.92±3.12	28.63±4.69	255.47±18.99	191.77±20.96	40.44±5.58	40.99±3.37
I in vivo	3000 ppm Iprodione							
hCG in vitro	0 IU hCG				0.005 IU hCG			
I in vitro	0 µg/mL	1 µg/mL	10 µg/mL	100 µg/ml	0 µg/mL	1 µg/mL	10 µg/mL	100 µg/mL
testosterone♪								
0 hr.	81.27±14.64	76.74±11.29	69.04±14.20	65.99±12.57	77.76±9.70	74.16±15.07	62.31±10.90	60.73±9.16
1 hr.	107.45±17.05	99.67±11.67	83.57±14.35	83.83±12.69	116.24±12.71	103.75±19.16	81.18±13.45	78.77±11.38
2 hr.	115.28±17.47	110.58±11.80	89.89±12.88	86.45±12.62	189.45±20.53	150.95±22.38	92.07±13.67	89.82±10.02
3 hr.	127.44±17.65	116.80±11.26	92.79±13.19	96.97±12.56	280.38±29.69	249.80±32.69	103.75±13.72	104.07±12.12
max. T [hr]♦	2.83±0.17	2.67±0.14	2.36±0.20	2.82±0.18	3.0±0	3.0±0	2.92±0.08	2.92±0.08
AUC♫	83.28±8.92	76.79±8.40	47.25±4.49	53.79±8.28	251.47±32.98	194.21±24.75	69.35±8.19	68.81±7.67

I = Iprodione; ♪ ng/g testis; ♫ AUC = area under curve; ♦ time of maximum testosterone level; data from Table B-6, pages 40-41 of the report

Table 8. Testicular Testosterone of Male Rats Following 14 Days of Iprodione Administration in Diet - In Vitro incubation wi Iprodione in Media [% change]

hCG in vitro	0 IU hCG				0.005 IU hCG			
I in vitro	0 µg/mL	1 µg/mL	10 µg/mL	100 µg/mL	0 µg/mL	1 µg/mL	10 µg/mL	100 µg/mL
I in vivo 0 ppm								
1 hr.	30.98±2.79	23.46±7.11	26.09±3.58	24.42±6.14	57.00±6.34	51.56±5.16	31.55±5.08	26.62±3.33
2 hr.	49.02±7.46	38.13±8.20	32.93±5.18	30.94±6.22	193.99±20.02	191.31±23.21	41.80±7.62	42.57±5.56
3 hr.	68.63±7.03	45.96±8.43	48.63±7.21	48.20±8.67	385.90±41.95	416.56±38.10	78.05±12.29	57.03±5.95
I in vivo 3000 ppm								
1 hr.	37.68±6.62	36.30±7.05	26.00±4.16	38.19±11.10	54.46±6.17	43.14±3.72	35.67±5.60	32.46±4.87
2 hr.	50.57±8.71	54.06±8.63	40.05±5.87	43.83±10.30	162.46±22.07	129.80±22.23	58.79±9.71	59.00±8.77
3 hr.	71.51±13.38	67.20±12.57	46.13±9.91	69.51±17.14	299.81±42.32	312.15±64.44	84.41±13.47	85.22±11.15

I = Iprodione; data from Table B-7, page 42 of the report

Table 9. Testicular Testosterone of Male Rats Following 14 Days of Iprodione Administration in Diet - In Vitro Incubation wi Iprodione in Media [% of control]

hCG in vitro	0 IU hCG				0.005 IU hCG			
I in vitro	0 µg/mL	1 µg/mL	10 µg/mL	100 µg/mL	0 µg/mL	1 µg/mL	10 µg/mL	100 µg/mL
I in vivo 0 ppm								
0 hr.	100	89	81	76	100	78	67	76
1 hr.	100	83	77	71	100	75	56	61
2 hr.	100	81	71	64	100	74	32	37
3 hr.	100	75	70	64	100	79	25	25
AUC	100	60	54	44	100	75	16	16
I in vivo 3000 ppm								
0 hr.	100	94	85	81	100	95	80	78
1 hr.	100	93	78	78	100	89	70	68
2 hr.	100	96	78	75	100	80	49	47
3 hr.	100	92	73	76	100	89	37	37
AUC	100	92	57	65	100	77	28	27

calculated by reviewer using data from Table B-6, pages 40-41 of the report

[IPRODIONE] Mechanistic Study/Testosterone Secretion/Rats [§ none]

Table 10a. Effect of hCG on Testicular Testosterone of Male Rat w/out In Vivo or In Vitro Iprodione Exposure		
Parameter/Treatment	0 IU hCG	0.005 IU hCG
testosterone [ng/g testis]		
0 hour	58.06±5.45	61.49±5.10
1 hour	75.57±7.01	94.48±6.67
2 hour	85.76±7.65	173.48±10.72
3 hour	96.50±8.19	282.44±17.79
Area Under Curve	64.43±6.19	255.47±18.99*

* P<0.05; data from Tables B-6 and B-8, pages 40 and 43 of the report

Table 10b. Effect of hCG on Testicular Testosterone of Male Rat w/ In Vivo but w/out In Vitro Iprodione Exposure		
Parameter/Treatment	0 IU hCG	0.005 IU hCG
testosterone [ng/g testis]		
0 hour	81.27±14.64	77.76±9.70
1 hour	107.45±17.05	116.24±12.71
2 hour	115.28±17.47	189.45±20.53
3 hour	127.44±17.65	280.38±29.69
Area Under Curve	83.28±8.92	251.47±32.98

data from Table B-6, page 41 of report

Table 11a. Effect of In Vitro Iprodione Exposure on Testicular Testosterone of Male Rats				
	0 ppm in vivo Iprodione			
	0 IU hCG		0.005 IU hCG	
Iprodione in vitro	0 µg	10 µg	0 µg	10 µg
testosterone [ng/g testis]				
0 hour	58.06±5.45	46.98±5.26	61.49±5.10	41.42±4.56
1 hour	75.57±7.01	57.82±4.93	94.48±6.67	53.01±4.58
2 hour	85.76±7.65	60.94±5.70	173.48±10.72	56.26±4.16
3 hour	96.50±8.19	67.25±5.79	282.44±17.79	69.44±4.53
Area Under Curve	64.43±6.19	34.92±3.12*	225.47±18.99	40.44±5.58*

* linear regression, p<0.001; data from Tables B-6 and B-10, pages 40 and 45 of the report

Table 11b. Effect of In Vitro Exposure on Testicular Testosterone of Male Rats Following In Vivo Exposure				
	3000 ppm in vivo Iprodione			
	0 IU hCG		0.005 IU hCG	
Iprodione in vitro	0 µg	10 µg	0 µg	10 µg
testosterone [ng/g testis]				
0 hour	81.27±14.64	69.04±14.20	77.76±9.70	62.31±10.90
1 hour	107.45±17.05	83.57±14.35	116.24±12.71	81.18±13.45
2 hour	115.28±17.47	89.89±12.88	189.45±20.53	92.07±13.67
3 hour	127.44±17.65	92.79±13.19	280.38±29.69	103.75±13.72
Area Under Curve	83.28±8.92	47.25±4.49*	251.47±32.98	69.35±8.19*

* linear regression, p<0.001; data from Tables B-6 and B11, pages 41 and 46 of the report

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[IPRODIONE] Mechanistic Study/Testosterone Secretion/Rats [§ none]

Table 12. Effect of In Vivo Iprodione Exposure via the Diet on Testicular Testosterone of Male Rats				
	0 µg in vitro Iprodione			
	0 IU hCG		0.005 IU hCG	
Iprodione in vivo	0 ppm	3000 ppm	0 ppm	3000 ppm
testosterone [ng/g testis]				
0 hour	58.06±5.45	81.27±14.64	61.49±5.10	77.76±9.70
1 hour	75.57±7.01	107.45±17.05	94.48±6.67	116.24±12.71
2 hour	85.76±7.65	115.28±17.47	173.48±10.72	189.45±20.53
3 hour	96.50±8.19	127.44±17.65	282.44±17.79	280.38±29.69
Area Under Curve	64.43±6.19	83.28±8.92♪	255.47±18.99	251.47±32.98

♪ p=0.0720; data from Tables B-6 and B-9, pages 40, 41, and 44 of the report

Table 13. Dose Relationship of In Vitro Iprodione on Testicular Testosterone [ng/g testis] W/ and W/out hCG				
AUC/Dose I in vitro	0 µg/mL I	1 µg/mL I	10 µg/mL I	100 µg/mL I
0 ppm in vivo I w/out hCG*	64.43±6.19	38.68±8.66	34.92±3.12	28.63±4.69
0 ppm in vivo I w/ hCG*	255.47±18.99	191.77±20.96	40.44±5.58	40.99±3.37
3000 ppm in vivo I w/out hCG	83.28±8.92	76.79±8.40	47.25±4.49♪	53.79±8.28
3000 ppm in vivo I w/ hCG	251.47±32.98	194.21±24.75	69.35±8.19♪	68.18±7.67

AUC=area under curve; data from Tables B-6, B-12, and B13, pages 40-41 and 47-48 of the report; I=Iprodione; * significant linear dose response on Arithmetic Scale; ♪ significantly different from control, linear regression [SUDAAN, Ver.7.0]

The author stated that the testes appear to be less responsive to hCG in vitro in the presence of Iprodione [Table 13].

C. Statistical Analysis of Testosterone

The statistical analyses of the results of the various combinations of variables tested is described below [from pages 143-144 of the report; pages 5-6 of the Statistical Analyses Report of the study].

Hypothesis Set #1 [relates to the main effects and interactions among in vivo Iprodione, hCG challenge, in vitro Iprodione dose, and time since administration],

Testosterone levels were reported as increased significantly in the 3000 ppm in vivo Iprodione and in the 0.005 IU hCG challenge when compared to their respective controls NOTE: It is not obvious to this reviewer which data were compared in this analysis or what is being compared. On page 27 of the report, there appears to be a contradiction of this assessment; i.e., it is stated that when Iprodione treatment in vivo is considered, neither the basal concentration or the hCG-stimulated release of testosterone was significantly affected by Iprodione pretreatment. The statistical analysis continues, stating that testosterone levels were reduced significantly in each of the in vitro Iprodione dose groups compared to the controls and were increased significantly in a linear trend across time [Table 7]. The 2-way interaction between in vitro Iprodione and hCG challenge was statistically significant also, and further testing showed that the effects of in vitro Iprodione and time

were larger in the presence of challenge than in the absence [although significant in both cases], but that the effects of in vivo Iprodione were stronger in the absence of challenge than in the presence [Table 8]. It is not apparent to this reviewer what this indicates or what the significance of this findings is.

The next model in this hypothesis set contained the main effects of all 4 covariates plus the 3-way interaction between in vitro Iprodione, hCG challenge, and in vivo Iprodione. The 3-way interaction effect was statistically significant, so the effects of in vitro Iprodione and time separately were explored within cross-classified levels of hCG challenge and in vivo Iprodione were examined. Both time and in vitro Iprodione dose were found to have the largest effects [not identified] in the presence of challenge, as observed above. It was noted that this effect was diminished, but still statistically significant, in the presence of in vivo Iprodione. Again, the significance of these findings are not evident, nor is there any indication of whether the "effects" observed occurred at all dose levels of in vitro Iprodione.

Hypothesis Set # 2 [tests the effect of in vivo Iprodione on testosterone levels at time = 0, when there is no exposure to either hCG challenge or in vitro Iprodione dose].

Testosterone was increased significantly in the 3000 ppm in vivo group when compared to the controls [Tables 7&13; control 58.06 vs 3000 ppm Iprodione 81.27]. TB II notes that the p-value [2-sided; page 9 of the statistical report (page 147 of the report)] is 0.1770.

Hypothesis Set # 3 [test the effect of in vitro Iprodione dose on testosterone levels at time = 0].

With respect to the effect of in vitro Iprodione separately within each of the 4 cross-classified levels of hCG challenge by in vivo Iprodione, it is stated that the results indicate that testosterone levels were decreased in each dose group vs control at each level of challenge by in vivo Iprodione, but the largest reductions occurred in the situation where challenge was present and in vivo Iprodione was absent. The 10 µg and 100 µg Iprodione dose levels were reported to displayed significantly lower testosterone levels than the controls for every combination of challenge and in vivo Iprodione, but the 1 µg Iprodione group was only significantly lower than controls in the absence of in vivo Iprodione [regardless of the presence or absence of challenge]. Also, with respect to the effect of in vitro Iprodione separately within each level of in vivo Iprodione at time 0, adjusting for the effect of hCG challenge [challenge was not administered at time 0, so the hCG challenge effect is basically a replicate effect here], the reductions in testosterone levels in each in vitro Iprodione dose group vs control were larger in the absence of in vivo Iprodione than in its presence. All 3 dose groups were reduced significantly compared to the controls in the absence of in vivo Iprodione, but only the 10 µg and 100 µg dose groups were said to be significantly different from controls in the presence of in vivo Iprodione. The effect of hCG challenge [a replicate effect at time 0] was not statistically significant. Finally, with respect to the effect of in vitro Iprodione at time = 0 adjusted simultaneously for both in

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vivo Iprodione and hCG challenge [a replicate effect], testosterone levels were significantly reduced in all 3 in vitro Iprodione dose groups when compared to controls, but the effect of in vivo Iprodione [observed increases in the 3000 ppm group vs controls] and hCG challenge [the replicate effect] was not statistically significant.

Hypothesis # 4 [tests the effects of hCG challenge and in vitro Iprodione dose on AUC testosterone (within levels of in vivo Iprodione)].

Results indicate that, although in vitro Iprodione has a significant effect on lowering the testosterone AUC with and without in vivo Iprodione, the effects were larger in the absence of in vivo Iprodione. The testosterone AUC was significantly lower in the 1 μg group compared to the controls only in the absence of in vivo Iprodione, but the 10 μg and 100 μg groups were significantly lower than controls both with and without in vivo Iprodione. The testosterone AUC was also significantly increased in the 0.005 IU hCG challenge group compared to the control, but the effect was larger in the absence of in vivo Iprodione [Table demonstrating this was not referenced].

Hypothesis # 5 [tests effects of hCG challenge and in vivo Iprodione within each level of in vitro Iprodione dose].

Testosterone AUC was significantly increased in the 0.005 IU hCG challenge group compared to the controls at every in vitro Iprodione dose, but the effects were largest in the 0 μg and 1 μg dose groups [Table 7]. Additionally, AUC was consistently increased in the 3000 ppm in vivo Iprodione group compared to controls, but significant only in the 10 μg and 100 μg in vitro Iprodione groups.

Hypothesis # 6 [tests the effects of in vivo Iprodione on AUC testosterone when all other effects are set to their control levels (hCG challenge = 0 IU and in vitro Iprodione dose = 0 μg)].

AUC was observed to be increased in the 3000 ppm Iprodione group compared to the controls, but the effect only approached statistical significance (p=0.0720) [Table 13].

III. **DISCUSSION**

A. Conclusions of Author: 1) Testosterone secretion from testicular sections, incubated in vitro, was significantly reduced in a dose-related manner by the addition of Iprodione to the media, with and without the hCG stimulus; 2) prior dietary exposure to Iprodione at 3000 ppm for 14 days appeared to have no effect on the secretion of testosterone from testicular sections in vitro, either with or without the hCG stimulus. The author states that the effects of Iprodione on testosterone secretion in vitro appear to be in contrast to the apparent minimal effects on plasma testosterone observed in vivo. The increases observed in plasma LH concentrations suggest to the author that Iprodione interferes with sex/steroid hormone regulation and that it appears that in vivo homeostatic mechanisms are able to rapidly compensate for any Iprodione-induced effects on testosterone secretion from Leydig cells.

[**IPRODIONE**] Mechanistic Study/Testosterone Secretion/Rats [§ none]

Analysis of data as presented in the report.

The Registrant states that the release of testosterone [on a ng/g of testis basis] into culture media, with or without hCG stimulation, was suppressed by Iprodione [in vitro] addition to the media and refers to report Tables B-6 [DER Table 7], B-7 [DER Table 8], and B-8 (sic) {B-8 does not present data regarding Iprodione} [DER Table 10a]. For all treatment combinations, it is shown that testosterone release into the media increased over time. Additionally, in all groups [both with and without hCG stimulation], the Registrant indicates that the presence of Iprodione in the media at 1, 10, and 100 µg/mL caused a reduction of testosterone release into the media and report Tables B-6 [DER Table 7], B-1 [DER Table 11], B-11 [DER Table 12], and B-12 [DER Table 13] are referenced. TB II notes that Table B-11 presents data for the in vivo exposed rats and questions why this table is referenced here, since later in the discussion of results, the in vivo data are presented. The report continues, stating that the total testosterone produced over the 4-hour period was 2.2-fold higher in control slices than for the high-dose, Iprodione-treated testes, and after hCG stimulation, the difference was 6.2-fold. The Registrant concludes from this that the testes appear to be less responsive to hCG in vitro in the presence of Iprodione {Tables B-6 (Section 1, AUC) [DER Table 7] and B-7 (% change at 3 hours) [DER Table 8] are referenced}. TB II notes that this does not appear to be the case at 1 µg/mL Iprodione. The report continues stating that when Iprodione treatment in vivo is considered, neither the basal concentration nor the hCG-stimulated release of testosterone was significantly affected by Iprodione pretreatment for 14 days at 3000 ppm {Tables B-7 [DER Table 8], B-8 [DER Table 10a,], and B-9 [DER Table 13] are referenced}. TB II notes that Table B-8 does not present any data regarding in vivo Iprodione.

Tables B-8 through B-13 of the report present mean ± SEM values for Area Under Curve [AUC], which the author states describes the sum of relative trapezoidal areas from 0 hour to 1 hour, plus 1 hour to 2 hour, etc. These data on testosterone release from testicular sections in vitro were used by the Registrant to answer "four simple questions":

1) Is there an effect of hCG on testosterone release into the media at the selected ED50 for hCG? Referring to Table B-8 [DER Table 10a], **YES** There was a statistically-significant difference [4-fold larger area represented by hCG-stimulated testosterone release compared to non-stimulated controls] over the 4-hour period in the AUC.

2) Does a 14-day exposure to Iprodione have any effect on the level of testosterone release into the media? Referring to Table B-9 [DER Table 13], **NO** the Registrant states that neither testosterone baseline secretion [without hCG] nor release [with hCG] was apparently affected by in vivo pretreatment with Iprodione. TB II notes that in the statistical analysis of the data, Hypothesis #2 concerns the effect of in vivo Iprodione on testosterone levels at time = 0 in the absence of in vitro Iprodione and hCG challenge. It was concluded in the statistical report that testosterone was significantly increased in the in vivo Iprodione group when compared to the controls. It is not clear to this reviewer whether Hypothesis #2 is addressing Question 2).

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[**IPRODIONE**] Mechanistic Study/Testosterone Secretion/Rats [§ none]

3) Is there an effect of Iprodione in vitro on testosterone secretion? Referring to Tables B-10 [DER Table 11a] and B-11 [DER Table 11b], **YES** In all four cases, baseline secretion [no hCG] and stimulated release [with hCG] are significantly reduced by the addition of Iprodione to the media. TB II points out that only the 10 µg/mL dose of in vitro Iprodione was referenced. There was no statement regarding whether a significant effect was observed at 1 µg/mL and 100 µg/mL DER Tables 12a-12d].

4) Is the in vitro effect of Iprodione dose related? Referring to Tables B-12 and B-13 [DER Table 14], **YES** Both baseline testosterone secretion [no hCG] and hCG-stimulated testosterone release were said to be significantly reduced in a dose-related manner. It was concluded that increasing concentrations of Iprodione in the media caused decreasing release of testosterone in both cases.

B. Reviewer's assessment: The objective of this study was to determine the effect of in vitro Iprodione exposure on basal testosterone secretion and stimulated release from testicular sections in culture media [in vitro Endocrine Challenge Test (ECT) using human chorionic gonadotropin (hCG)]. The effects of prior in vivo exposure of the male rats via the diet [3000 ppm for 14 days] was also evaluated. Testicular sections obtained from male CD® Sprague-Dawley rats administered Iprodione via the diet for 14 days at dose levels of 0 ppm or 3000 ppm were incubated with 0, 1, 10, or 100 µg/ml Iprodione for one hour. Half of these testicular sections from each in vitro treatment group were challenged with human chorionic gonadotrophin and the other half of the sections were monitored for basal testosterone secretion. Media testosterone concentrations were monitored at hourly intervals for 3 hours after challenge.

There was a dose-related reduction in testosterone secretion from testicular sections incubated in vitro with Iprodione, with and without hCG stimulation. Prior exposure of the rats to Iprodione in vivo for 14 days appeared to have little effect on the secretion of testosterone, with and without hCG stimulation, from testicular sections incubated in vitro other than a slight increase at t=0. Plasma testosterone was not significantly affected in the rats at sacrifice following the 14-day exposure period to Iprodione, but plasma LH concentrations were significantly increased compared to the control.

Additional information: 1) The in vivo dose of Iprodione used in this study [3000 ppm, ~175 mg/kg/day] is greater than the high dose used in the chronic toxicity/ carcinogenicity study in rats [1600 ppm, 69 mg/kg/day in males] in which the incidence of Leydig cell tumors was increased compared to the concurrent control. The Registrant indicated that the dose to the males during the first 2 weeks of the chronic toxicity/carcinogenicity study was 191 mg/kg/day. The CPRC considered the dose levels in the rat carcinogenicity study to be adequate; there was no statement regarding MTD, but it was not stated that the dose levels were considered excessive. Ketoconazole has been shown to be a testosterone biosynthesis inhibitor, but it has not been reported to induce Leydig cell tumors, although it has not been tested at an MTD.

2) Plasma testosterone and LH levels were not monitored in the rat

chronic toxicity/carcinogenicity study. In the 30-day gavage study cited above, peripheral plasma LH was significantly increased above control following 15 days of exposure to 600 mg Iprodione/kg/day. In the 30-day exposure group of that study, plasma testosterone was significantly decreased compared to the control in both the Iprodione and pair-fed groups [comparable decrease]. During the 10-hour sampling period, LH values were initially decreased in both the pair-fed and Iprodione groups compared to the control in the 30-day study; thereafter, increases in LH alternated with decreases compared to control values, with the total mean and maximum LH values being comparable to the control for both groups. With respect to the necropsy sample in the 30-day study, a non-significant increase in LH [130% of control] and a non-significant decrease in testosterone [64% of control] were observed in the Iprodione group, and a non-significant decrease in LH [80% of control] and a significant decrease [28% of control] in testosterone were observed in the pair-fed group. In the current study, no statistically-significant difference was reported in peripheral plasma testosterone [132% of control] following Iprodione exposure for 14 days, but the LH value was significantly increased [210% of control] at necropsy compared to the control. In the current study, a pair-fed group was not included.

3) LH levels were shown to be elevated after a 15-day [gavage] and a 14-day [dietary] exposure period but not after a 30-day [gavage] exposure period. In the subchronic and chronic rat studies, LH was not monitored.

4) Iprodione administered to pregnant rats via gavage on days 6-15 of gestation and 2 hours after the final dose on gestation day 15, plasma levels of Iprodione were reported to be 2.7 µg/mL.

5) Adrenal weight was significantly increased [127% of control] over control in a 30-day gavage study [MRID 43535002] at 600 mg Iprodione/kg/day and in the current study [127% of control]. Adrenal weight was not reported affected in the chronic toxicity/carcinogenicity rat study, although focal and generalized enlargement of cells of the zona glomerulosa were observed at the 300 ppm and 1600 ppm dose levels. In the 90-day rat study, females at the 3000 ppm dose level displayed decreased absolute and adjusted adrenal weight and the relative adrenal weight was comparable to the control, although there was a 30% deficit in body weight at the 3000 ppm dose level. Microscopically, enlargement of the cells of the zona glomerulosa were observed in both sexes at the 2000 and 3000 ppm dose levels, and fine vacuolation of the zona fasciculata was observed in males at these dose levels and in one female at 3000 ppm.

The data demonstrate that Iprodione reduces the secretion of testosterone from testicular sections incubated with Iprodione in vitro. Prior in vivo exposure to Iprodione via the diet [3000 ppm for 14 days] had no significant effect on either the basal secretion or stimulated release of testosterone in vitro. There are several questions not addressed in this study whose answers would be helpful to the interpretation the various results from the mechanistic studies that have been performed on Iprodione. These include (1) how do the levels of Iprodione utilized in the in vitro phase of this study relate to levels attained within the body [plasma, testes, etc.]

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following long-term in vivo exposure to Iprodione via the diet [rat chronic toxicity/carcinogenicity study (150, 300, 1600 ppm)]; (2) does a dose level of 1600 ppm in the rat diet result in increased LH levels in the plasma; (3) is there an increase in LH in the rat dosed via the diet at 300 ppm; (4) what is the in vitro dose of Iprodione where no reduction in testosterone secretion is observed; (5) is there a maximum level of reduction in testosterone secretion that does not result in an increase in LH; (6) if 1600 ppm in the diet results in increased LH levels in the plasma, when does the increase occur and is it sustained; (7) what effect does decreased body weight/gain/food consumption have on the various parameters in question over an extended period; (8) what does the Registrant consider indicative of an adverse sustained effect that ultimately results in tumor formation. The homeostatic mechanism involved is a normal physiological occurrence; the fact that homeostatic regulation may function throughout a lifespan does not necessarily indicate that it is detrimental or that it will result in an increased tumor incidence. This is supported by the consensus conclusions reached at a recent workshop on rodent Leydig cell adenomas and human relevance, where it was determined that the role of chronic LH stimulation of Leydig cells in Leydig cell tumorigenesis remains conjectural.

B. Study deficiencies TB II notes an apparent discrepancy in the interpretation of the data; i.e., on page 143 of the report [page 5 of the statistical analysis report], it states that the second hypothesis concerns the effect of in vivo Iprodione on testosterone levels at time = 0 in the absence of in vitro Iprodione and hCG challenge. The analyses indicate that testosterone was significantly increased in the 3000 ppm in vivo Iprodione group when compared to controls. However, on page 27 of the report, it states that when Iprodione treatment in vivo is considered, neither the basal concentrations nor the hCG-stimulated release of testosterone was significantly affected by Iprodione pretreatment for 14 days at 3000 ppm. And under question (2) on page 27, it states that neither testosterone baseline secretion [without hCG] nor release [with hCG] is apparently affected by in vivo pretreatment with Iprodione at 3000 ppm in feed. In question (4) on page 27 of the report [is in vitro effect of Iprodione dose related?], the author answers "yes", refers to Tables B-12 and B-13 of the report [reproduced below as Table], and states that both the baseline testosterone secretion [no hCG] and the hCG-stimulated testosterone release were significantly reduced in a dose-dependent manner; increasing concentrations of Iprodione in the media caused decreasing release of testosterone in both cases. TB II notes that this is not exactly true for the hCG-stimulated release, where the 100 µg/mL group is greater than/nearly comparable to the 10 µg/mL group; one must consider the fact that the 10 µg/mL level of Iprodione may be the level of maximum response and higher levels will not exert a greater response. The study author/Registrant have not performed a definitive analysis of all of the results in this study. There are alot of data produced in this study, but the report does not provide a rigorous assessment of the data available; i.e., the statistical analyses were not put into terms that relate to an interpretation of a possible mechanism of tumorigenesis.

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[**IPRODIONE**] Mechanistic Study/Testosterone Secretion/Rats OPPTS/§ none

EPA Reviewer: Linda L. Taylor, Ph.D. 2/4/97
Review Section II, Toxicology Branch II (7509C)
EPA Secondary Reviewer: K. Clark Swentzel 2/5/97
Review Section II, Toxicology Branch II (7509C)

DATA EVALUATION RECORD

STUDY TYPE: Mechanistic Study [rat testicular sections]; OPPTS/§ none

DP BARCODE: D232064 SUBMISSION CODE: S481268

P.C. CODE: 109801 TOX. CHEM. NO.: 470A

TEST MATERIAL (PURITY): Iprodione [97.3%]

CHEMICAL: 3-(3,5-dichlorophenyl)-N-isopropyl-2,4-dioxoimidazolidine-1-carboxamide

SYNONYMS: Iprodione, Glycophene, Promidione, RP 26019

CITATION: Fail, P.A., Anderson, S.A., and Pearce, S.W. (1996). Toxicity Testing of a Fungicide, Iprodione: Endocrine Toxicology Studies of Testes From Adult Male CD®Sprague-Dawley Rats Exposed to Iprodione In Vivo. Laboratory of Reproductive and Endocrine Toxicology/Center for Life Sciences and Toxicology/Chemistry and Life Sciences/Research Triangle Institute. RTI ID No. 65C-6169; Rt95-IPDA; RTI-527. November 26, 1996. MRID 44171904. Unpublished.

SPONSOR: Rhone-Poulenc Ag Company/Research Triangle Park, NC

EXECUTIVE SUMMARY: In an in vivo study [MRID 44171904], no changes in testicular function, as assessed by measuring testosterone levels in plasma and testicular homogenates from 15 male Sprague-Dawley rats administered Iprodione [97.3%] via the diet at doses levels of 0 ppm and 3000 ppm for 2, 7 or 14 days, were observed. Decreased body weight [95% of control after 2 days, 90-91% of control after 7 days, and 87% of control after 14 days], body-weight gain [negative gain after 2 days, 32% of control after 7 days, 44% of control after 14 days], and food consumption were observed following all exposure intervals. Organ-weight effects included decreased absolute liver, kidney, epididymis, and total accessory sex organs [TASO]; increased absolute and relative adrenal; and decreased relative TASO. The objective of this study was to assess the effects of in vivo Iprodione exposure on plasma and testicular homogenate testosterone concentrations in the male rat following a human chorionic gonadotrophin [hCG] Endocrine Challenge Test (ECT). There were no significant differences in either peripheral plasma or testicular homogenate testosterone levels observed in samples collected one hour after human chorionic gonadotrophin [hCG] challenge. Under the conditions of this study, Iprodione did not produce alterations in testicular function following dietary exposure at 3000 ppm for up to 14 days.

This study is classified Acceptable [non-guideline].

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided. No flagging statement was submitted.

[**IPRODIONE**] Mechanistic Study/Testosterone Secretion/Rats OPPTS/§ none

A. MATERIALS

1. Test Material: Iprodione
Chemical name: 3-(3,5-dichlorophenyl)-N-isopropyl-2,4-dioxoimidazolidine-1-carboxamide
Description: off-white powder consisting of large spherical granules
Lot #: 8906201;
Purity: 97.3%
Stability: stable [from Material Safety Data Sheet]
CAS #: 36734-19-7
Structural formula: $C_{13}H_{13}Cl_2N_3O_3$
[Structure]

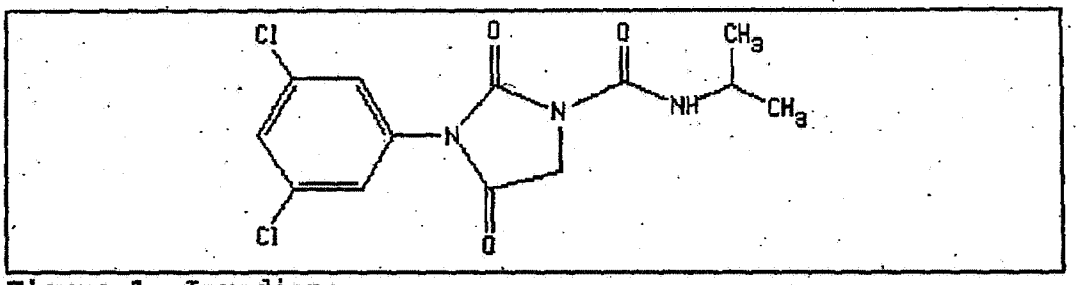

Figure 1 Iprodione

Formula: (1) $C_{13}H_{13}Cl_2N_3O_3$;
Source: Rhone-Poulenc [Research Triangle Park, NC]

2. Vehicle: #5002 Purina Certified Ground Rodent Chow; Batch #: not provided.

3. Test animals: Species: rat
Strain: virus antibody free [VAF/Plus®], Cesarean-derived (CD®), Sprague-Dawley (SD), barrier reared (BR), outbred albino [Crl:CD® (SD) BR]
Age on arrival: ~8 weeks old
Weight: 263.36-324.49 grams at randomization
Source: Charles River Laboratories, Inc. (Crl), Raleigh, NC.
Housing: individual cages
Diet: #5002 Purina Certified Ground Rodent Chow ad libitum
Water: deionized/filtered tap ad libitum
Environmental conditions: standard laboratory conditions, except that room was maintained on a 14:10 hour light:dark cycle
Acclimation period: 7 days

B. STUDY DESIGN and METHODS

1. In life dates - initiated: July 26, 1995; completed: August 9, 1995

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[**IPRODIONE**] Mechanistic Study/Testosterone Secretion/Rats OPPTS/§ none

2. Animal assignment: Fifteen adult virgin male rats were assigned to one of two experimental groups [either 0 and 3000 ppm Iprodione] by a randomization process and stratified by body weight; at study initiation, the body weights of both groups were homogeneous by statistical analysis.

3. Dose preparation and analysis - Iprodione was ground into a fine powder, and a known amount of this was added to a known amount of feed and mixed thoroughly. Test diets were analyzed for adequacy of mixing, stability under both freezer [-20°C] and study conditions [room temperature], and attained concentration. The test diets were prepared once at study initiation and stored under freezer conditions. Fresh diet was provided on days 1 and 7 of the 14-day feeding study.

RESULTS

The results of the storage stability study of diets containing 3000 ppm Iprodione indicate that the test diets were stable for a period of 125 days when stored under freezer conditions and for 9 days at room temperature [results shown on pages 118 and 119 of report (APPENDIX II)]. TB II notes that on page 120 of the report [APPENDIX II], it states that test diets can be stored for periods of time up to 35 days at freezer temperature [0 to -20°C]. The mixing procedure was shown to be adequate [page 111 of report, APPENDIX II]. The test diets were found to contain the appropriate amount of test material [106% of nominal concentration; results shown on page 135 of the report].

4. Preliminary experiment to determine hCG challenge parameters - This experiment was performed to establish an effective dose and time response regimen to detect a testosterone response in plasma and testicular homogenates. Fifteen groups of 6 untreated male rats [same supplier/strain/age of those used in definitive study] were utilized. Five dose levels of hCG [0, 0.2, 0.4, 0.8, and 8.0 IU/100 g body weight] were injected via the tail vein, and plasma and testicular samples were collected following sacrifice via rapid decapitation without anesthesia at 1, 3, and 6 hours post injection of hCG. Samples of the left testis were stored at ≈-20°C until homogenization and prior to homogenization were thawed, and the tunica albuginea was removed. The testis was homogenized on ice in a PBS buffer [0.01 M sodium phosphate plus 0.85% (w/v) sodium chloride, pH 7.4. Following homogenization, a sample of the testicular homogenate was diluted at 1:20 in a PBS-gel buffer [0.1 M sodium phosphate plus 0.85% (w/v) sodium chloride with 0.1% (w/v) sodium azide and 0.1% (w/v) gelatin, pH 7.4. The diluted homogenate was stored at ≈-20°C analysis for testosterone concentrations.

RESULTS

Testosterone concentrations were increased in all hCG dose groups in both the plasma and testicular homogenates compared to the controls at one or more time points [Table 1]. There was a dose-related increase in mean plasma testosterone concentration at 1 hour, and the increases

were statistically significant at doses of 0.4 IU/100 g BW and above. At all other time points, increases in mean plasma testicular concentrations were observed at all dose levels of hCG, but a dose-response was not observed. At 3 hours, all dose levels displayed a statistically significant increase in plasma testosterone concentration compared to the control. There was a dose-related increase in mean testosterone concentrations in testicular homogenates at each time point, with statistical significance being attained at all dose levels at 3 hours and at doses of 0.4 IU/100 g BW and above at 1 and 6 hours. Based on these data, the dose and sampling time for the definitive study were selected to be 0.8 /100 g BW and 1 hour, respectively.

Table 1. Preliminary Study to Define Dose and Time Response

hCG IU/100 g BW	Testosterone [ng/mL]				
	0	0.2	0.4	0.8	8.0
Plasma					
1 hour	3.23±1.12	4.12±0.93	9.72±2.76*	14.51±1.37*	24.68±2.15*
3 hour	1.57±0.44	8.22±1.64*	10.10±0.68*	15.27±2.47*	13.32±0.81*
6 hours	3.33±0.84	4.11±0.44	7.31±0.80*	12.01±1.04*	10.91±0.22*
Testicular Homogenate					
1 hour	14.83±4.07	26.61±8.36	48.01±8.66*	81.69±7.65*	113.82±3.56*
3 hour	8.13±3.59	36.92±7.72*	42.60±4.77*	65.19±8.64*	73.74±7.01*
6 hour	16.11±4.61	17.62±0.89	32.66±3.58*	77.96±12.14*	150.04±12.83*

* p<0.05; data from Table MA-1, page 19 of the report

5. Endocrine measurements - Hormone concentrations were determined in the plasma using radioimmunoassays [RIA] validated [using male CD rat plasma or homogenized testis, and cross-reactivity of Iprodione was checked at 28 µg/mL (maximum possible blood level in rats treated with 3000 ppm Iprodione)] for male rats at RTI. **Rat LH Mini RIA** - It was stated that the protocol incorrectly listed LH analysis, and the assay was performed but, due to cross reactivity of hCG in the LH assay, the results were not considered scientifically valid and are not reported. **Testosterone RIA** - A no-extraction, solid-phase ^{125}I-RIA was used, which utilized testosterone-specific, antibody-coated tubes and ^{125}I-testosterone [details from pages 22-23 of the report]. The standard curve was prepared in a PBS-Gel buffer [0.1 M sodium phosphate + 0.85% (w/v) sodium chloride with 0.1% (w/v) sodium azide and 0.1% (w/v) gelatin, pH 7.4]. Testosterone controls were prepared in the same matrix as unknown samples by adding known concentrations of testosterone to plasma or homogenized testis, as appropriate. For the RIA procedure, the sample [plasma or homogenized testis] was pipetted directly to the bottom of the tube, ^{125}I-testosterone was added, and the tubes were vortexed and then incubated in a ≈37°C water bath for 3 hours. After incubation, the supernatant was aspirated, and the tubes were counted in a gamma counter [ICN 10/600 Plus].

Assay characteristics are presented in Table 2. **Testosterone:** Testosterone was more readily recovered from the plasma than from the

testicular homogenate [≈90% vs ≈70%; respectively]. Possible interference with recovery from the testicular homogenate was thought to include the presence of more androgen-binding components or metabolism in testicular tissues. Intra-assay variations for blank and spiked samples were ≈12% or less for plasma and ≈6% or less for testicular homogenates. From the control values, the intra- and inter-assay coefficient of variation, % recovery, and the index of parallelism for the assays were determined. The sensitivity of this assay was 3.5 pg.

Table 2. Characteristics of RIAs Validated for Determination of Testosterone from Male Rats

Parameter	Hormone Assay	
	plasma testosterone	testicular homogenate
sensitivity [pg]	3.5	3.5
intra-assay variation♪ blank mass added	 0/8.7% 2/11.8% 8/5.2%	 0/7.6% 12.5/5.5% 25/4.4% 50/4.2%
interassay variation♪ # of assays blank mass added	 1 NA NA	 1 NA NA
% recovery of added mass♫	2/92.5% 8/94.3%	12.5/60.7% 25/68.8% 50/88.5%
index of parallelism (%)♦	127.1%	112.6%
cross reactivity of Iprodione (%)	0%	NT

♪ #s are mass added [ng/mL]/% variation [for intra-assay variation, # of samples assayed was 10 in each case; NT = not tested; NA = not applicable;
♫ #s are mass added [ng/mL]/% recovered; ♦ concentration of low volume ÷ concentration of high volume x 100; data from Table MA-3, page 23, of the report

6. Definitive study design -**In Vivo Phase** Two groups of 15 male rats each were administered Iprodione via the diet at dose levels of 0 ppm and 3000 ppm for 2, 7, or 14 consecutive days, during which time daily clinical signs, body weight [days 1 and 3, 1 and 7, and 1, 7, and 14, respectively], feed and water consumption [same time points as above], and mortality were monitored. The rats were tested using an in vivo hCG endocrine challenge test [ECT] for testicular responsiveness on the day of sacrifice [day 3, 8, or 15]. The ED_{50} of hCG was administered intravenously via a tail vein, and the rats were sacrificed one hour later via rapid decapitation, and trunk blood was collected for the determination of plasma testosterone. A complete necropsy was performed on each rat, and the liver, paired adrenals, paired kidneys, each epididymis, each testis, and total accessory sex organs [TASOs] weighed as a unit with fluids [seminal vesicles, prostate, coagulating glands; bladder removed] were weighed. The left

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and right testicles were removed, trimmed of fat, and the left testis was frozen until analysis for testicular testosterone concentration.

7. Statistics: Parametric statistical procedures were applied to parametric data. Appropriate General Linear Models [GLM] procedures (SAS Institute Inc.) for the Analyses of Variance [ANOVA] or Student's T-test were employed using SAS/STAT® software [SAS® Version 6.08]. Prior to GLM analysis, which was used to determine whether significant dose effects had occurred for selected measures, a $\log_{10}$ transformation was performed on all testosterone data [Snedecor and Cochran, 1967] to allow use of parametric methods. GLM analysis was used to determine whether significant dose effects had occurred for selected measures [ANOVA]. An F' [folded] statistic was used to test for equality of variances, and univariate procedure was used to test for normality of distribution. A two-tailed test was used for all pairwise comparisons, body and organ weight parameters, and feed consumption. The null hypothesis, that the relationship between testosterone in the testis and testosterone in the blood does not vary by dose group or length of exposure, was tested via a series of linear regression models in SAS GLM. Analysis of the preliminary study utilized GLM procedures for the ANOVA and Dunnett's test for pairwise comparisons of treatment groups back to the control.

II. **RESULTS**

A. In Vivo Phase

1. Survival/observations/body weight/food consumption - All rats survived until study termination, and there were no apparent treatment-related signs. The Iprodione rats displayed decreased body weights by days 3, 7, and 7 [95%, 91%, and 90% of control values, respectively] of the 2-day, 7-day, and 14-day exposure studies. A decrease in overall body-weight change was observed in each Iprodione exposure group compared to the control groups [Table 3]. The Iprodione rats also ate less food than the controls [Table 3]. Water consumption was comparable between the control and treated rats in the 2- and 7-day exposure groups but slightly lower [94% of control] in the Iprodione group compared to the control value throughout the study. On a mg/kg/day basis, the amount of Iprodione ingested was (1) 153.04 by the 2-day exposure rats, (2) 172.37 by the 7-day exposure rats, and (3) 186.16 [week 1]/204.00 [week 2] by the 14-day exposure rats.

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[IPRODIONE] Mechanistic Study/Testosterone Secretion/Rats OPPTS/§ none

Table 3. Body-Weight and Food-Consumption Data

Exposure Days	2		7		14	
Parameter/Group	0 ppm	3000 ppm	0 ppm	3000 ppm	0 ppm	3000 ppm
Body Weight [g]/Day						
1	316.3	316.5	317.0	314.9	316.5	315.6
3	332.8	315.2*[95]♦	-	-	-	-
7	-	-	364.8	330.5*[91]	362.9	326.9*[90]
14	-	-	-	-	411.7	357.1*[87]
Body-Weight Change [g]	16.6	-1.2*	47.8	15.5*	95.2	41.5*
Food Consumption [g/day]	24.9	16.1 [65]	26.3	18.6 [71]	26.3(wk. 1) 27.4(wk. 2)	19.9*[76](wk. 1) 23.3*[85](wk. 2)

- not measured; * p<0.05; ♦ [% of control]; data from Table A-1, page 33-34 of the report

2. Organ weights - Compared to the control rats, the Iprodione rats displayed decreased liver [all exposure groups], kidney [7- and 14-day exposures], TASO [7- and 14-day exposures], and right/left/total epididymides weights [7- and 14-day exposures] and increased adrenal weights [3- and 14-day exposures]. The relative adrenal weights were significantly increased in the 2-day and 14-day exposure rats compared to their respective control values, and the relative testes weights were significantly increased in the 7- and 14-day groups. The relative TASO weight was decreased in the Iprodione rats exposed for 7 and 14 days compared to their respective controls [Table 4].

Table 4. Organ-Weight Data

Exposure Duration	2 days		7 days		14 days	
Organ/Group	0 ppm	3000 ppm	0 ppm	3000 ppm	0 ppm	3000 ppm
liver [g]	13.5	12.4* [92]♦	15.3	13.3* [87]	16.0	14.7* [92]
kidneys [g]	2.52	2.52	2.72	2.45* [90]	2.83	2.58* [91]
adrenals [mg]	54.6	65.6* [120]	63.8	62.7	54.5	69.1* [127]
right testis [g]	1.48	1.43	1.55	1.52	1.54	1.54
left testis [g]	1.44	1.42	1.57	1.52	1.54	1.54
total testes [g]	2.92	2.84	3.11	3.04	3.08	3.09
right epididymis [mg]	358.7	360.0	425.3	355.3* [84]	450.8	411.7* [91]
left epididymis [mg]	330.4	359.0	429.7	352.2* [82]	441.8	403.6* [91]
total epididymes [mg	689.0	719.6	856.6	707.5* [83]	892.6	815.4* [91]
TASO [g]	1.49	1.48	1.81	1.28* [71]	2.11	1.54* [73]
relative♦						
liver [g]	40.63	39.24	41.99	40.16	38.88	41.18* [106]
kidneys [g]	7.58	7.99	7.45	7.41	6.88	7.25
adrenals [mg]	0.16	0.21* [131]	0.18	0.19	0.13	0.19* [146]
right testis [g]	4.44	4.53	4.24	4.59* [108]	3.75	4.33* [115]
left testis [g]	4.34	4.51	4.29	4.62 [108]	3.73	4.33* [116]
total testes [g]	8.78	9.04	8.53	9.21* [108]	7.48	8.67* [116]
right epididymis [mg]	1.08	1.15	1.17	1.08 [92]	1.10	1.16 [105]
left epididymis [mg]	0.99	1.14	1.18♪	1.07* [91]	1.08	1.13 [105]
total epididymes [mg	2.07	2.29	2.34♪	2.15* [92]	2.17	2.29 [106]
TASO [g]	4.47	4.70	4.97	3.88* [78]	5.14	4.31* [84]
Body Weight [g]	332.9	315.2* [95]	364.8	330.5* [91]	411.7	357.1* [87]

* p<0.05; ♦ [% of control]; ♦ mean organ weight [mg]/g body weight; ♪ n=14
data from Table A-3 & A-4, pages 35-37 of the report

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homogenates collected one hour after hCG challenge following any of the exposure scenarios. The release of testosterone into blood and total testicular tissues was similar among the exposure times and between control and Iprodione-treated rats [Table 5].

Table 5. Plasma and Testicular Testosterone Values

Sample/Exposure Length/Dose	0 ppm	3000 ppm
2 Days		
plasma testosterone [ng/mL]	19.71±1.22	21.57±2.06
testicular testosterone [ng/g testis]	814.64±44.32	844.68±63.93
testicular testosterone [ng/mL]♪	116.33±22.16	120.09±36.06
7 Days		
plasma testosterone [ng/mL]	19.53±1.07	21.02±1.89
testicular testosterone [ng/g testis]	867.58±28.89	928.55±70.75
testicular testosterone [ng/mL]♪	135.51±20.54	142.40±45.29
14 Days		
plasma testosterone [ng/mL]	17.72±0.96	18.04±2.48
testicular testosterone [ng/g testis]	938.30±45.64	908.82±108.56
testicular testosterone [ng/mL]♪	143.23±22.74	140.58±66.73

♪ calculated by reviewer using data from Table AA-16 [pages 62-64]; data from Table A-5, page 38 of the report

III. DISCUSSION

A. The objective of this study was to assess the effects of <u>in vivo</u> Iprodione exposure for 2, 7, and 14 days on plasma and testicular homogenate testosterone concentrations in the male rat following a human chorionic gonadotrophin [hCG] Endocrine Challenge Test (ECT). Also reported were the results of the preliminary studies performed to (1) validate the testosterone radioimmunoassay [RIA] for use with testicular homogenates and (2) establish an effective dose of hCG and appropriate collection times for detecting a testosterone response in plasma and testicular homogenates following an injection of hCG into a tail vein.

No changes in testicular function, as assessed by measuring testosterone levels in plasma and testicular homogenates from male Sprague-Dawley rats following oral administration <u>via</u> the diet at doses levels of 0 ppm and 3000 ppm Iprodione for 2, 7 or 14 days, were observed. As in another mechanistic study on Iprodione of similar duration and at the same 3000 ppm dose level [MRID 44171903], decreased body weight, body-weight gain, and food consumption were observed following Iprodione exposure for 2, 7, and 14 days <u>via</u> the diet. Similar organ-weight effects [↓ absolute liver, kidney, epididymis, and TASO; ↑ absolute and relative adrenal; ↓ relative TASO] were observed in the current study as were observed in the other study also. There were no significant differences in either peripheral plasma or testicular homogenate testosterone levels observed in samples collected one hour after hCG challenge. In the other study cited above, plasma testosterone levels were not found to be statistically significantly different from the control values, although an increase was observed [1.16±0.16 <u>vs</u> 1.53±0.38 (132% of control)]. In a 30-day gavage study [MRID 43535002], increased plasma testosterone levels were observed in rats administered 300 mg/kg/day

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[IPRODIONE] Mechanistic Study/Testosterone Secretion/Rats OPPTS/§ none

[not statistically significant] but not in those administered 120 or 600 mg/kg/day. In the 15-day exposure phase of that study, decreased plasma testosterone levels were observed in both the Iprodione [600 mg/kg/day] and pair-fed control rats compared to the vehicle control rats. In in vitro studies using rat testicular sections, Iprodione was shown to decrease testosterone secretion, which differs with the findings of the current study, suggesting that homeostatic mechanisms were utilized to compensate for effects induced by Iprodione on testosterone secretion from Leydig cells.

B. Study deficiencies None that would affect interpretation of the study.

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