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

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OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

TO: Larry Schnaubelt, Product Manager #12
Insecticide-Rodenticide Branch
Registration Division (TS-767)

FROM: Roger Gardner, Toxicologist
Toxicology Branch
Hazard Evaluation Division (TS-769) Roger Gardner 9-24-87

THRU: Judith Hauswirth, Ph. D., Acting Head
Review Section 6
Toxicology Branch
Hazard Evaluation Division (TS-769) Judith W. Hauswirth 8/24/87 for WBS 8/26/87

SUBJECT: Experimental Use Permit (EPA Reg. No. 45639-EUP-GG) and Temporary Tolerances for residues of APOLLO® on Pears, Almonds, Peaches and Nectarines Petition Nos. 7G3465. Tox. Chem. No. 593A; Tox. Project Nos. 1705, 1706, 1707, and 1708.

and

Permanent Tolerances for residues of APOLLO® on fresh apples at 1 ppm (Petition No. 6F3392) and in cattle (fat, meat and meat by products) and milk at 0.01 ppm, kidney at 0.05 ppm, and liver at 0.1 ppm (Petition No. 6H5500). Tox. Project No. 7-0211.

Actions Requested

1. Experimental Use Permit for a 50 SC formulation (45639-EUP-GG) on pears, almonds, peaches, and nectarines.

2. Temporary tolerances on pears (0.25 ppm), almonds (0.02 ppm), peaches (1.0 ppm), and nectarines (1.0 ppm).

3. Extension of the following temporary tolerances previously approved under Petition No. 3H5412: meat and meat by products of cattle (0.01 ppm), liver (0.1 ppm), kidney (0.05 ppm), and milk (0.01 ppm).

4. Permanent Tolerances for residues of APOLLO® on fresh apples at 1 ppm (Petition No. 6F3392) and in cattle (fat, meat and meat by products) and milk at 0.01 ppm, kidney at 0.05 ppm, and liver at 0.1 ppm (Petition No. 6H5500).

5. Review of studies listed in Appendix II below.

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Recommendations and Conclusions

1a. Acute oral and dermal toxicity studies suggest that technical grade clofentezine should be classified into Toxicity Category III. An acute inhalation toxicity study with an 80 WP formulation suggests that the technical grade material should also be classified into Toxicity Category III for acute inhalation. Results of a primary dermal irritation study indicated that technical grade clofentezine should be classified into Toxicity Category IV, and an eye irritation study on the 80 WP formulation suggests that technical clofentezine should be classified into Category IV with respect to eye irritation. The technical material is also a skin sensitizer. See Sections I. B. 1. and II. A. 1., and Appendix I below for additional information.

1b. Acute oral and dermal toxicity studies on the 50 SC formulation indicate that it should be classified into Toxicity Category IV. Studies with the formulation indicate that it should be placed in Category IV for skin and eye irritation. See Sections I. B. 1. and II. A., and Appendix I below for further information.

2. The lowest no-observed-effect level (NOEL) was established at 50 ppm (1.25 mg/kg/day) in a one-year dog feeding study. Observed effects included elevated serum cholesterol and triglyceride levels in animals given diets containing 1000 or 20,000 ppm. The LEL was established at 1000 ppm (25 mg/kg/day). See Section I. B. 2. and Appendix I below.

3. A chronic feeding study in rats indicated that the NOEL is 40 ppm (2 mg/kg/day), and the LEL is 400 ppm (20 mg/kg/day; highest dose tested). The effects included increased liver weights and liver-to-body weight ratios, increased incidence of centrilobular hepatocyte hypertrophy, and elevated free thyroxine levels in 400 ppm dose group males. These results are consistent with subchronic study results (see Section I. B.). Section II. A. 2. a., and Appendix II contain additional information.

4. The incidence of thyroid follicular cell tumors was increased in male rats given clofentezine in the diet at a level of 400 ppm for 27 months. The only tumors that were apparently increased by clofentezine in the diet of mice were liver cell adenomas and carcinoma in females. These results have been referred to the Toxicology Branch Peer Review Committee for a determination of clofentezine's oncogenic potential to humans. Additional information is found in Section II. A. 2. and Appendix II.

5a. Maternal toxicity (slight centrilobular hepatocellular enlargement) was observed in pregnant rats given 3200 mg/kg/day during gestation. The NOEL for that effect was 1280 mg/kg/day. No teratogenic or fetotoxic effects were reported at any of the doses tested, and the NOEL was reported to be the highest dose tested (3200 mg/kg/day). The adult-to developmental toxicity ratio (A:D ratio) in rats is less than one (Section I. B. 4.).

5b. In rabbits, slight maternal toxicity (reduced food consumption and body weight gain) as well as slight fetotoxicity (reduced litter and fetal weights) were observed at the 3000 mg/kg/day dose level (highest dose tested). The NOEL for these effects was 1000 mg/kg/day. No compound related increases in developmental effects were observed in the study. The A:D ratio suggested by these results is one (Section I. B. 4.).

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6. Results of a multigeneration reproduction study suggest that APOLLO® has no reproductive toxicity in rats at dietary levels of 4, 40, or 400 ppm See Section I. A. 5., and Appendix II for additional information on teratology and reproductive toxicity studies.

7a. Apollo did not induce point mutations in bacteria (Salmonella typhimurium). There were no dominant lethal mutations induced by the chemical in rats, and no induction of micronuclei was observed in treated mice. There were also no gene conversion and mitotic recombination effects in yeast exposed to Apollo. See Section I. B. 6. below for details.

7b. No point mutations were induced in mammalian cells in vitro (mouse lymphoma cells), but the study did not contain specific historical control data to completely support the conclusions of the investigators. See Section II. A. 3. and Appendix II. below.

8a. Treated rats readily absorbed single oral doses of clofentezine with maximum accumulation of residues in the liver and kidney. Those residues were subsequently cleared within 24 hours after treatment.
Almost all of a single oral dose is excreted within 24 to 48 hours after administration regardless of its amount, and the major route of excretion is the feces. Metabolites identified in the urine of treated rats and baboons were free and conjugated hydroxylated derivatives of clofentezine. No fecal metabolites have been identified (see Section I. A. 7.).

8b. Repeated dosing of rats demonstrated that tissue levels of clofentezine residues reach a plateau after 5 to 15 daily doses. Residue concentrations in the liver and kidneys were 2 to 4 times those reported after a single dose. One study also indicated that the chemical does not readily pass across the placenta in pregnant rats (see Section I. A. 7.).

9. A dermal absorption study showed that absorption was low (less than 1%) during the 10 hours that followed application of a simulated 50 SC formulation to the skin of rats. Section II. A. 5. and Appendix II. contain more information.

10. Data gaps include an acute inhalation study or data to indicate that the formulation is not in a respirable form under conditions of normal application, identification of fecal metabolites of clofentezine, data on historical control cultures from the laboratory where the mouse lymphoma mutation assay was conducted, and an assay to assess clofentezine's potential to cause DNA damage (see Section II. B.).

11. Using the NOEL established in the one-year dog study and a Safety Factor of 100, an Acceptible Daily Intake (ADI) of 0.0125 mg/kg/day is derived (see Section II. C. below).

12. The results mentioned in point 4. will be considered by the Toxicology Branch's Peer Review Committee in order to classify clofentezine's oncogenic potential. Recommendations on the proposed tolerances on apples, pears, almonds, peaches and nectarines and the requested extension of temporary tolerances on meat and meat by products of cattle, liver, kidney, and milk can not be made until the peer review is completed. Relevant information has been provided to the Committee for consideration.

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I. Background

A. General Information

APOLLO® is proposed for use as a miticied on apples, pears, almonds, peaches, and nectarines. Its common name is clofentezine, and its chemical name is 3,6-bis[2-chlorophenyl]-1,2,4,5-tetrazine. The pesticide is formulated for the proposed uses as a suspension concentrate (50% active ingredient). The inert ingredients have been cleared for food use (Memorandum dated June 18, 1984. From: R. Gardner. Subject:Experimental Use Permits (EPA Reg. Nos. 45639-EUP-T and 45639-EUP-RU) and Temporary Tolerances for Residues of NC 21314 in/on Fresh Apples (1 ppm), Apple Pomace (20 ppm), Cattle (fat, meat and meat by products; 0.01 ppm), Liver (0.1 ppm), Kidney (0.05 ppm), and Milk (0.01 ppm). Petition Nos. 2G2719 and 3H5412. Tox. Chem. No. 593A. To: J. Ellenberger, Registration Division.)

The formulation, which is called APOLLO MITICIDE (50% SC), is to be applied at the rate of 4 to 8 oz./A according to the label, and the seasonal application rate is not to exceed one pint per acre. The miticide is to be sprayed with ground equipment.

B. Summary of Previously Submitted Data

Appendix I includes the Toxicology Branch "One-Liners" for the studies described in this Section.

1. Acute Toxicity

The acute LD_{50} values for technical grade and 50 SC formulations of chlofentezine are summarized as follows:

	Toxicity Categories	
Type of Toxicity	Technical grade	APOLLO® 50 SC
Acute oral	III	IV
Acute dermal	III	IV
Acute inhalation	--*	--**
Primary skin irritation	--**	--**
Primary eye irritation	--†	--**

*Based on a study with an 80% WP formulation (see Appendix I below).

**These studies are discussed in Section II. A. on page 7 below. Previously, data on the 80 WP formulation were used and suggested a Toxicity Category of IV for primary eye and skin irritation and Category III for acute inhalation toxicity.

†These data are unavailable on the technical grade material. However, a study on an 80 WP formulation (80% a. i.) suggested a Toxicity Category of IV.

The most frequently observed compound-related effect reported in all but the dermal studies was the discoloration of feces in treated animals. The test substance was described as a magenta colored powder, and the feces of treated animals

appeared pink in color for 25 to 30 hours after treatment (84 hours after i. p. injection). At necropsy in an intraperitoneal study, precipitate of the test material was observed in the abdomen of treated rats.

2. Subchronic Toxicity

In the 90-day rat feeding study a no-observed-effect level (NOEL) of 40 ppm (2 mg/kg/day) was demonstrated, and the lowest-effect-level (LEL) was reported to be 400 ppm (20 mg/kg/day; highest dose tested). Effects noted at that dose level were centrilobular hepatocellular enlargement, elavated mixed-function oxidase enzyme activities and a slight increase in liver weights.

In a 90-day dog study, a NOEL was not established with respect to increased liver weights or changes in electrocardiograms. The lowest dose tested was 3200 ppm (80 mg/kg/day).

Discoloration of the urine and feces by clofentezne was also noted in treated animals in these subchronic studies.

3. Chronic Toxicity and Oncogenicity

An interim report described observations of animals that died or were sacrificed during the first 18 months of a long-term rat feeding study. Results of the final report are discussed in Section II. A. (page 7).

In a one-year dog feeding study using levels of 0, 50, 1000, or 20,000 ppm, a NOEL with respect to elevated serum cholesterol and triglyceride levels was established at 50 ppm (reported daily intake ranged from 1.52 to 2.47 mg/kg/day). Electrocardiograms were not affected in dogs given the 20,000, 1000, or 50 ppm diets. Based on these results a NOEL of 50 ppm (1.25 mg/kg/day) and an LEL of 1000 ppm (25 mg/kg/day) is suggested for dogs.

4. Developmental Toxicity

Maternal toxicity (slight centrilobular hepatocellular enlargement) was observed in pregnant rats given 3200 mg/kg/day (highest dose tested) during gestation. The NOEL for that effect was 1280 mg/kg/day. No teratogenic or fetotoxic effects were reported at any of the doses tested, and the NOEL was reported to be the highest dose tested (3200 mg/kg/day).

In a study with rabbits, slight maternal toxicity (reduced food consumption and body weight gain) as well as slight fetotoxicity (reduced litter and fetal weights) were observed at the 3000 mg/kg/day dose level (highest dose tested). The NOEL for these effects was 1000 mg/kg/day. No compound-related increases in teratogenic effects were observed in the study.

Treated animals in these studies again to exhibited discoloration of feces as mentioned above.

5. Reproduction Toxicity

There were no reproductive effects observed in rats given diets containing 0, 4, 40, or 400 ppm clofentezine. The 400 ppm diet caused centrilobular hepato-

cyte hypertrophy in the male rats in the study, and the NOEL with respect to that effect was 40 ppm.

6. Mutagenicity

No mutagenic effects were reported in bacterial point mutation assays, gene conversion and mitotic recombination studies in yeast, a mouse micronucleus test, or a dominant lethal study in rats.

7. Metabolism

Studies with rats, mice, dogs, rabbits, and baboons demonstrated that the feces was the major route of excretion (70-80% of the administered dose in all species).

Almost all of the administered dose was recovered within 24 to 48 hours after administration, and in rats the proportion of fecal excretion at high doses (1000 mg/kg) increased to 95-98% of the dose.

The metabolites identified in the urine of treated rats and baboons were free and conjugated hydroxylated derivatives which accounted for approximately 70-80% of the radiolabel extracted. The feces were not analyzed for identification of residues.

In the rat, intravenous administration resulted in approximately the same proportions of excreted residues in the feces and urine as were noted after oral dosing suggesting excretion in the bile as the major route. Whole body radiography of treated rats also demonstrated the accumulation of residues in the liver and kidney which were subsequently cleared within 24 hours after treatment.

Repeated dosing of rats at 20 mg/kg/day demonstrated that tissue levels of clofentezine residues reached a plateau after 5 to 15 daily doses. Residue concentrations in those two organs were 2 to 4 times those reported after a single dose.

The fetuses of pregnant rats treated with 3200 mg/kg/day doses of clofentezine during gestation had residue concentrations which were only one-fifth that of maternal blood or tissues which indicates that the chemical does not readily pass across the placenta.

II. Discussion

A. New Toxicology Data

1. Acute Studies

An acute oral LD_{50} >3230 mg/kg (highest dose tested) was established for technical grade clofentezine in male and female rats. These results indicated that the technical grade should be classiffied into Toxicity Category III.

The investigators noted that the aerosol jets used in the acute inhalation study of Apollo® 50 SC were clogged by the viscous test substance shortly after generation of test atmospheres was begun. Changes in the rate of air flow or delivery of the test material to the atomizer did not overcome the problem, and

the authors concluded that a test atmosphere could not be sustained for a 4-hour exposure period. The label for Apollo 50 SC states that the formulation may be added to water and sprayed on the crop to be treated. This suggests that the test substance may require dilution with water before an aerosol is generated for an acute inhalation test. The authors noted that exposure is unlikely during mixing of the test substance, but no statement is made regarding exposure to spray during application. On that basis, the report does not clearly demonstrate that a test atmosphere is impractical for evaluation of potential acute toxicity resulting from inhalation exposure.

An eye irritation study indicated that APOLLO® 50 SC is practically non-irritating to the eyes of rabbits. The formulation should be classified into Toxicity Category IV for eye irritation.

Results of skin irritation studies with technical grade clofentezine and the 50 SC formulation indicated that they should be classified into Toxicity Categories IV and III, respectively.

Clofentezine caused dermal sensitization in two of twenty treated female guinea pigs which suggested that the chemical is a weak sensitizer.

2. Long-Term Feeding Studies

a. Rats

Clofentezine was administered in the diet at levels of 0, 10, 40, or 400 ppm to male and female Charles River Crl:CD Sprague-Dawley BR strain rats for up to 27 months.

Toxicity: There was no effect of treatment on survival or body weight in the study. Liver weights and weight ratios for males and liver-to-body weight ratios for females given the 400 ppm diet were slightly elevated above that for control group animals. Those observations for the control and high dose groups are summarized as follows:

	Dose level (ppm)			
	Males		Females	
Observation	0	400	0	400
Body weight (g)	563	589	415	430
Liver weight (g)	13.74	17.05†	10.12	11.37*
Liver/body weight ratio (%)	2.44	2.90 **	2.45	2.66**

*Statistically significantly different from controls ($p<0.05$)
**Statistically significantly different from controls ($p<0.01$)
†Statistically significantly different from controls ($p<0.001$)

Dose-related incidences of histological changes occurred in the liver of male rats (see Table 1 below).

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Table 1

Summary of selected non-neoplastic lesions exhibiting statistically significant differences between groups or a dose-related trend in the liver of male rats.

Observation	Dose level (ppm) 0	10	40	400
Centrilobular hepatocyte enlargement				
Interim sacrifice	0/20	0/20	0/20	18/20*
Intercurrent deaths	1/26	0/26	0/23	3/29
Terminal sacrifice	0/24	0/24	0/27	10/21*
Overall	1/70	0/70	0/70	31/70
Centrilobular hepatocyte vacuolation				
Interim sacrifice†	3/20	6/20	1/20	10/20**
Intercurrent deaths	0/26	2/26	1/23	0/29
Terminal sacrifice	0/24	0/24	0/27	1/21
Overall†	3/70	8/70	2/70	11/70**

*Statistically significantly different form controls ($p<0.001$).
**Statistically significantly different form controls ($p<0.001$).
†Statistically significant positive dose-related trend ($p<0.01$)/

The 400 ppm diet was associated with a slight increase in free thyroxine levels in blood taken from males after 27 months. The group mean for control male rats was 13.4 pmol/l while that for the 400 ppm dose group was 19.7 pmol/l ($P<0.05$; t test). The incidence of follicular cell hyperplasia is summarized as follows:

Observation	Dose level (ppm) 0	10	40	400
Follicular cell hyperplasia				
Interim sacrifice	0/20	0/20	2/20	1/20
Intercurrent deaths	1/26	0/26	1/23	1/29
Terminal sacrifice	1/24	2/24	5/27	3/21

Based on the liver and thyroid effects, a no-effect level was indicated in the experiment at 40 ppm (2 mg/kg/day), and the lowest-effect level was 400 ppm (20 mg/kg/day).

Oncogenicity: According to the investigators, the incidence of thyroid follicular cell tumors in male rats exhibited a statistically significant positive dose-related trend primarily because of the highest dose group's slightly higher incidence in comparison to that in the control group.

The incidence of benign and/or malignant follicular cell tumors in male rats is summarized as follows:

Observation	Dose level (ppm) 0	10	40	400
Type of tumor				
Benign	1/70	1/70	0/70	3/70
Malignant	1/70	1/70	2/70	5/70
Total*	2/70	2/70	2/70	8/70

*Statistically significant positive dose-related trend ($p<0.05$).

The first follicular cell tumor was observed in a control group male that died during week 88 of the study. The other thyroid tumor in that group was found during week 114. The only other follicular cell tumor observed during the study was in one male of the mid dose group at week 114. The remainder of the follicular cell tumors were observed in rats sacrificed at the end of the study, including all eight tumors observed in the high dose group.

If reported incidences are adjusted by censoring those animals sacrificed at 12 months and those dying before the first diagnosis was made in the control group (week 88), the incidences would be 2 of 47, 2 of 41, 2 of 40, and 8 of 40 for the control, low, mid, and high dose groups, respectively. A Fisher's Exact test on the control and high dose group incidences shows that the difference between the groups is statistically significant ($p = 0.024$). The adjusted results also show a significant positive trend ($p < 0.005$; Cochran-Armitage test).

b. Mice

Clofentezine was administered in the diet to male and female Charles River CD-1 Swiss mice for up to 105 weeks at levels of 0, 50, 500, or 5000 ppm.

Toxicity: Treatment was associated with statistically significant reductions in group mean body weight (approximately 7% below control means) and body weight gains (approximately 15 to 22% less than that for control group) for males at 5000 ppm. These differences were observed during the first three months of the feeding period.

An increase in mortality was observed in the high dose group females when compared to the control group for the last six months of the study. These results are summarized with results for male mice as follows:

Dose (ppm)	Mortalities during weeks: Males 0-52	52-78	78-104	Termination	Females 0-52	52-78	78-104*	Termination
0	4	13	22	13	6	9	12	25
50	7	9	19	17	1	8	15	28
500	7	8	21	16	1	6	18	27
5000	6	12	22	11	7	13	22	10

*Statistically significant trend (dose-related decrease in survival) by the Cox and generalized K/W tests (according to a draft analysis provided by the Toxicology Branch Biostatistics Team).

The highest dose also increased the incidence of altered hepatocytes in male mice (areas or foci of eosinophilic hepatocytes). In female mice given the 5000 ppm diet for up to two years, there were small increases in liver weights, altered hepatocytes (eosinophilic and/or basophilic areas or foci), and increased mortality during the last six months of the study. Those deaths were attributed to amyloidosis.

Oncogenicity - male mice: The investigators noted that liver cell tumors were a contributing factor to deaths of some treated male mice during the study. However, the incidence of those tumors was not dose-related.

Based on an independent review of the individual animal reports for male mice in the study, the incidence of liver tumors (malignant and benign combined) can be summarized as follows:

Dose (ppm)	No. with tumor/no. examined 1-52	53-106†	Termination†	Total†
0	1/4	10/35	8/13	19/52
50	0/6	12/29	8/17	20/52
500	1/7	19/29**	12/16	33/52*
5000	0/6	17/35*	8/11	25/52

*Statistically significantly different from controls ($p<0.05$, Fisher's Exact test).
**Statistically significantly different from controls ($p<0.01$, Fisher's Exact test).
†No significant trend (Cochran Armitage test).

Diagnoses of the first liver tumor in the control, low, mid, and high dose groups of males were made during the 48th, 72nd, 43rd, and 67th weeks, respectively. However, most of the tumors in each test group were diagnosed in the last 6 months or at terminal sacrifice. When animals dying before the week of the first diagnosis are eliminated from consideration, the incidence of liver tumors in male mice can be summarized as follows:

Dose (ppm)	No. with tumor/no. examined 43-74	75-106	Termination	Total
0	4/13	7/24†	8/13	19/50
50	1/8	11/21	8/17	20/46
500	3/9	17/23**	13/16	33/48**
5000	1/11	16/26*	8/11	25/48

*Statistically significantly different from control ($p < 0.05$ by Fisher's Exact test).
*Statistically significantly different from control ($p < 0.01$ by Fisher's Exact test).
†No statistically significant trend (Cochran-Armitage test $0.1 < p < 0.5$).

The distribution of type of liver cell tumors is summarized as follows:

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Type of Tumor	Dose level (ppm) 0	50	500	5000
Benign only	5	4	9	6
Malignant only	13	13	20	17
Benign and malignant	1	3	4	2
Benign and/or malignant	19	20	33	25

These results do not indicate a dose-related increase in malignant liver cell tumors in male mice bearing hepatocellular tumors of any type.

Historical control data for male Charles River CD-1 mice from six other studies indicated the following mean incidence and range (expressed as %) for liver cell tumors:

	Intercurrent deaths		Terminal Sacrifice	
Tumor type	Mean %	Range	Mean %	Range
Benign	10.0	3.4 - 21.2	20.8	10.0 - 28.0
Malignant	15.7	6.1 - 25.8	13.2	4.8 - 28.0
		Benign Tumors		
Control	15.4	----	7.7	----
50 ppm	11.4	----	17.6	----
500 ppm	22.9	----	29.4	----
5000 ppm	12.2	----	27.3	----
		Malignant Tumors		
Control	17.9	----	53.8	----
50 ppm	0	----	29.4	----
500 ppm	37.1	----	47.1	----
5000 ppm	34.0	----	45.5	----

Oncogenicity - female mice: The incidence of liver tumors in female mice occurred in a statistically significant manner as follows:

Observation	Dose level (ppm) 0	50	500	5000
Benign ††	4/52	3/52	3/52	7/52
Malignant	0/52	0/52	0/52	1/52
Benign and/or malignant combined††	4/52	3/52	3/52	7/52**

**Statistically significantly different from control ($p<0.05$ by Chi square test).

††Statistically significant positive trend ($p<0.001$), but the trend was not significant when the high dose group was omitted.

Diagnoses of the first liver tumor in the control, low, mid, and high dose groups of female mice were made during terminal sacrifice, the 98th, 95th, and 93rd weeks, respectively. When animals dying before the week of the first

diagnosis are eliminated from consideration, the incidence of liver tumors in female mice can be summarized as follows:

Dose (ppm)	No. with tumor/no. examined 93-106*	Termination*	Total**
0	0/7	4/25	4/32
50	1/7	2/29	3/36
500	1/11	2/27	3/38
5000	4/13†	3,10†	7/23†

*Marginally significant trend ($0.05 < p < 0.1$; Cochran-Armitage trend test).
**Statistically significant trend ($p < 0.01$; Cochran-Armitage trend test).
†Not significantly different from control ($p > 0.05$; Fisher's Exact test).

Only one of the 17 liver tumors found in female mice was classified by the investigators as malignant, and that was diagnosed in a high dose group female at the terminal sacrifice.

The incidence (expressed as %) of liver tumors in female mice from the Apollo study is presented along with historical control results from 6 studies of comparable duration as follows:

	Intercurrent deaths		Terminal Sacrifice	
Tumor type	Mean %	Range	Mean %	Range
Benign	2.0	0.0 - 6.7	3.1	0.0 - 8.0
Malignant	2.2	0.0 - 8.0	1.5	0.0 - 4.7
		Benign Tumors		
Control	0.0	----	0.0	----
50 ppm	4.2	----	7.1	----
500 ppm	4.0	----	7.4	----
5000 ppm	7.1	----	30.0	----
		Malignant Tumors		
Control	0.0	----	0.0	----
50 ppm	0.0	----	0.0	----
500 ppm	0.0	----	0.0	----
5000 ppm	2.3	----	0.0	----

3. Mutagenicity

L5178Y mouse lymphoma cell cultures were treated with concentrations of 15, 30, 70, 100, and 128 ug/ml without metabolic activation or 2, 10, 30, 80, 100, and 128 ug/ml with metabolic activation. Although a dose-related increase in mutation frequency was noted, the investigators stated that the highest frequency (11.1 per 10^5 cells at 128 ug/ml) was not twice that of the control cultures (6.6 per 10^5 cells) and was within the range historically observed at the laboratory (4.4 to 15.3 per 10^5 cells). Based on these two criteria, the

investigators concluded that the apparent increase in mutation frequency was not biologically significant. However, the report did not contain specific historical data to demonstrate the distribution of mutation frequencies usually seen by the investigators.

4. Metabolism

The submitted experiment demonstrated that a single 1000 mg/kg dose of clofentezine administered to male and female rats by gavage was readily absorbed. Approximately 40% of the total radioactivity found in the plasma after dosing was attributed to metabolites of clofentezine. Peak levels of radioactivity were observed in the plasma 6 to 8 hours after dosing and were reported to be 15.6 and 14.1 mg clofentezine per liter of plasma for males and females, respectively. By the 24-hour observation, plasma levels of radioactivity declined to 3.3 and 3.1 mg/l for males and females, respectively, and the plasma half life was calculated to be 3.6 hours. There were adequate data to indicate that uptake and elimination of radiolabelled clofentezine was similar in male and female rats.

5. Thyroid Studies

A series of three experiments was conducted to determine the relationship between treatment of rats with clofentezine in the diet and thyroid effects observed in chronic and subchronic toxicity studies.

In the first study, thyroid residue accumulation was evaluated. Male and female rats were given radiolabelled clofentezine by gavage twice a day (20 mg/kg/day) for one or ten days. Determination of tissue residue levels after those periods of treatment indicated no preferential accumulation of clofentezne in the thyroid.

The second study investigated the effects of the pesticide on thyroxine half-life. Male rats were dosed twice daily with aqueous sodium iodide for 7 days. On the fourth day the animals received an intravenous injection of 50 ul radiolabelled thyroxine solution. The rats were then given diets containing 0 or 30,000 ppm clofentezine for 29 days at which time the 7-day regimen of sodium iodide and ^{125}I-thyroxine dosing was repeated. The mean thyroxine half-life in rats on control diet changed from 16.70 to 17.61 hours after a month. The respective values for animals receiving the 30,000 ppm diet were 17.05 and 16.42 hours after a month. Statistical analysis (Mann Whitney test) showed no significant differences between the pre- and post-treatment observations in each group, but statistical analysis of the individual animal results indicated significant treatment-related decreases in the thyroxine half-live (approximately 9%).

The third experiment evaluated the effects of clofentezine on thyroid iodine uptake. Groups of male and female rats or mice were given diets containing 0 or 30,000 ppm clofentezine for 4 weeks. At the end of the 4-week period the animals received an intraperitoneal dose of ^{131}I-sodium iodide. There was a significant and rapid increase in thyroid uptake of iodine in clofentezine treated rats following intraperitoneal dosing with [^{131}I]-sodium iodide. At six hours after administration of radiolabelled sodium iodide, the level in treated males was 1.7 times that for controls, and in females the radiolabel was found at levels 2.7 times that for control group females. Blood iodine levels were significantly reduced in both rats and mice six hours after iodine administra-

tion. At the 24-hour observation, higher levels of radiolabelled iodine were found in the thyroids of both species than were seen at the 6-hour observation. The blood levels observed at that time in the rat remained lower than those seen in the control group rats. Blood levels in the treated and control mice were comparable at 24 hours.

Finally, groups of male and female rats were fed diets containing 0, 400, or 30,000 ppm clofentezine for 6 weeks.

There were no mortalities or clinical signs observed in the study, and the only effect on body weight noted was a 28% decrease in the group mean weight of females given the 30,000 ppm diet for 6 weeks when compared to controls.

The test substance increased absolute liver weights and liver-to-body weight ratios for both sexes at the 30,000 ppm level and for males given the 400 ppm diet.

Increases in thyroxine, free thyroxine index, thyrotrophin, progesterone, and dehydroepiandrosterone were seen in both sexes at 30,000 ppm. Males given the highest dose also showed an increased level of tri-iodothyroxine. At the 400 ppm dose level, males also had increased thyroxine levels, and females had increased dehydroepiandrosterone levels. These changes are summarized as follows:

Observation	Dose level (ppm) 0	400	30,000
	Males		
Tri-iodothyroxine (nmol/l)	0.9	1.0	1.1*
Thyroxine (nmol/l)	54	62*	69***
Thyroxine binding capacity	0.77	0.79	0.71
Free thyroxine index†	71	84	98**
Thyrotrophin (ng/ml)	5.4	5.7	9.0*
Progesterone (nmol/l)	14.8	17.7	40.0**
Dehydroepiandrosterone (umol/l)	0.52	0.57	1.41***
	Females		
Thyroxine (nmol/l)	51	54	67***
Thyroxine binding capacity	0.77	0.78	0.65*
Free thyroxine index†	68	71	105***
Thyrotrophin (ng/ml)	3.5	3.7	7.3***
Progesterone (nmol/l)	89.1	90.7	134.7*
Dehydroepiandrosterone (umol/l)	0.44	0.66*	2.22***

*Statistically significantly different from control ($p<0.05$; t test).
**Statistically significantly different from control ($p<0.01$; t test).
***Statistically significantly different from control ($p<0.001$; t test).
†Calculated from thyroxine/thyroxine binding capacity.

6. Dermal Absorption

Radiolabelled clofentezine in a 50 SC formulation was applied to the skin of male rats at dose levels of 4.8. 44, or 180 mg per kg body weight to evaluate the dermal absorption of the active ingredient. There were adequated data presented to indicate that absorption was low (less than 1%) during the 10 hours that followed application of all doses.

B. Data Gaps

In Section II. A. 1. (page 7), technical difficulties with generation of test atmospheres were described as the reason an acute inhalation LC_{50} could not be determined. Efforts to overcome the difficulties did not include dilution of the test substance with water as is recommended on the proposed label for use of APOLLO® 50 SC in the field. The authors noted that exposure is unlikely during mixing of the test substance, but no statement was made regarding exposure to spray during application. On that basis, the report does not clearly demonstrate that a test atmosphere is impractical for evaluation of potential acute toxicity resulting from inhalation exposure. Therefore, an acute inhalation study or data to indicate that the formulation is not in a respirable form under conditions of normal application is needed.

Section II. A. 3. (page 12) indicates that the highest mutation frequency observed in mouse lymphoma cell cultures treated with clofentezine was within a normal range of 4.4 to 15.3 per 10^5 cells. However, no specific information regarding the range such as an average mutation frequency, the number of control groups used to obtain the range, or individual control group values were not included. These specific data are needed before the report can be considered complete.

Section I. B. 7. on page 6 states that the major route of excretion of clofentezine residues in treated rats is the feces, but metabolites are identified only in the urine. Based on the importance of bile and feces as a route of excretion and the results of long-term feeding studies in rats and mice, identification of fecal metabolites is needed to support a complete assessment of clofentezine toxicity.

C. Tolerance Assessment

A previous Toxicology Branch memorandum (dated September 10, 1985. From: R. Gardner. To: J. Ellenberger, Registration Division. Subject: Experimental Use Permit (EPA Reg. No. 45639-EUP-RO) and a Temporary Tolerance for Residues of NC 21314 (APOLLO™) in/on Strawberries (3.0 ppm; Petition No. 5G3203). Tox. Chem. No. 593A) calculated an Acceptable Daily Intake (ADI) on the basis of a NOEL from the one-year dog study (see Section I. B. 2. above) and a Safety Factor of 100. The calculated PADI is 0.0125 mg/kg.

In view of the results of long-term rat and mouse feeding studies described in Section II. A. 2. (pages 7-12), proposed tolerances on apples, pears, almonds, peaches and nectarines will not be considered until a Toxicology Branch Peer Review is conducted for clofentezine. Relevant information has been provided to the Peer Review Committee for consideration.

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APPENDIX I

Toxicology Branch "One-Liners" for
Clofentezine (Tox. Chem. No. 593A)

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Study/Lab/Study #/Date	Material	EPA Accession No.	Results: LD_{50}, LC_{50}, PIS, NOEL, LEL	TOX Category	CORE Grade/ Doc. No.
Teratology-rat; FBC Limited, Report no. TOX/82/167-34; August 20, 1982	Technical 100 ± % Pure	071085	Teratogenic NOEL > 3200 mg/kg (HDT) Fetotoxic NOEL > 3200 mg/kg/day Maternal NOEL = 1280 mg/kg/day Maternal LEL = 3200 mg/kg/day (differential staining and slight enlargement of the centrilobular hepatocytes) Levels tested by gavage in Charles River COBS CD Sprague-Dawley strain - 0, 320, 1280 and 3200 mg/kg/day		Minimum 003879
Teratology-rabbit, FBC Limited, Report no. TOX/83/167-42, January, 1983	Technical	071714	Teratogenic NOEL > 3000 mg/kg/day (HDT) Fetotoxic NOEL = 1000 mg/kg day Fetotoxic LEL = 3000 mg/kg/day (body wt reduction) Maternal NOEL = 1000 mg/kg/day Maternal LEL = 3000 mg/kg/day (body wt reduction, reduced food intake) Levels tested by gavage in New Zealand strain - 0, 250, 1000 and 3000 mg/kg/day		Minimum 003879
2-Week feeding-rat, FBC Limited; report no. METAb/83/23; 6/15/83	Technical	071914	After two weeks, the 40 and 400 ppm diets increased the cytochrome P_{450} and b_5 concentrations in rats. The 10 ppm diet had no effect on the liver enzymes of the rats. The test diets contained 0, 10, 40, or 400 ppm.		003879

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Study/Lab/Study #/Date	Material	EPA Accession No.	Results: LD_{50}, LC_{50}, PIS, NOEL, LEL	TOX Category	CORE Grade/ Doc. No.
8-Week feeding-mice; FBC Limited, report no.METAB /82/20, 4/82	Technical 100%	070965	Specific effects of diets containing 0, 400, or 27,000 ppm on mixed function oxicase activity in the mouse liver were evaluated. The highest dose cuased a 3-fold increase in cytochrome P_{430} and b_5 concentrations, a 2-fold increase in aminopyrene activity, and no change in the aniline hydroxylase activity. Liver weights were increased by approximately 33% by the high dose. The 400 ppm level increased the aminopyrene demethylase activity.		003879
8-Week feeding-rat; FBC Limited; report no. METAB/82/2; 1/82	Technical	071914	After two weeks on untreated diets, the rats previously fed 27,000 ppm diets showed only slightly elevated cytochrome b_5 levels (see report no. METAB/81/31 above).		003879
8-Week feeding-rat; BFC Limited, report no. METAB/81/31; 11/9/81	Technical 100%	070965	The study specifically evaluated the effects of dietary concentrations of 40 or 27,000 ppm on hepatic mixed-function oxidase activity. The NOEL is 40 ppm, and 27,000 ppm elevated cytochromes P_{450} and b5 as well as aniline hydroxylase activity		003879

Study/Lab/Study #/Date	Material	EPA Accession No.	Results: LD_{50}, LC_{50}, PIS, NOEL, LEL	TOX Category	CORE Grade/ Doc. No.
56-Day oral - baboon; FBC Limited, Report no. TOX/83/167-55, May 19, 1983	Technical	071719	One animal of each sex was used, and the dosage was varied during the experiment. Doses of Emesis in the female when the dose was elevated to four daily doses of 400 mg/kg by gavage. Feces was colored pink as the result of treatment in both animals. No other signs of toxicity were reported. In the context of other studies listed herein this study can be considered as supplementary since its results are consistent with those in other species.		Supplementary 003879
Metabolism - baboon; FBC Limited, Report no. METAB/83/24; 6/10/83	47.7 uCi/g spec. activ. purity unspecified	071714	After a single oral dose of 10 mg/kg urinary metabolites were identified as free and glucuronide conjugated 4-OH NC 21314 (75%). Urinary residues accounted for 5% of the administered dose. No fecal residues were identified.		003879
90-Day feeding-rat; FBC Limited, Report no. TOX/81/147-22; 1/82	Technical 99%	070962	NOEL < 3,000 ppm (LDT) (increase in liver weight, gross changes in livers, and centrilobular enlargement, elevated serum cholesterol and triglyceride levels) The effects were found to be reversible after the 4-week "regression" period. Levels tested in Charles River COBS CD Sprague-Dawley - 0, 3000, 9000 and 27,000 ppm		Supplementary 003879

Study/Lab/Study #/Date	Material	Accession No.	Results: LD_{50}, LC_{50}, PIS, NOEL, LEL	TOX Category	CORE Grade/ Doc. No.
90-Day feeding-rat; FBC Limited; report no. TOX/ 81/167-23/1; study #81019; 12/81	Technical 99%	070963	The 400 and 4000 ppm diets increased the serum cholesterol levels in male and female rats. A NOEL for centrilobular hepatocellular enlargement was not established according to the original report (see Report no. TOX/ 83/167-44 and Huntingdon Res. Centre studies below) Levels tested in Charles River COBS CD Sprague-Dawley - 0, 40, 400 and 4000 ppm FIRST ADDENDUM #TOX/83/167/-44; Jan 26,1983		Minimum (with addenda) 003879
		071384	Histological examinations of livers from treated rats indicates that 40 ppm is the NOEL with respect to centrilobular hepatocellular enlargement (see report no TOX/81/167-23/1 above) SECOND ADDENDUM; Huntingdon Res.		
		071859	Histopathology of liver treated rats indicates that the 40 ppm level is the NOEL with respect to centrilobular hepatocellular enlargement, and that 400 ppm is the LEL		
90-Day feeding-dog; FBC Limited, report no. TOX/ 81/167-21/1; 12/81	Technical	070964	The NOEL for increased liver weight or electrocardiogram changes was not established (3200 ppm in the diet was the lowest dose tested). No other effects were noted.		Supplementary 003879

Study/Lab/Study #/Date	Material	EPA Accession No.	Results: LD_{50}, LC_{50}, PIS, NOEL, LEL	TOX Category	CORE Grade/ Doc. No.
3-Generation reproduction - rat; FBC Limited, Report No. TOX/84/167-66, August 30, 1984	Technical	257994	Reproductive NOEL >400 ppm Systemic NOEL = 40 ppm Systemic LEL = 400ppm (centrilobular hepatocyte hypertrophy, increased liver weight) Dietary levels tested = 0, 4, 40, 400 ppm		Minimum 004651
Chronic-rat; FBC Limited; Report No. TOX/83/167-62; December, 1983	Technical	257993	Interim Report After 18 months of feeding: NOEL = 40 ppm LEL = 400 ppm (slightly increased liver weight and liver-to-body weight ratio, hepatocellular hypertrophy) Dietary levels tested = 0, 10, 40, and 400 ppm (Includes 12-month sacrifice and histopath. on 20 rats/sex/group)		Supplementary 004651
Oncogenic-mice; FBC Limited; Report no. TOX/83/167-64: December, 1983	Technical (98.7% ± 1.3%)	257995	Interim Report No effects observed after 12 months of the study. Dietary levels tested = 0, 50, 500, and 5000 ppm		Supplementary 004651

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Study/Lab/Study #/Date	Material	EPA Accession No.	Results: LD_{50}, LC_{50}, PIS, NOEL, LEL	TOX Category	CORE Grade/ Doc. No.
1-Year feeding-dog; FBC Limited, report no. TOX/83/167-57, 6/23/83	Technical 98.2%	071714 257493	NOEL = 50 ppm LEL = 1000 ppm (hepatocyte enlargement with eosinophilic cytoplasm; increased liver weight, thyroid & adrenals, elevated serum cholesterol, triglycerides, & alkaline phosphates). Levels tested in beagles: 0, 50, 1000 & 20,000 ppm.		Supplementary 003879 Minimum 004423
Metabolism-rat ; FBC Limited; # METAB/81/26; July, 1981	>98% (sp. act. 86.4 uCi/mg)	070965	50-60% of the an oral dose (0.1 mg/kg) is recovered in the feces of rats during the first 17 hours after dosing. Approximately 20% is recovered during that time in the urine.		003879
Metabolism-rat; FBC Limited; Report no. METAB/83/14; May 3, 1982	>98% (sp. act. 86.4 uCi/mg)	070965	60-70% of an intravenous dose (0.1 mg/kg) is excreted in the feces during the first 24 hours after dosing. Approximately 20-25% is recovered in the urine during that time.		003879
Metabolism-rat; FBC Limited; Report no. METAB/81/38; December, 1981	>98% (sp. act. 86.4 uCi/mg)	070965	The rats were fed a diet containing 27000 ppm for 10 weeks prior to the administration of 0.1 mg/kg i. v. The excretion profile is similar to that reported in study no. METAB/83/14 above.		003879
Metabolism-rat, FBC Limited; Report no. METAB/82/1; January, 1982	>98% (sp. act. 86.4 uCi/mg)	070965	60-70% of an oral dose (10 mg/kg) was excreted in the feces during the first 24 hours after treatment. Approximately 20% was excreted in the urine during that time. The liver and the kidney were found to have the highest tissue concentrations 72 hours after dosing.		003879

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Study/Lab/Study #/Date	Material	EPA Accession No.	Results: LD_{50}, LC_{50}, PIS, NOEL, LEL	TOX Category	CORE Grade/ Doc. No.
Metabolism - rat; FBC Limited, Report no. METAB/83/27, June 16, 1983.	>98% purity 86.4 mCi spec. activ.	071714	The rats were given a 10 mg/kg dose of radiolabelled test substance. 35% of radioactivity recovered in urine was described as a monochloro-hydroxylated derivative of the parent found free or conjugated. Approximately 34% of the recovered activity was identified as free or conjugated 3- or 4-hydroxy NC 21314. 9% of activity associated with unidentified metabolites and 3% was associated with unmetabolized parent		003879
Metabolism - rat; FBC Limited; Report no. METAB/82/22; March, 1982	>98% purity, spec. activ. 86.4 mCi/g	070965	1000 mg/kg dose. Majority excreted in the feces (98% of administered dose; approximately 1 to 2% of dose excreted in urine. Highest tissue concentrations in the liver and plasma. Moderate levels were found in adrenals and fat. These levels were slightly above those found in all other tissues.		003879
Metabolism - rat; FBC Limited, Report no. METAB/81/32; 11/81	>98% purity 86.4 mCi/g spec. activ.	070965	1-25 consecutive daily doses of 20 mg/kg administered by gavage to rats Tissue levels rose to a plateau in kidney, liver, heart (females only), skin, and ovaries after 5 to 15 days These levels were 2 to 4 times those found after a single dose.		003879
Metabolism - rat; FBC Limited, Report no. METAB/82/23, 4/82	>98% purity 86.4 uCi/g spec. activ.	070965	Whole-body autoradiography of rats given a single 10 mg/kg dose of radiolabelled test substance indicated that there is poor gastrointestinal absorption and organs and tissues are cleared of residues within 48 hours after dosing.		003879

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Study/Lab/Study #/Date	Material	EPA Accession No.	Results: LD_{50}, LC_{50}, PIS, NOEL, LEL	TOX Category	CORE Grade/ Doc. No.
Metabolism - rat; FBC Limited, Report no. METAB/81/19; 4/81	>98% purity 10 uCi/g spec. activ.	070965	Residues in fetuses of rats given a single oral dose of 20 mg/kg were one-fifth the level found in the maternal blood 6 hours after dosing. 24 hours after treatment, the liver, kidneys, and fat had 1 ppm concentrations of radioactive residues. No other tissues contained significant residue concentrations. The test substance did not readily cross the placenta, and is rapidly cleared from pregnant rats.		003879
Metabolism - mice, FBC Limited, Report no. METAB/82/11; 1/82	>98% purity 86.4 uCi/g spec. activ.	070965	Approx. 65% of a single 10 mg/kg oral dose is excreted in the feces of treated mice. Almost all of the administered dose is excreted within 48 hours after treatment.		003879
Metabolism - dog; FBC Limited; Report no. METAB/82/6; January, 1982	>98% purity 86.4 uCi/g spec. activ.	070965	94-97% of the 10mg/kg oral dose was excreted by treated dogs within 48 hours after treatment.		003879
Metabolism - dog; FBC Limited; Report no. METAB/81/37; 12/81	>98% purity 86.4 uCi/g spec. activ.	070965	After an i. v. injection of a single dose of 0.1 mg/kg approximately 70% of the administered radioactivity was recovered in the feces and 20% was found in urine. Most of the excretion was noted during the first 48 hours after dosing. Tissue residues were at or below the level of detection (0.01 ppm).		003879

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Study/Lab/Study #/Date	Material	EPA Accession No.	Results: LD_{50}, LC_{50}, PIS, NOEL, LEL	TOX Category	CORE Grade/ Doc. No.
Metabolism -rabbit, FBC Limited, Report no. METAB/82/21; 4/82	>98% purity 86.4 uCi/g spec. activ.	070965	Approximately 60% of a single oral dose of 10 mg/kg to rabbits was recovered in the feces, and 40% was found in the urine. Almost all of the recovered residues were found during the first 48 hours after dosing. At 96 hours after treatment from 0.5 to 1.43 ppm was found in the stomach, bile, and large intestine of treated rabbits.		003879
Mutagenic - Ames test -Salmonella, FBC Limited; Report no. TOX/80/167-3	Technical 98.5%	070961	No mutagenic activity was found in bacteria with or without metabolic activation at doses ranging from 3.3 mg/plate to 10 ug/plate		003879
Mutagenic (gene conversion and mitotic recombination)-yeast; FBC Limited, Report no. TOX/83/167-56, June 6, 1983	Technical	071714	The vehicle (dimethylformamide/ethanol) was found to be cytotoxic and a reduced concentration had to be used. Concentrations >125 ug per ml culture medium were insoluble. No increases in gene conversions or the frequency of mitotic recombinations were observed at 12.5, 25, 100, or 200 ug/ml above that in vehicle controls was found.		003879
Mutagenic - micronucleus - mice; FBC Limited; report no. TOX/82/167-32; 4/82	Technical	071085	There was no compound-related increase in the number of micronuclei in mice given 800, 1600, or 3200 mg/kg doses above that observed in untreated control mice.		003879
Photosensitization - no species; FBC Limited; Report no. TOX/81/167-17; 5/81	Technical	070961	No data were presented. A theoretical discussion of the potential of the test substance to be phototoxic was presented.		003879

Study/Lab/Study #/Date	Material	EPA Accession No.	Results: LD_{50}, LC_{50}, PIS, NOEL, LEL	TOX Category	CORE Grade/ Doc. No.
Mutagenic - dominant lethal -rat; FBC Limited, report no. TOX/83/167-45; 3/29/83	Technical	071714	No dominant lethal mutations were induced in rats after 10 weeks of feeding diets containing 0, 4, 40, or 400 ppm. Slightly elevated cholesterol levels, absolute and relative liver weights were observed in rats given the 400 ppm diet.		003879
Acute oral LD_{50}-mice; FBC Limited, Report no. TOX/80/167-7, September, 1980	Technical 99.1%	070961	LD_{50} >3200 mg/kg (only dose tested)	III	Supplementary 003879
Acute oral LD_{50}-dog; FBC Limited, Report no. TOX/80/167-10, June, 1980	Technical 99.1%	070961	LD_{50} >2000 mg/kg (HDT) (only two doses tested and one dog per dose)	III	Supplementary 003879
Acute oral LD_{50}-hamster, FBC Limited, Report no. TOX/80/167-7; September, 1980	Technical 99.1%	070961	LD_{50} >3200 mg/kg (only one dose tested)	III	Supplementary 003879
Acute intraperitoneal LD_{50}-rat; FBC Limited; Report no. TOX/80/167-5, September, 1980	Technical 99.1%	070961	LD_{50} >800 mg/kg		003879
Acute dermal LD_{50}-rat; FBC Limited; Report no. TOX/80/167-4: August, 1980	Technical 98.5%	070961	LD_{50} >1330 mg/kg (only one dose was tested and al animals had intact skin). Sufficiently high doses to clearly establish a Toxicity Category classification were not used.		Supplementary 003879
Acute oral LD_{50} - rat; FBC Limited, Report no. TOX/81/167-15, April, 1981	80 WP (80% a. i.)	070961	LD_{50} > 5000 mg/kg (no mortality)	IV	Minimum 003879

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Study/Lab/Study #/Date	Material	EPA Accession No.	Results: LD_{50}, LC_{50}, PIS, NOEL, LEL	TOX Category	CORE Grade/ Doc. No.
Acute inhalation LC_{50} - rat, FBC Limited, Report no. TOX/82/167-24; 1/82	80 WP (80% a. i.)		LC_{50} > 11.35 mg/L/6 hrs (nominal conc) (no mortality)	III	Minimum 003879
Acute dermal LD_{50} - rabbit; FBC Limited; Report no. TOX/81/167-20; 8/81	80 WP (80% a. i.)	070961	LD_{50} > 20,000 mg/kg (abraded skin)	IV	Minimum 003879
Primary dermal irritation - rabbit, FBC Limited; report no. TOX/81/167-13; 6/81	80 WP (80% a. i.)	070961	Only slight irritation was observed 24 hours after application. Treated skin appeared normal at 72 hours following treatment. (aqueous saline vehicle used)	IV	Minimum 003879
Primary eye irritation - rabbit, FBC Limited, report no. TOX/81/167-19, 6/81	80 WP (80% a. i.)	070961	The formulation caused mild reversible irritation in washed and unwashed eyes of female rabbits. Conjunctival irritation was the most prominant effect.	IV	Minimum 003879
Acute oral LD_{50} - rat, FBC Limited, Report no. TOX/81/167-12; February, 1981	50 WP (50% a. i.)	070961	LD_{50} > 5000 mg/kg (no mortality)	IV	Guideline 003879
Acute oral LD_{50} - rat; FBC Limited; Report no. TOX/82/167-40; January 19,1983	50 SC (42% a. i.)	071914	LD_{50} > 5000 mg/kg (no mortality)	IV	Guideline 003879
Acute dermal LD_{50} - rabbit; FBC Limited; report no. TOX/83/167-48; 3/7/83	50 SC (42% a. i.)	071714	LD_{50} > 2.4 g/kg (no mortality) (abraded skin)	III	Minimum 003879

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Study/Lab/Study #/Date	Material	EPA Accession No.	Results: LD_{50}, LC_{50}, PIS, NOEL, LEL	TOX Category	CORE Grade/ Doc. No.
Acceptable Daily Intake- EPA/ OPP/ HED TOx.	TECH		PADI = 0.013 mg/kg/day Safety Factor = 100 Dated: Updated: Study: 1-Year Feeding Dog Study NOEL: 50 ppm Lab.: FBC Limited Study No.: TOX/83/167-57 Study Date: 6/23/83 Doc.No.: 005096 Comments: Carcinogenicity: Not determined, final report on mouse and rat pending.		005096

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APPENDIX II

DATA EVALUATION RECORDS
for New Toxicology Data

Mallyon, B. A., and Sanderson, D. M. (1980) The Acute Oral Toxicity of Unformulated NC 21314 to the Male and Female Rat. (Unpublished study no. TOX/80/167-2 received April 3, 1986 under Petion Nos. 6F3392 and 6H5500 prepared by FBC Limited, submitted by NOR-AM Chemical Co., Wilmington, DE; Acc. No. 262259)

Greenough, R. J.; McDonald, R. (1985) Apollo 50 SC Formulation: Atmosphere Generation for an Acute Inhalation Study. (Unpublished study TOX 85064 received April 3, 1986 under Petion Nos. 6F3392 and 6H5500 prepared by Inveresk Research International, submitted by NOR-AM Chemical Co., Wilmington, DE; Acc. No. 262259)

Cuthert, J. A.; Carr, S. M. A. (1983) NC 21314 50 SC Formulation: Primary Eye Irritancy Study in Rabbits. (Unpublished study no. TOX/83/167-46 received April 3, 1986 under Petion Nos. 6F3392 and 6H5500 prepared by Inveresk Research International, submitted by NOR-AM Chemical Co., Wilmington, DE; Acc. No. 262259)

Crome, S. J.; Sanderson, D. M.; and Brooks, P. N.; (1980) The Primary Skin Irritancy of Unformulated NC 21314 (CR 20099/5) to the Guinea Pig. (Unpublished study no. TOX/80/167-6 received April 3, 1986 under Petion Nos. 6F3392 and 6H5500 prepared by FBC Limited, submitted by NOR-AM Chemical Co., Wilmington, DE; Acc. No. 262259)

Cuthert, J. A.; Carr, S. M. A.; and O'Arcy-Burt, K. J. (1983) NC 21314 50 SC Formulation Primary Skin Irritancy Study in Rabbits. (Unpublished study no. TOX/83/167-47 received April 3, 1986 under Petion Nos. 6F3392 and 6H5500 prepared by Inveresk Research International, submitted by NOR-AM Chemical Co., Wilmington, DE; Acc. No. 262259)

Teale, N. J. (1980) Delayed Dermal Sensitization Study in the Guinea Pig: NC 21314 Technical. (Unpublished study TOX 82027 received April 3, 1986 under Petion Nos. 6F3392 and 6H5500 prepared by FBC Limited, submitted by NOR-AM Chemical Co., Wilmington, DE; Acc. No. 262259)

Ginnocchio, A. V.; and Mallyon, B. A. December 17, 1985. The Oncogenicity and Chronic Toxicity of Technical NC 21314 (Chlofentezine in the Diet to the Rat (Final Report). Unpublished report no. TOX/ 84/167-70 prepared by Huntingdon Research Centre. Submitted by FBC Limited; Nor-Am Cehmical Co. EPA Acc. Nos. 262261 and 262262.

Lloyd, G. K., D. J. Spencer-Briggs, R. Heywood, C. Gopinath, and C. P. Cherry. November, 1985. Technical NC 21314: Oncogenicity in the Diet to the Mouse (Final Report). Unpublished report no. TOX/ 85/167-80 prepared by Huntingdon Research Center, Huntingdon, Cambridgeshire, UK. Submitted by FBC Limited; Nor-Am Chemical Co. EPA Acc. Nos. 262263 and 262264

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APPENDIX II

DATA EVALUATION RECORDS
(continued)

Bootman, O., and R. Rees. October 14, 1982. Technical NC 21314: Investigation of Mutagenic Activity in the Tk +/- Cell Mutation System. Unpublished report no. TOX/82/167-38 prepared by Life Science Research, Stock, Essex, England. FBC Limited. Submitted by Nor-Am Chemical Co.; EPA Acc. No. 262268.

Campbell, J. K., and D. Needham. March 15, 1985. Concentration of (^{14}C) Clof - entezine and Its Metabolites in the Plasma of Rats Dosed Orally with Clofentezine at the Rate of 1000 mg/kg Bodyweight. Unpublished report no. METAB/85/2 prepared by by FBC Limited. Submitted by Nor-Am Chemical Co.; EPA Acc. No. 262268.

Challis, I. R., and C. L. Creedy. December 20, 1985. The Effects of Thyroid Function. Unpublished report no. METAB/85/36 prepared by by FBC Limited. Submitted by Nor-Am Chemical Co.; EPA Acc. No. 262268.

Saunders, P. C., and B. A. Mallyon. January 27, 1986. Technical Clofentezine: 6 Week Dietary Investigation of Thyroid Function in the Rat. Unpublished report no. TOX/85/167-77 prepared by FBC Limited. Submitted by Nor-Am Chemical Co.; EPA Acc. No. 262268.

Campbell, J. K., and D. Needham. February 3, 1986. Dermal Absorption of [^{14}C]-Clofentezine by Male Rats Given a Topical Application of 50 SC Formulation. Unpublished report no. METAB/86/1 prepared by by FBC Limited. Submitted by Nor-Am Chemical Co.; EPA Acc. No. 262268.

DATA EVALUATION RECORD 006232

1. CHEMICAL NAME: NC 21314, Clofentezine, APOLLO® 3,6-bis[2-chlorophenyl]-1,2,4,5-tetrazine

2. TEST MATERIALS: Technical grade NC 21314 (99 ± 2% a. i.)

3. STUDY/ACTION TYPE: Acute oral toxicity - rat (§81-1)

4. STUDY IDENTIFICATION: Mallyon, B. A., and Sanderson, D. M. (1980) The Acute Oral Toxicity of Unformulated NC 21314 to the Male and Female Rat. (Unpublished study no. TOX/80/167-2 received April 3, 1986 under Petion Nos. 6F3392 and 6H5500 prepared by FBC Limited, submitted by NOR-AM Chemical Co., Wilmington, DE; Acc. No. 262259)

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6. APPROVED BY:

Name: Judith Hauswirth, Ph. D.
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Organization: Review Section 6 Date: 8/21/87
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7. DISCUSSION AND CONCLUSIONS: The results of the study indicated that the acute oral LD_{50} in male and female rats is >3230 mg/kg suggesting that NC 21314 should be classified into Toxicity Category III.

Core classification: Minimum

8. MATERIALS AND METHODS

Test species: Male and female CFY SD strain rats were used. The body weights for males ranged from 192 to 230 g, and that for females ranged from 173 to 218 g. at the start of the experiment.

Experimental procedure: Groups of 6 male and 6 female rats was given a single oral doses of 0, 800, 1131, 1600, 2261, or 3200 mg test substance per kg body weight. The test substance was dissolved in 0.5% aqueous gum tragacanth (w/v) and administered by gavage. The rats were observed for mortality and appearance of toxicological signs on the day of dosing and once each day during the 14 days that followed dosing. Surviving animals were weighed on the day of dosing and weekly therafter, and they were sacrificed at the end of the observation period. Postmortem examinations included gross observations, weighing of the liver, kidneys, spleen, adrenals, brain, thymus, and pituitary, and preparation of tissues from the liver, kidneys, liver, kidneys, esophagus, stomach, duodenum, urinary bladder, and spleen for microscopic examination.

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8. MATERIALS AND METHODS (continued)

Statistically analysis was confined to the two-sample test of Wilcoxon for body and organ weight data. Differences were considered to be significant when $p<0.05$. Because no deaths were observed in treated animals, a method for calculation of the LD_{50} was not presented.

9. REPORTED RESULTS

No signs of compound-related toxicity were observed by the investigators. The report stated that all male rats given the two highest doses and all treated female rats had pink colored feces 20 to 22 hours after dosing. The authors noted that the color was similar to that of the test substance itself.

There were no other dose-related effects on body weight, organ weights, gross necropsy observations, or histopathology according to the report.

Based on the results of the experiment, the investigators concluded that the oral LD_{50} in male and female rats is >3200 mg/kg (the highest dose tested).

006232

DATA EVALUATION RECORD

1. CHEMICAL NAME: NC 21314, Clofentezine, APOLLO® 3,6-bis[2-chlorophenyl]-1,2,4,5-tetrazine

2. TEST SUBSTANCE: Apollo 50 SC (42% active ingredient); density = 1.20 g/ml; viscosity = 13,700 C.P.S.; surface tension = 50 dynes/cm on a 0.08% w/v formulation at ambient temperature

3. STUDY/ACTION TYPE: Acute inhalation toxicity - rat (§81-3)

4. STUDY IDENTIFICATION: Greenough, R. J.; McDonald, R. (1985) Apollo 50 SC Formulation: Atmosphere Generation for an Acute Inhalation Study. (Unpublished study TOX 85064 received April 3, 1986 under Petion Nos. 6F3392 and 6H5500 prepared by Inveresk Research International, submitted by NOR-AM Chemical Co., Wilmington, DE; Acc. No. 262259)

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7. DISCUSSION AND CONCLUSIONS: The label for Apollo 50 SC states that the formulation may be added to water and sprayed on the crop to be treated. This suggests that the test substance may require dilution with water before an aerosol is generated for an acute inhalation test. The authors noted that exposure is unlikely during mixing of the test substance, but no statement is made regarding exposure to spray during application. On that basis, the report does not clearly demonstrate that a test atmosphere is impractical for evaluation of potential acute toxicity resulting from inhalation exposure.

Core classification: Supplementary. Other methods should be tried before concluding that an acute inhalation toxicity study is not needed.

8. Materials and methods

Test species: None (see below).

Experimental Procedure: The objective of the study was to determine test conditions for a subsequent acute inhalation toxicity study with the test material.

According to the report, a Watson-Marlow pump was used to generate test atmospheres fitted to a glass concentric jet atomizer at the top of an aluminum

8. Materials and methods (continued)

inhalation chamber. Atomizer jet orifices of 0.2 mm or 1.0 mm were tested. Speed of the pump and airflow rate were adjusted to change the concentration of the test substance inside the inhalation chamber.

Air concentrations in the chamber were determined gravimetrically using filters through which the test atmosphere was drawn at a rate of 1.0 liter/min for a measured time. Each filter was weighed before and after sampling and the difference in weights was used in the calculation of test substance concentration.

Particle size distribution was determined with an Anderson Mini Sampler which according to the report, consisted of 4 impaction plates with a fiberglass filter backup in an anodized aluminum can. Air was drawn through the sampler at a rate of 1.4 liters/min for a specific time period. Impaction plates and filter were weighed before and after sampling, and the weight difference was used in determination of the particle size distribution.

4. REPORTED RESULTS

The investigators noted that the aerosol jets used in the experiment were clogged by the viscous test substance shortly after generation of test atmospheres was begun. Changes in the rate of air flow or delivery of the test material to the atomizer did not overcome the problem, and the authors concluded that a test atmosphere could not be sustained for a 4-hour exposure period.

006232

DATA EVALUATION RECORD

1. CHEMICAL NAME: NC 21314, Clofentezine, APOLLO® 3,6-bis[2-chlorophenyl]-1,2,4,5-tetrazine

2. TEST SUBSTANCE: NC 21314 50 SC (lot no. 16244/1; 42.0% active ingredient).

3. STUDY/ACTION TYPE: Primary eye irritation - rabbit (§81-4)

4. STUDY IDENTIFICATION: Cuthert, J. A.; Carr, S. M. A. (1983) NC 21314 50 SC Formulation: Primary Eye Irritancy Study in Rabbits. (Unpublished study no. TOX/83/167-46 received April 3, 1986 under Petion Nos. 6F3392 and 6H5500 prepared by Inveresk Research International, submitted by NOR-AM Chemical Co., Wilmington, DE; Acc. No. 262259)

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Date: 8/21/87

7. DISCUSSION AND CONCLUSION: The report included adequate information to support the conclusion that NC 21314 50 SC is practically non-irritating to the eyes of rabbits. It should be classified into Category IV for eye irritation.

Core classification: Guideline

8. MATERIALS AND METHODS

Test species: Albino rabbits were used (sex and strain were not specified).

Experimental procedure: Nine rabbits previously examined and found without signs of corneal damage were used in the experiment. One-tenth ml of the test substance was instilled into one eye of each rabbit, and the eyelids were gently held together for one second. Twenty to thirty seconds after the instillation, the treated eyes of 3 rabbits were washed for one minute with warm water. The eyes of the 6 remaining rabbits were not washed.

All eyes were examined 24, 48, and 72 hours after instillation of the test substance and 4 and 7 days after treatment. Ocular reactions were scored according to the following scales:

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8. MATERIALS AND METHODS (continued)

Corneal opacity

Degree of density

1 - scattered or diffuse area, details of iris visible
2 - easily discernible translucent areas, details of iris slightly obscured
3 - opalescent areas, no details of iris visible, size of pupil barely discernible
4 - opaque, iris invisible

Area of cornea involved

1 - one-quarter (or less but not zero)
2 - greater than one-quarter to less than one-half
3 - greater than one-half to less than threequarters
4 - greater than three-quarters

score = score for degree x score for extent x 5; maximum = 80

Iris

1 - folds above normal, congestion, swelling, circumcorneal injection (any one or a combination of these), iris still reacting to light (sluggish reaction is positive)
2 - no reaction to light, hemorrhage, gross destruction (any one or all of these)

score = score for iris x 5; maximum score = 10

Conjunctivae

Redness

1 - vessels definitely injected above normal
2 - more diffuse, deeper crimson red, individual vessels not discernible
3 - diffuse beefy red

Chemosis

1 - any swelling above normal (including nictitation membrane
2 - obvious swelling with parital eversion of the lids
3 - swelling of lids about half closed
4 - swelling of lids about half to completely closed

Discharge

1 - any amount different from normal (does not include small amount in inner canthus of normal animals)
2 - discharge with moistening of the lids and hairs just adjacent to the lids
3 - discharge with moistening of the lids and considerable area around the eye

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3. MATERIALS AND METHODS (continued)

Score = sum of values for redness, chemosis, and discharge multiplied by 2.
Maximum = 20

4. REPORTED RESULTS

According to the report, there were no treated eyes with corneal opacity or iritis. Only one of the three eyes washed after treatment and 3 of the 6 unwashed eyes exhibited conjunctival irritation (grade 1) 24 hours after instillation. All treated eyes appeared normal at the 48-hour observation.

The weighted mean scores for rabbits with washed eyes was 0.66 and that for rabbits with unwashed eyes was 1.33 at the 24 hour observation The investigators noted that a weighted mean maximum score was calculated for each of the two groups of rabbits (with or without washed eyes), and the results were sufficient to classify the test substance as practically non-irritating (score ranging from 0.5 to 2.5).

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DATA EVALUATION RECORD

1. CHEMICAL NAME: NC 21314, Clofentezine, APOLLO® 3,6-bis[2-chlorophenyl]-1,2,4,5-tetrazine

2. TEST SUBSTANCE: Technical grade NC 21314 (99.1 ± 1.5% a. i.)

3. STUDY/ACTION TYPE: Primary dermal irritation - rabbit (§81-5)

4. STUDY IDENTIFICATION: Crome, S. J.; Sanderson, D. M.; and Brooks, P. N.; (1980) The Primary Skin Irritancy of Unformulated NC 21314 (CR 20099/5) to the Guinea Pig. (Unpublished study no. TOX/80/167-6 received April 3, 1986 under Petion Nos. 6F3392 and 6H5500 prepared by FBC Limited, submitted by NOR-AM Chemical Co., Wilmington, DE; Acc. No. 262259)

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7. DISCUSSION AND CONCLUSIONS: The results of the study indicated that technical grade NC 21314 is not a skin irritant and should be classified into Toxicity Category IV.

Core classification: Minimum

8. MATERIALS AND METHODS

Test species: Female Dunkin-Hartley strain guinea pigs were used.

Experimental procedure: Before the experiment, a dorsal skin area was shaved, and a 333 mg/ml suspension of the test substance in 0.5% gum tragacanth was prepared for application to the prepared skin.

Two-tenths ml of the test substance suspension was placed under 15 cm square gauze patches at two sites on each animal. One site was left untreated under a gauze patch, and another was treated with the 0.5% aqueous gum tragacanth vehicle and covered with a patch. The patches were covered with foil and secured with plaster.

Twenty-four hours after application of the test substance the dressings were removed, and the test sites were rinsed and gently wiped clean. The test sites were examined for signs of skin irritation. and they were examined daily for up to 7 days after treatment. At the end of the 7-day observatin period the animals were sacrificed and subjected to gross post-mortem examinations.

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8. MATERIALS AND METHODS (continued)

Samples of the skin at test sites were also prepared for microscopic examination.

9. REPORTED RESULTS

According to the report, no animal showed signs of irritation during the study, and no deaths were observed. The authors noted that sites where test substance was applied appeared pink in color when occlusive dressings were removed. The color disappeared from 5 to 7 days after application. There were no other treatment related effects noted after post-mortem examinations, and the microscopic examination of skin samples did not indicate any differences between treated and untreated sites.

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DATA EVALUATION RECORD

1. CHEMICAL NAME: NC 21314, Clofentezine, APOLLO® 3,6-bis[2-chlorophenyl]-1,2,4,5-tetrazine

2. TEST SUBSTANCE: NC 21314 50 SC (42.0% a. i.); a liquid that is bright pink in color

3. STUDY/ACTION TYPE: Primary dermal irritation - rabbit (§81-4)

4. STUDY IDENTIFICATION: Cuthert, J. A.; Carr, S. M. A.; and O'Arcy-Burt, K. J. (1983) NC 21314 50 SC Formulation Primary Skin Irritancy Study in Rabbits. (Unpublished study no. TOX/83/167-47 received April 3, 1986 under Petion Nos. 6F3392 and 6H5500 prepared by Inveresk Research International, submitted by NOR-AM Chemical Co., Wilmington, DE; Acc. No. 262259)

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7. DISCUSSION AND CONCLUSIONS: There was adequate information presented in the report to support the conclusion that NC 21314 50 SC is less than a moderate skin irritant, and it should be classified into Toxicity Category III with respect to primary skin irritation (see "4. REPORTED RESULTS" section below).

Core classification: Minimum

8. MATERIALS AND METHODS

Test species: Male and female New Zealand White rabbits were used.

Experimental procedure: Before the experiment, the back and flanks of each animal were clipped, and two of four test sites were abraded (penetrating the stratum corneum without causing bleeding). A 0.5 ml of the test substance or a 10% aqueous solution of sodium lauryl sulfate (positive control) was applied to intact and abraded skin sites. Each test site was covered with a 2.5 X 2.5 cm piece of chromatography paper. The patches were covered with occlusive tape.

Twenty-four hours after application of the test substance the dressings were removed, and the test sites were rinsed and gently wiped clean. The test sites were examined for signs of skin irritation daily for up to 7 days after treatment.

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8. MATERIALS AND METHODS (continued)

Erythema and eschar formation as well as edema were scored on a 5-point scale (0-4) with a maximum possible score of 8 for any site. Scoring was done according to the following classifications:

Erythema and eschar		Edema	
No erythema	0	No edema	0
Slight erythema	1	Very slight edema	1
Well-defined erythema	2	Slight edema	2
Moderate to severe erythema	3	Moderate edema	3
Severe erythema to slight eschar formation	4	Severe edema	4

The report noted that test sites were not scored for erythema because of the discoloration caused by the test substance. The authors noted that severe erythema could have been detected if present.

4. REPORTED RESULTS

According to the report, test sites were bright pink at the 24 hour observation, and the residue left on the site after wiping with a tissue obscured the skin for scoring of erythema. Edema was assessed by touch, and one animal exhibited edema with a score of 1 at the intact and abraded sites 24 hours after application of the test substance. At 72 hours the skin remained discolored, and there was no edema observed.

Results with the positive control substance indicated that it is a moderate skin irritant (grades 1 to 3 for erythema and 2 to 3 for edema). The erythema and erythema persisted for 72 hours.

The investigators concluded:

> Because of the bright pink color of the test material no erythemal assessment was possible although a severe erythemal response, if present, would have been noted...
>
> The positive control material is a moderate irritant. Comparison of sites treated with NC 21314 50 SC and sodium lauryl sulfate shows a marked difference in edema,...Based on this and the absence of obvious severe erythema it is concluded that NC 21314 50 SC is less than a moderate skin irritant and is likely at the most to be a mild skin irritant.

006232

DATA EVALUATION RECORD

1. CHEMICAL NAME: NC 21314, Clofentezine, APOLLO® 3,6-bis[2-chlorophenyl]-1,2,4,5-tetrazine

2. Test substance: Technical grade NC 21314 (batch no. CR20099/8, purity unspecified)

3. STUDY/ACTION TYPE: Dermal sensitization - guinea pig (§81-6)

4. STUDY IDENTIFICATION: Teale, N. J. (1986) Delayed Dermal Sensitization Study in the Guinea Pig: NC 21314 Technical. (Unpublished study TOX 82027 received April 3, 1986 under Petion Nos. 6F3392 and 6H5500 prepared by FBC Limited, submitted by NOR-AM Chemical Co., Wilmington, DE; Acc. No. 262259)

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7. CONCLUSIONS: NC 21314 was shown to cause dermal sensitization in two of twenty treated female guinea pigs under the test conditions described below which indicates that the chemical is a weak sensitizer.

Core classification: Minimum

8. MATERIALS AND METHODS

Test species: According to the report, young female Duncan Hartley strain guinea pigs weighing from 300 to 500 g were used.

Range-finding Procedure: The hair was clipped from the flanks of four test animals previously given an injection of Complete Freund's Adjuvant (CFA). Four concentrations of the test material in ethanol were evaluated for irritation on each animal. The test concentrations were 25 and 50% suspensions, a saturated solution (0.08%), and the test material moistened with 0.5 ml ethanol), and each concentration was applied to a 2 cm^2 filter paper patch. The patches were secured by adhesive tape and an elastic bandage around the animal's torso. After 24 hours, the dressings were removed.

Treated sites were scored 24 and 48 hours after application according to the following scale:

0 = no reaction
1 = scattered mild redness
2 = moderate and diffuse redness
3 = intense redness and swelling (edema)

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8. MATERIALS AND METHODS (continued)

Results from the preliminary study were the basis for chosing 5 and 50% concentrations for the primary phase of the main study.

Main Experiment: The protocol was described as a maximization test.

In the induction phase of the study, the hair on the shoulders of 20 animals was clipped. Three test solutions were intradermally injected in a row on each side of the center of the prepared skin area. The injections were 0.1 ml of a saturated solution of the test substance (0.08% in ethanol), 0.1 ml of CFA without test substance, or 0.1 ml of saturated solution of the test substance with CFA. Patches of 0.5 g test substance moistened with 0.5 ml ethanol were then applied over the injection sites and secured as described above. The patches were left in contact with the injection sites for 48 hours. A week after the injections a second topical application of the ethanol moistened test substance was made using the same procedure, and the occlusive dressings were removed after the patches had been in contact with the test site for 48 hours.

A control group of 10 guinea pigs was treated in the same manner as the test group with the exception that no test substance was used.

Two weeks after the last induction application, the flanks of the test animals were clipped in preparation for challenge applications. A patch with the ethanol moistened test substance was secured to one flank, and a patch with the 50% suspension was applied to the other flank. These patches were removed after 24 hours, and the test sites were scored for irritation according to the scale described above. Application sites were scored for irritation 24 and 48 hours after patches were removed.

The report noted that interpretation of results was based on the proportion of animals exhibiting sensitization. The following scale was used to grade the potency of the test substance:

Sensitization rate	Grade
0 - 8%	I
9 - 28%	II
29 - 64%	III
65 - 80%	IV
81 - 100%	V

9. REPORTED RESULTS

According to Table 2 in the report, there were two animals in the test group exhibiting a reaction after topical application of the test substance. Those reactions were scored as scattered mild redness (score of 1), and they were seen in animal numbers 8 and 18 at the 24-hour observation after the challenge application. These reactions were absent at the 48-hour observation.

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10. DISCUSSION

There was enough information presented in the report to support the conclusion that NC 21314 is a weak sensitizer. However, the conclusion was not consistent with tabulated results. The authors' conclusion stated that a reaction was observed only in one animal after the challenge in contrast to the two animals listed in Table 2 of the report. The investigators also noted that the results in the control group did not indicate that the test substance caused irritation, so they concluded that it is a weak sensitizer since there was a reaction after the challenge dose was administered.

006232

DATA EVALUATION RECORD

CHEMICAL NAME: NC 21314, Clofentezine, APOLLO® 3,6-bis[2-chlorophenyl]1,2,4,5-tetrazine

STUDY/ACTION TYPE: Subchronic feeding study (6 weeks) - rat

STUDY IDENTIFICATION: Saunders, P. C., and B. A. Mallyon. January 27, 1986. Technical Clofentezine: 6 Week Dietary Investigation of Thyroid Function in the Rat. Unpublished report no. TOX/85/167-77 prepared by FBC Limited. Submitted by Nor-Am Chemical Co.; EPA Acc. No. 262268.

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DISCUSSION AND CONCLUSIONS: Male and female rats were fed diets containing 0, 400, or 30,000 ppm clofentezine for 6 weeks in an experiment to investigate the pesticide's effect on thyroid function.

The test substance increased absolute liver weights and liver-to-body weight ratios for both sexes at the 30,000 ppm level and for males given the 400 ppm diet.

Increases in thyroxine, free thyroxine index, thyrotrophin, progesterone, and dehydroepiandrosterone were seen in both sexes at 30,000 ppm. Males given the highest dose also showed an increased level of tri-iodothyroxine. At the 400 ppm dose level, males also had increased thyroxine levels, and females had increased dehydroepiandrosterone levels.

Based on the liver weight and hormone observations, a no-observed-effect level (NOEL) was not established in this experiment.

Background: This study was conducted to investigate effects observed at the end of a long-term feeding study in rats given the highest does tested (400 ppm) [Ginnocchio, A. V.; and Mallyon, B. A. December 17, 1985. The Oncogenicity and Chronic Toxicity of Technical NC 21314 (Chlofentezine in the Diet to the Rat (Final Report). Unpublished Report No. TOX/ 84/167-70 prepared by Huntingdon Research Centre and FBC Limited. Submitted by Nor-Am Cehmical Co. EPA Acc. No. 262268.]. No attempt was made to establish a NOEL.

Core Classification: Supplementary based on the stated purpose of the study which was to investigate the specific nature of clofentezine's effect on thyroid function.

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I. MATERIALS AND METHODS

A. MATERIALS

1. Test species: Male and female Charles River CR1: COBS CD (SD) BR Sprague-Dawley rats were used. They were 28 days of age on arrival at the laboratory conducting the experiment, and body weights ranged from 66 to 85 g for males and 52 to 74 g for females.

2. Test substance: Technical grade clofentezine (Batch no. 20099/14, 99.3 ± 1.0% purity) were used.

3. Diet preparation: Basal diet consisted of Modified SQC Expanded Rat and Mouse Maintenance Diet No. 1 supplied by Special Diets Services, Ltd.,Stepfield, Witham, Essex, UK. Test diets were prepared weekly and stored at room temperature.

B. STUDY DESIGN

1. Animal assignment: Animals were randomly assigned to test groups as follows:

Test groups No.	Designation	Dose (ppm)	Animals/sex
1	Control	0	40
3	Low (LDT)	400	40
4	High (HDT)	30,000	40

2. Observations schedule

Type of observation	Number of animals per sex per group	Frequency
Mortality	All	Twice a day*
Signs of toxicity	All	Twice a day*
Blood samples	10	At 6 weeks
Necropsy	10	At termination (6 weeks)

C. METHODS

1. Observation of blood samples: Blood was collected from the abdominal aorta of anesthetized animals after 6 weeks on the test diets. Observations included the following:

Total tri-iodothyroxine (Total T_3)	Free T_4 index (FT_4I)	Estradiol
Thyroxine	Thyrotrophin	Progesterone
T_4-binding capacity (TBI)	Testosterone	Dehydroepiandrosterone

2. Necropsy The first 10 animals of each sex from each group were anesthetized and blood samples were collected. The livers of each animal were weighed.

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D. STATISTICAL ANALYSIS

1. Continuous variables: (body weight, clinical chemistry, and liver weight.

Statistical procedure	Purpose
Bartlett's Test	Determine homogeneity of variance.*
One-way analysis of variance	Determine significance of variability among all groups.**
Students "t" test	Determine significance of differences between the control and each treatment group.**
Kruskal-Wallis Test	Detect any significant group differences.***

*If variances are not homogeneous ($p>0.05$), the data are transformed (log transformation is used)
**Used on transformed or untransformed data only when variances are shown to be homogeneous or equal.
***Nonparametric test performed on data with heterogenous variances.

II. REPORTED RESULTS

The report stated that there were no mortalities or clinical signs observed in the study. The only effect on body weight noted was a decrease in the weight of females given the 30,000 ppm diet after 6 weeks. The high dose group mean weight was reported to be 197 g, and that for the control group was 274 g. These values were statistically significantly different ($p<0.001$).

There were statistically significant increases in absolute and relative liver weights for both sexes given the 30,000 ppm diet as well as for males given the 400 ppm diet when compared with controls (see Addendum I below).

Addendum II shows group mean clinical chemistry results. The report noted significant increases in thyroxine, free thyroxine index, thyrotrophin, progesterone, and dehydroepiandrosterone in both sexes at 30,000 ppm. The males given the highest dose also showed a significantly increased level of triiodothyroxine. At the 400 ppm dose level, males exhibited a significant increase in thyroxine levels, and females had increased dehydroepiandrosterone levels (see Addendum II below).

ADDENDUM I

Summary (as reported) of Body Weight, Liver Weight,
and Liver-to-Body Weight Ratio Results

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CLOFENTEZINE TOXICOLOGY REVIEW

Page _____ is not included in this copy.

Pages 49 through 52 are not included in this copy.

The material not included contains the following type of information:

___ Identity of product inert ingredients

___ Identity of product impurities

___ Description of the product manufacturing process

___ Description of product quality control procedures

___ Identity of the source of product ingredients

___ Sales or other commercial/financial information

___ A draft product label

___ The product confidential statement of formula

___ Information about a pending registration action

X FIFRA registration data

___ The document is a duplicate of page(s) _____

___ The document is not responsive to the request

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

006232

DATA EVALUATION RECORD

1. CHEMICAL NAME: NC 21314, Clofentezine, APOLLO® 3,6-bis[2-chlorophenyl]-1,2,4,5-tetrazine

2. TEST MATERIALS: ^{14}C-labelled clofentezine (Batch no. CFQ 2847, specific activity 47.7 mCi/g, >99% radiochemical purity) and unlabelled clofentezine (Batch no. 20099/12, 98.8 ± 1.4% purity were used.

3. STUDY/ACTION TYPE: Metabolism in rats.

4. STUDY IDENTIFICATION: Campbell, J. K., and D. Needham. March 15, 1985. Concentration of (^{14}C) Clofentezine and Its Metabolites in the Plasma of Rats Dosed Orally with Clofentezine at the Rate of 1000 mg/kg Bodyweight. Unpublished report no. METAB/85/2 prepared by by FBC Limited. Submitted by Nor-Am Chemical Co.; EPA Acc. No. 262268.

5. REVIEWED BY:

Name: Roger Gardner
Title: Toxicologist
Organization: Review Section 6
Toxicology Branch
Signature: Roger Gardner
Date: 8-7-87

6. APPROVED BY:

Name: Judith Hauswirth, Ph. D.
Title: Section Head
Organization: Review Section 6
Toxicology Branch
Signature: Judith W. Hauswirth
Date: 8/21/87

7. DISCUSSION AND CONCLUSIONS: The experiment demonstrated that a single 1000 mg/kg dose of clofentezine administered to male and female rats by gavage was readily absorbed. Approximately 40% of the total radioactivity found in the plasma after dosing was attributed to metabolites of clofentezine. Peak levels of radioactivity were observed in the plasma 6 to 8 hours after dosing and were reported to be 15.6 and 14.1 mg clofentezine per liter of plasma for males and females, respectively. By the 24-hour observation, plasma levels of radioactivity declines to 3.3 and 3.1 mg/l for males and females, respectively, and the plasma half life was calculated to be 3.6 hours. There were adequate data to indicate that uptake and elimination of radiolabelled clofentezine was similar in male and female rats.

Core classification: Acceptable

8. MATERIALS AND METHODS

Test species: Male and female Sprague-Dawley CD rats (24 per sex) were used in the atudy. Weight ranges were given for males as 144-167 g., and 125-152 g. for females at the beginning of the experiment.

Sampling and analysis: Blood samples were collected by cardiac puncture from anesthetized animals and centrifuged to separate the plasma from red cells. Total radioactivity was determined by mixing 100 ul plasma with 100 ul Fisofluor

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8. MATERIALS AND METHODS (continued)

MPC scintillation cocktail and counting the diluted samples with a Beckman LS250 liquid scintillation counter.

The plasma concentration of radiolabelled clofentezine in plasma samples was determined by high pressure liquid chromatography (HPLC) and a 4.6 X 250 mm Zorbax ODS column. Control plasma was spiked with clofentezine (0-15 ppm) to prepare a standard curve. The plasma was extracted twice with 1-chlorobutane, and the pooled extracts were evaporated to dryness under a stream of nitrogen at 40° C. The residues were taken up in 100 ul aliquots of methanol for HPLC. According to the report, the clofentezine peaks were collected as they eluted and assayed for radioactivity to determine extraction efficiency.

Five-hundred ml samples of plasma from test animals were extracted in the same manner as control plasma samples were in the calibration procedure except that no clofentezine was added to the experimental samples. The concentration of test substance in plasma of treated animals was then determined by comparison of the peak heighths of test samples with those of the standard curves.

Preparation of dosing suspensions: Clofentezine was suspended in aqueous gum tragacanth at a specific activity of 0.024 mCi/g.

Experimental design: Groups of 24 male and 24 female rats were given doses of 1000 mg/kg by gavage, and blood samples were taken 1, 2, 4, 6, 8, 10, 18, and 24 hours after dosing. There were three animals of each sex for each sample time.

9. REPORTED RESULTS

The Addendum below shows the results as reported. Based on those observations, the investigators concluded that approximately 40% of the total radioactivity in the plasma after dosing was associated with metabolites of clofentezine. Peak levels of radioactivity were observed in the plasma 6 to 8 hours after dosing and were reported to be 15.6 and 14.1 mg clofentezine per liter of plasma for males and females, respectively. By the 24-hour observation, plasma levels of radioactivity declined to 3.3 and 3.1 mg/l for males and females, respectively, and the plasma half life was calculated to be 3.6 hours.

ADDENDUM

Reported Plasma Concentrations of Radiolabelled Clofentezine after a Single Oral Dose of 1000 mg/kg in Male and Female Rats

Page _____ is not included in this copy.

Pages 56 through 57 are not included in this copy.

The material not included contains the following type of information:

___ Identity of product inert ingredients

___ Identity of product impurities

___ Description of the product manufacturing process

___ Description of product quality control procedures

___ Identity of the source of product ingredients

___ Sales or other commercial/financial information

___ A draft product label

___ The product confidential statement of formula

___ Information about a pending registration action

X FIFRA registration data

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The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

006232

DATA EVALUATION RECORD

1. CHEMICAL NAME: NC 21314, Clofentezine. APOLLO® 3,6-bis[2-chlorophenyl]-1,2,4,5-tetrazine

2. TEST MATERIALS: ^{14}C-labelled clofentezine (Batch no. CFQ 2874, specific activity 47.7 mCi/g, 100% radiochemical purity) and unlabelled clofentezine (Batch no. 20099/5, 98.6 ± 1.0% purity were used. Radiolabelled clofentezine was mixed with unalabelled material to obtain a specific activity of 5.18 (± 0.05) mCi/g. A 1.05467 g aliquot of that mixture was added to 1.6 ml blank formulation (without active ingredient) to make up the radiolabelled formulation to be tested.

3. STUDY/ACTION TYPE: Dermal absorption in rats.

4. STUDY IDENTIFICATION: Campbell, J. K., and D. Needham. February 3, 1986. Dermal Absorption of [^{14}]-Clofentezine by Male Rats Given a Topical Application of 50 SC Formulation. Unpublished report no. METAB/86/1 prepared by by FBC Limited. Submitted by Nor-Am Chemical Co.; EPA Acc. No. 262268.

5. REVIEWED BY:

Name: Roger Gardner
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Organization: Review Section 6 Date: 8-7-87
Toxicology Branch

6. APPROVED BY:

Name: Judith Hauswirth, Ph. D.
Title: Acting Head Signature: Judith W. Hauswirth
Organization: Review Section 6 Date: 8/21/87
Toxicology Branch

7. DISCUSSION AND CONCLUSIONS: Radiolabelled clofentezine in a 50 SC formulation was applied to the skin of male rats at dose levels of 4.8, 44, or 180 mg per kg body weight to evaluate the dermal absorption of the active ingredient. There were adequated data presented to indicate that absorption was low (less than 1%) during the 10 hours that followed application of all doses.

Core classification: Acceptable

8. MATERIALS AND METHODS

Test species: Male Sprague-Dawley CD rats were used in the atudy. Weights ranged from 189 to 236 g., and the animals were approximately 8 weeks of age at the beginning of the experiment.

Dose preparation: The report stated that a final volume of 2.2 ml test solution contained 455 mg active ingredient per ml. This dose suspension was first diluted with water to obtain a clofentezine concentration of 100 mg/ml, and a second dilution was performed to obtain a concentration of 10 mg/ml.

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8. MATERIALS AND METHODS (continued)

Experimental design: According to the report, three groups each containing six male rats had the hair shaved from test sites on their backs 24 hours prior to dosing. Each group received a measured volume of one of the three suspensions described above, and the dose was spread over a 26 cm^2 area of the prepared skin. Following application and drying of the test material on the skin, application sites were covered with two layers of gauze which was held in place with fabric elastoplast. The rats were then housed individually in metabolism cages.

Dose suspensions were sampled at the time of dosing, and urine, feces, and cage washings were collected 10 hours after dosing for radioassay. Dressings covering the test sites, gauze used to wash the test sites, and pipets used in dosing were also collected 10 hours following dosing, and radioactivity was recovered by extraction with methanol.

Treated skin was washed with soft soap at necropsy according to the report, and washed skin from treated sites, gastrointestinal tract, and carcass were collected and prepared for subsequent radioassay. Blood samples were collected from the abdominal aorta. All of these samples were also collected 10 hours after application of test suspensions.

Preparation and analysis of samples: Blood samples were oxidized, and resulting $^{14}CO_2$ was assayed for radioactivity by liquid scintillation counting. Samples of skin, gastrointestinal tract and carcass were solubilized in a mixture of sodium hydroxide (80 g), Triton X 405 (100 g.), methanol (300 ml.) and water (600 ml.). Aliquots of these preparations were neutralized and counted by liquid scintillation. The scintillation cocktail used was FHV multi-phase cocktail.

Feces were homogenized in 9 volumes of water, and aliquots (approximately 1 g.) were digested for counting in the same manner as tissue and carcass samples. Cage washings were analyzed in the same manner as the fecal homognates.

Aliquots of urine were counted directly in scintillation cocktail (see above).

9. REPORTED RESULTS

Particle size distribution for the commercial formulation and test substance wwer presented as follows:

Formulation	Test sample
Not less than 40% below 2 u	43% below 2.4 u
Not less than 80% below 5 u	83.7% below 5 u
Not less than 90% below 8 u	96.6% below 8.2 u

The report described an error in the preparation of doses as follows:

> ...the concentration of the dose suspension for group 3 (undiluted) was 269 mg/ml instead of 455 mg/ml...too much blank formulation had

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9. REPORTED RESULTS (continued)

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been added. It was not feasible to increase the volume of dose applied to the group 3 animals to compensate for the reduced clofentezine concentration but the dilutions for groups 2 and 1 were altered so as to bring the dose suspension closer to the nominal concentration. Consequently, Groups 2 and 1 received the correct dose.

The report stated that nominal doses for Groups 1, 2, and 3 were 3, 30, and 300 mg/kg respectively, and actual doses were determined to be 4.8, 44, and 180 mg/kg for Groups 1, 2, and 3, respectively.

The reported distribution (group mean % of dose) of the applied dose is shown as follows:

Location	Actual dose (mg/kg) 180	44	4.8
On dressing	43.01 ± 20.05	47.89 ± 16.34	58.61 ± 6.46
In wash	46.57 ± 18.44	20.69 ± 6.67	7.76 ± 2.18
On application site	10.37 ± 4.38	32.11 ± 11.81	31.16 ± 5.05
In urine	<0.01	0.01 ± 0.01	0.06 ± 0.02
In feces	<0.01	<0.01	<0.01
In GI tract	<0.01	0.03 ± 0.01	0.32 ± 0.06
In carcass	0.08 ± 0.06	0.09 ± 0.06	0.23 ± 0.20
Recovery	100.22 ± 1.62	100.76 ± 1.82	98.14 ± 0.48

The report also stated that no radioactivity was found in blood.

The investigators noted that a significant proportion of the dose remained on the skin after washing, but the percentage of the dose absorbed was small for all three groups (0.61, 0.08, and 0.09% in groups 1, 2, and 3, respectively).

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DATA EVALUATION RECORD

1. CHEMICAL NAME: NC 21314, Clofentezine, APOLLO® 3,6-bis[2-chlorophenyl]-1,2,4,5-tetrazine

2. TEST MATERIALS: ^{14}C-labelled clofentezine (Batch no. CFQ 2874, specific activity 47.7 mCi/g, >99% radiochemical purity) and unlabelled clofentezine (Batch no. 20099/12, 98.8 ± 1.0% purity; and batch no. 20099/14, 99.3 ± 1.0% purity) were used.

   Radiochemicals also used in the studies included: ^{131}I-sodium iodide (supplied in sodium hydroxide solution with specific activity of 40 mCi/ml and radiochemical half-life of 8.04 days), ^{125}I-thyroxine (supplied in ethanol/water (3:1) solution with a specific activity of 200 uCi/ml with a radiochemical half-life of 60 days).

3. STUDY/ACTION TYPE: Metabolism (thyroid function assays in rats)

4. STUDY IDENTIFICATION: Challis, I. R., and C. L. Creedy. December 20, 1985. The Effects of Thyroid Function. Unpublished report no. METAB/85/36 prepared by by FBC Limited. Submitted by Nor-Am Chemical Co.; EPA Acc. No. 262268.

5. REVIEWED BY:

   Name: Roger Gardner
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   Toxicology Branch

6. APPROVED BY:

   Name: Judith Hauswirth, Ph. D.
   Title: Acting Head Signature: Judith W. Hauswirth
   Organization: Review Section 6 Date: 8/24/87
   Toxicology Branch

7. CONCLUSIONS: A series of three experiments was conducted to determine the relationship between treatment of rats with clofentezine in the diet and thyroid effects observed in chronic and subchronic toxicity studies. The experiments are generally accepted as additional information in the assessment of results of the other feeding studies.

   Tissue residue accumulation study: Male and female rats were given radiolabelled clofentezine by gavage twice a day for 1 or 10 days. Determination of tissue residue levels after 1 and 10 days of treatment indicated no preferential accumulation of clofentezne in the thyroid.

   Thyroxine half-life study: Male rats were dosed twice daily with aqueous sodium iodide for 7 days. On the fourth day the animals received an intravenous injection of 50 ul radiolabelled thyroxine solution. The rats were then given diets containing 0 or 30,000 ppm clofentezine for 29 days at which time the 7-day regimen of sodium iodide and ^{125}I-thyroxine dosing was repeated. A slight decrease in thyroxine half-life was observed in treated

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7. CONCLUSION (continued)

rats when compared to control animals which exhibited a slight increase in thyroxine half-life after the one-month treatment period.

Thyroid iodine uptake study: Groups of male and female rats or mice were given diets containing 0 or 30,000 ppm clofentezine for 4 weeks. At the end of the 4-week period the animals received an intraperitoneal dose of ^{131}I-sodium iodide. There was a significant and rapid increase in thyroid uptake of iodine in clofentezine treated rats following intraperitoneal dosing with [^{131}I]-sodium iodide. This effect was not seen in mice.

8. BACKGROUND

According to the report, the experiments described below were conducted because of results observed in previous studies (references). Those results included:

1. A higher incidence of thyroid follicular cell tumors observed in male rats after 27 months on a diet containing 400 ppm clofentezine. The male rats also exhibited elevated serum levels of thyroixne at 27 months in the chronic study (reference).

2. Observed increases in serum thyroxin and thyrotrophin levels in male rats treated with a dietary level of 30,000 ppm clofentezine for six weeks, and increased thyroxin levels in male rats given diets containing 400 ppm in the same study (reference).

9. MATERIALS AND METHODS

Test species: Male and female Sprague-Dawley CD rats and male and female CD1 mice were used in the assays. Weight ranges were given in the report as follows:

Tissue residue accumulation study	Rats -	Males: 205 - 230 g. Females: 160 - 185 g.
Thyroxine half-life study	Rats -	Males: 169 - 193 g.
Thyroid iodine uptake study	Rats -	Males: 144 - 163 g. Females: 138 - 156 g.
	Mice -	Males: 25 - 34 g. Females: 19 - 28 g.

Sampling and analysis: Bone samples were weighed and combusted in an oxidizer. The radiolabelled CO_2 was trapped in Carbosorb/Permafluor V for scintillation counting.

Other tissues including ovaries, thyroids, pituitaries, eyes, muscle, and fat were solubilized in 1 ml of a solution of sodium hydroxide (80 g), Triton X405 (100 ml), methanol (300 ml), and water (600 ml). The samples were completely solubilized at 60° C in a sonic bath, and after completion of that process, PCS scintillant was added. The samples were then counted. In the iodine uptake

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9. MATERIALS AND METHODS (continued)

study, whole thyroids were placed in plastic containers and counted with a gamma counter.

All other tissues were homogenized in water, and 0.5 ml aliquots of the homogenate (10% w/v) was solubilized and counted as described above.

For the tissue residue accumulation study, 25 to 100 ul aliquots of blood were decolorized with hydrogen peroxide and counted directly in PCS scintillant. Twenty ul aliquots of blood were collected in capillary tubes and counted in glass or plastic tubes with a gamma counter. Blood samples (1 ml for rats and 20 ul for mice), taken in the iodine uptake experiments, were counted in the same manner.

Calculations: All samples in the tissue residue accumulation experiment were counted in triplicate where possible. Residue levels were calculated as mg/kg in tissue and mg/l in blood and plasma samples.

Counts per minute (cpm) in the thyroxine half-life study were plotted against time on a log linear scale, and the best fit straight line was determined from these data by the least squares method. The thyroxine half-life for each rat was calculated from the resulting line. Group mean half-life values were determined from the individual lines for each rat in a group, and group means were tested for significant differences by the Mann Whitney test. Statistical differences were tested by the Mann Whitney test in the iodine uptake study also.

Experimental design - Tissue residue accumulation study: Ten male and 10 female rats were given radiolabelled test substance (suspension in 0.5% gum tragacanth, 4 uCi/ml, 1 ml/200 g body weight) by gavage twice a day for 1 (5 rats per sex) or 9 days (5 rats per sex).

After the first day of dosing, blood samples were taken from 5 anesthetized rats per sex by cardiac puncture. Then the animals were sacrificed and liver, kidney, spleen, gonads, lung, heart, adrenals, fat, muscle, eyes, brain, bone, thyroid, and pituitary samples were collected for analysis. Aliquots of blood were taken for analysis also; some were centrifuged for plasma.

The remaining animals were dosed for 9 days, and evaluated in a similar manner on the 10th day of the experiment.

Experimental design - Thyroxine half-life study: Thirty-two male rats were dosed twice daily with aqueous sodium iodide for 7 days. The report stated that on the fourth day the animals received an intravenous injection (via the tail vein) of 50 ul radiolabelled thyroxine solution. Blood samples were collected in glass capillaries from snipped tails of 26 rats 2, 4, 6, 8, 17, 21, 24, 26, 43, 46, 48, 50, 67, and 72 hours after thyroxine was administered. Immediately after the last blood sample was collected, 13 of the rats were placed on a diet containing 30,000 ppm clofentezine, and the remaining 13 rats were given untreated diet. (All rats received untreated diet during the first 7 days of the experiment.)

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9. MATERIALS AND METHODS (continued)

The rats were maintained on the test diets for an additional 29 days before the 7-day regimen of sodium iodide and ^{125}I-thyroxine dosing was performed again. Blood samples were collected as before, but only 10 animals from each group receiving the control or treated diet were used.

Experimental design - Thyroid iodine uptake study: Groups of 20 male and 20 female rats or mice were given diets containing 0 or 30,000 ppm clofentezine for 4 weeks. At the end of the 4-week period the animals received an intraperitoneal dose of ^{131}I-sodium iodide solution (100 ul, 50 uCi/ml), and six and 24 hours after the injection, 10 animals from each group, sex, and species were anesthetized for collection of blood samples. The animals were then sacrificed and the thyroid glands were removed for analysis.

10. REPORTED RESULTS

Tissue residue accumulation study: Addendum I shows the results as reported. The investigators noted that one day after dosing maximum residues were found in the liver and kidney (1.5 mg clofentezine/kg tissue). Levels in other tissues were reported to range in male rats from 0.04 mg/kg in brain to 1.4 mg/kg in fat and from 0.1 to 0.4 mg/kg in blood-rich tissues such as heart, lungs, spleen, adrenals, and thyroid in males. The investigators noted that the residue levels found in the thyroid and pituitary (0.3 - 0.4) were similar to levels found in blood indicating that rapid accumulation of clofentezine residues in these tissues was not occurring.

The report stated that after 10 days' dosing, the residue levels in liver and kidneys rose to 3.6 to 5.1 mg/kg tissue with a corresponding rise in other tissues. The mean rise in residue levels after 10 days of dosing (expressed as the ratio of the 10-day level and the 1-day level) was 2.7 for males and 3.0 for females (see Addendum I below). The report pointed out the similarity between the rise of residues in the thyroid and pituitary to the mean for all tissues. Based on these results, the investigators concluded that there was no apparent accumulation of clofentezne residues after 10 twice-daily 20 mg/kg doses in the thyroid and pituitary glands that could be correlated to observed thyroid effects (see 8. BACKGROUND above).

Thyroxine half-life study: According to the report, the mean thyroxine half-life in rats on control diet changed from 16.70 to 17.61 hours after a month. The respective values for animals receiving the 30,000 ppm diet were 17.05 and 16.42 hours after a month. Statistical analysis (Mann Whitney test) showed no significant differences. The investigators noted that statistical analysis of the individual animal results indicated significant treatment-related decreases in the thyroxine half-live (approximately 9%). Based on these results, the investigators described the changes as slight and variable, but they concluded that the changes may be significant over longer periods of time (see Addendum II below for additional information).

Thyroid iodine uptake study: The report stated that there was a rapid uptake of iodine in rats given the 30,000 ppm diet in comparison to the uptake observed for control group rats. At six hours after administration of radiolabelled sodium iodide, the level in treated males was 1.7 times that for controls, and in females the radiolabel was found at levels 2.7 times that for control group

9. MATERIALS AND METHODS (continued)

females (see Addendum III below). There was no effect on iodine uptake observed in mice. Blood iodine levels were significantly reduced in both species six hours after iodine administration, and the authors suggested that this observation may be the result of increased iodine excretion or increased uptake by the thyroid (in rats). The report noted that at the 24-hour observation, higher levels of radiolabelled iodine were found in the thyroids of both species than was seen at the 6-hour observation. The blood levels observed at that time in the rat remained lower than those seen in the control group rats. Blood levels in the treated and control mice were comparable at 24 hours.

11. DISCUSSION

The authors concluded:

> ...extensive and preferential accumulation of clofentezine in the thyroid does not appear to be responsible for any observed toxicological effect in subchronic and chronic studies.
>
> Dietary treatment of male rats with clofentezine at 30,000 ppm for 1 month resulted in a slight decrease in thyroxine half-life compared to control animals where a slight increase occurred over this time period.
>
> ...in rats there was a significant and rapid increase in thyroid uptake of iodine (compared to control animals) following intraperitoneal dosing with [^{131}I]-sodium iodide. This effect was not seen in mice.

There were adequate data presented in the report to support the conclusions of the investigators.

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ADDENDUM I

Results as Reported on the Thyroid Residue
Accumulation Study in Rats

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CLOFENTEZINE TOXICOLOGY REVIEW

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___ Identity of product inert ingredients

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The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

R. H. 8-25-87

006232

Reviewed by: Roger Gardner
Section 6, Toxicology Branch (TS 769C)
Secondary Reviewer: Judith Hauswirth, Ph. D. JH 5/25/87
Section 6, Toxicology Branch (TS 769C)

DATA EVALUATION RECORD

STUDY TYPE: Chronic feeding/Oncogenicity (Guideline §83-5)

ACCESSION NUMBER: 262261, 262262

TEST MATERIAL: Technical grade Clofentezine (CR 20099/12, purity 98.7%) was used. It was described as a magenta colored crystaline substance.

SYNONYMS: Clofentezine; NC 21314; APOLLO®; 3,6-bis(2chlorophenyl)-1,2,4, 5-tetrazine

STUDY NUMBER(S): TOX/ 84/167-70

SPONSOR: FBC Limited; Nor-Am Cehmical Co.

TESTING FACILITY: Huntingdon Research Centre

TITLE OF REPORT: The Oncogenicity and Chronic Toxicity of Technical NC 21314 (Chlofentezine in the Diet to the Rat (Final Report).

AUTHOR(S): Ginnocchio, A. V.; and Mallyon, B. A.

REPORT ISSUED: December 17, 1985

CONCLUSIONS: Clofentezine was administered in the diet to male and female Charles River Crl:CD Sprague-Dawley BR strain rats for up to 27 months at levels of 0, 10, 40, or 400 ppm. Clofentezine treatment was associated with a slight increase in free thyroxine levels in blood taken from males at 27 months. Liver weights and weight ratios for high dose group males were increased above those values for controls (by approximately 20%) and for high dose group females (by approximately 10%). Those group differences were statistically significant. Histological changes occurred in the liver of males in a dose-related manner, and they included centrilobular hepatocyte hypertrophy and vacuolation, focal cystic degeneration of hepatocytes, and diffuse distribution of fat deposits in the livers of high dose group males.

Based on these observations, a no-effect level was established in the experiment at 40 ppm (2 mg/kg/day), and the lowest-effect level was 400 ppm (20 mg/kg/day).

The incidence of mammary tumors in female rats decreased with dose, and according to the report, the incidence of thyroid follicular cell tumors in male rats exhibited a statistically significant positive dose-related trend ($p<0.05$) primarily because of the highest dose group's higher incidence in comparison to that in the control group. Reported incidences were adjusted by censoring those animals that died before the first diagnosis was made (week 88), and a

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CONCLUSIONS (continued)

Fisher's Exact test on the control and high dose group incidences showed the difference between the groups was statistically significant ($p = 0.024$). The adjusted results also showed a significant positive trend ($p < 0.005$; Cochran-Armitage test).

Core classification: Minimum

I. PROTOCOL

A. MATERIALS

1. Test compound: The test compound is described as a magenta crystaline substance with unspecified purity of 98.7%.

2. Test species: Male and female 4-week old Charles River Crl:CD Sprague-Dawley BR strain rats were used. The males weighed from 57 to 79 g, and the females weighed from 53 to 78 g on receipt at the laboratory. The animals were approximately 6 weeks of age when placed on test diets.

3. Diet preparation: Basal diet consisted of Modified SQC Expanded Rat and Mouse Maintenance Diet No. 1 supplied by Special Diets Services, Ltd.,Stepfield, Witham, Essex, UK. Test diets were prepared weekly and stored at room temperature. Samples of test diets were analyzed for stability, homogeneity and accuracy of test concentration before the study was begun. Diets were analyzed for concentration of test substance during weeks 1, 2, 3, 4, 8, 11, 15, 16, 18, 22, 25, 29, 30, 33, 37, 41, 45, 49, 53, 57, 61, 65, 69, 73, 77, 81, 85, 89, 93, 97, 101, 105, 108, 112, 116, and 120.

B. STUDY DESIGN

1. Animal assignment: Animals were randomly assigned to test groups as follows:

Test groups		Dose	Animals per sex		
No.	Designation	(ppm)	Main study*	Pre-test	Interim Sacrifice**
1	Control	0	50	10	20
2	Low (LDT)	10	50	10	20
3	Mid	40	50	10	20
4	High (HDT)	400	50	10	20

*30 months.
**At 12 months

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2. Observations schedule

Type of observation	Number of animals per sex per group	Frequency
Mortality	All	Twice a day*
Signs of toxicity	All	Twice a day*
Body weight	All	On day of arrival at laboratory, at pre-test during randomized group assignment, at weekly intervals through the first 13 weeks, biweekly thereafter, and on the day of necropsy.
Food consumption	All	Weekly**
Water consumption	All	Weekly**
Ophthalmology	50†	At 12 months
Blood samples	10***	Pre-test
	10	At 6, 12, 18, and 27 months.
Urine samples		
Necropsy	Animals found dead or moribund	When found.
	20†	At 12 months
	50†	At 27 months

*The report stated that observations were made only once each day on weekends and holidays. The signs, their severity, time of onset, and duration were recorded.
**For each cage of 5 animals.
***These animals were discarded from the study.
†All survivors in the main study group only.

C. METHODS

1. Observation of blood samples: Blood was collected from the retro-orbital sinus, and the animals were fasted overnight prior to sampling.

a. Hematology

X Hematocrit	X Differential white cell counts
X Hemoglobin	X Mean corpuscular hemoglobin concentration
X Red cell count	X Mean cell volume
X Platelet count	X Mean corpuscular hemoglobin
X Total white cell count	

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C. METHODS (continued)

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1. Observation of blood samples (continued)

b. Blood chemistry

X Total protein	X Uric acid	X Alkaline phosphatase (AP)
X Albumin	X Glucose	X Lactate dehydrogenase (LDH)
X Total globulins	X Total cholesterol	X Triglycerides
X Albumin/globulin ratio	X Total bilirubin	
X Blood urea nitrogen	X Aspartate aminotransferase (AST)	
X Electrolytes	X Alanine aminotransferase (ALT)	
X Creatinine		

c. Additional tests: The following tests were conducted after the final sacrifice (27 months).

Total tri-iodothyroxine (Total T_3)	T_4-binding capacity (TBC)	Testosterone
Thyroxine	Free T_4 index (FT_4I)	Estradiol
	Thyrotrophin	Progesterone

2. Urine observations

X Volume	X glucose	X occult blood	X specific gravity
X pH	X ketones	X urobilinogen	X microscopic examination of centrifuged deposits
X protein	X bilirubin		

3. Necropsy Gross lesions were noted.

a. Weighed organs

X Liver	X Spleen	X Adrenals	X Brain
X Kidneys	X Heart	X Gonads	X Pituitary

b. Tissues examined microscopically

X Adrenals	X Liver	X Kidneys
X Aorta	X Lungs	X Spleen
X Bone and marrow	X Lymph nodes	X Spinal cord
X Brain	X Mammary glands	X Stomach
X Cecum	X Esophagus	X Testes/ovaries
X Colon	X Pancreas	X Thymus
X Duodenum	X Pituitary	X Thyroid with parathyroid
X Ears	X Prostate	X Trachea
X Epididymides	X Rectum	X Urinary bladder
X Eyes with Harderian gland	X Salivary gland	X Uterus with cervix
X Heart	X Seminal vessicles	X Vagina
X Ileum	X Sciatic nerve	X All macroscopic abnormalities
X Jejunum	X Skeletal muscle	
	X Skin and subcutis	

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D. STATISTICAL ANALYSIS

1. Continuous variables: (body weight, hematology, clinical chemistry, organ and weights.

Statistical procedure	Purpose
Bartlett's Test	Determine homogeneity of variance.*
One-way analysis of variance	Determine significance of variability among all groups.**
Students "t" test	Determine significance of differences between the control and each treatment group.**
Kruskal-Wallis Test	Detect any significant group differences.***

*If variances are not homogeneous ($p>0.05$), the data are transformed (log transformation is used)
**Used on transformed or untransformed data only when variances are shown to be homogeneous or equal.
***Nonparametric test performed on data with heterogenous variances.

2. Frequency data (gross and microscopic observations at necropsy)

( ) One-tailed or (X) two-tailed probability values were calculated.

Statistical procedure	Purpose
Fisher's Exact Test Chi-square Tests with Yates' correction	Determine significance of differences between individual groups, overall variations, or trends
Kruskal-Wallis Test	Determine significance of differences for graded responses.

3. Other methods: (data adjustments or transformations, criteria of significance, etc)

II. REPORTED RESULTS 006232

An 18-month interim report (Ginnocchio and Mallyon, 1983) on this study was reviewed previously (see Addendum I below). Therefore, the results described in this section are primarily from observations made during the 18th through 27th months of the study.

A. Mortality and Signs of Toxicity: No treatment-related effects were observed according to the report. Mortality during the last 9 months of the study is summarized as follows:

Dose (ppm)	Mortalities* during weeks Males 78-104	Males 104-120	Females 78-104	Females 104-120
0	13	8	19	6
10	15	7	18	9
40	13	4	14	7
400	16	3	18	4

*Excludes those animals sacrificed at 12 months and during weeks 52 through 77.

Soiling of the eyes and nose, hair loss, and abrasions were the most frequently observed clinical signs, but the report stated that these occurred at a low incidence which was unrelated to treatment.

- Body Weight, Food and Water Consumption: These observations in treated groups were described as comparable to those in the control groups throughout the study.

- Test Substance Intake: The average daily intake for treated animals was reported as follows:

Dose (ppm)	Daily dose (mg/kg/day) Males	Females	Both sexes
10	0.43	0.55	0.49
40	1.72	2.18	1.95
400	17.3	22.1	19.7

Clinical Pathology

Hematology: The investigators noted that there were sporadic statistically significant differences between treated and control groups, but the differences were not dose-related, and they were within normal ranges. A decrease in hemoglobin and mean cell hemoglobin concentration was noted for the 400 ppm females when compared to those values in the control females. The group mean hemoglobin values for the control and high dose group females were 14.9 and 14.1 g/dl, respectively ($p<0.01$) at 18 months. The respective group means at 27 months were 15.8 and 13.8 g/dl. The mean cell hemoglobin concentrations at 18 months were 19.9 pg in the control group and 19.7 pg for the high dose group (no statistically significant difference). At 27

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1. Hematology (continued):

months the control and high dose group means were 15.8 and 14.8 pg, respectively ($p<0.05$).

2. Clinical chemistry: The only significant difference noted by the authors at 27 months was in the group mean thyroxine level of 400 ppm males in comparison to that of control group males. The group mean for the control group was reported as 13.4 pmol/l while that for the 400 ppm dose group was 19.7 pmol/l ($P<0.05$).

Although the investigators noted other statistically significant differences for clinical chemistry parameters, these were described as unrelated to dose or not occurring consistently during the course of the experiment.

3. Urinalysis: The only observation the authors described as unusual was discoloration of the urine from female rats given the 400 ppm diet. The urine from these animals appeared bright orange, and the color was attributed to the test substance or its metabolites in the urine.

E. Ophthalmology: There were no treatment-related effects observed according to the report.

F. Necropsy

1. Organ weights: The only treatment related effect on organ weight was observed for the liver. Group mean values for those animals sacrificed at 27 months are summarized as follows:

	Dose level (ppm)			
	Males		Females	
Observation	0	400	0	400
Body weight (g)	563	589	415	430
Liver weight (g)	13.74	17.05†	10.12	11.37*
Liver/body weight ratio (%)	2.44	2.90 **	2.45	2.66**

*Statistically significantly different from controls ($p<0.05$)
**Statistically significantly different from controls ($p<0.01$)
†Statistically significantly different from controls ($p<0.001$)

. Gross observations: There were no gross observations reported at necropsy of animals after the interim or terminal sacrifice that the investigators could associate with treatment.

. Histopathology:

. Non-neoplastic observations

) General observations: Several lesions were observed to occur in statistically significant patterns such as trends, apparently treatment related effects, or a difference between one treated group and an appropriate control group (see Tables 1a and b for examples). However, most of these observations

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a. Non-neoplastic observations (continued)

were not associated by the investigators with the test substance because trends were not observed consistently at the interim and terminal sacrifices or in animals that died during the experiment, and trends and differences were not significant when overall incidences (total of all animals) were analyzed.

Table 1a

Summary of non-neoplastic lesions reported to exhibit statistically significant differences between groups or a dose-related trend in male rats.

Observation	Dose level (ppm) 0	10	40	400
Epididymis:				
Reduced spermatozoa				
Intercurrent deaths†††	5/26	4/26	13/23**	8/29
Kidney:				
Chronic interstitial nephrosis				
Interim sacrifice†	0/20	3/20	0/20	4/20
Hydronephrosis				
Interim sacrifice	3/20	2/20	1/20	1/20
Intercurrent deaths††	4/26	7/26	2/23	1/29
Terminal sacrifice	1/24	3/24	3/27	1/31
Overall	8/70	10/70	6/70	2/70
Pyelitis				
Overall****	0/70	3/70	8/70	2/70
Increased fat deposition (cortical tubules				
Intercurrent deaths††	3/26	3/26	1/23	0/29

*Statistically significant negative dose-related trend ($p<0.01$).
**Statistically significantly different from controls ($p<0.05$).
***Overall group variation statistically significant ($p<0.05$).
****Overall group variation statistically significant ($p<0.01$).
†Statistically significant positive dose-related trend ($p<0/05$).
††Statistically significant negative dose-related trend ($p<0.05$).

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Table 1b

Summary of non-neoplastic lesions reported to exhibit statistically significant differences between groups or a dose-related trend in female rats.

Observation	Dose level (ppm) 0	10	40	400
Adrenal medulla:				
Focal hyperplasia				
Interim sacrifice	0/20	0/20	0/20	0/20
Intercurrent deaths†	1/25	5/31	3/28	0/27
Terminal sacrifice	5/20	4/19	5/20	5/23
Overall	6/65	9/70	8/68	5/70
Kidney:				
Glomerular tubular nephropathy				
Interim sacrifice	1/20	3/20	0/20	0/20
Intercurrent deaths††	6/30	7/31	7/29	14/27*
Terminal sacrifice	6/20	7/19	8/29	8/23
Overall	6/70	7/70	8/69	22/70
Pelvic mineralization				
Interim sacrifice	6/20	5/20	6/20	8/20
Intercurrent deaths	23/30	15/31	18/29	17/27
Terminal sacrifice†††	12/20	7/19	14/20	20/23
Overall	41/70	27/70	38/69	45/70
Increased fat deposition (cortical tubules)				
Intercurrent deaths††	1/30	2/31	5/29	6/27
Liver:				
Foci/areas of telangiactasis				
Intercurrent deaths††	2/30	3/31	3/30	6/27
Terminal sacrifice††	10/20	9/19	11/20	16/23
Parathyroid:				
Hyperplasia				
Terminal sacrifice	3/20	8/18*	8/17*	2/22
Overall	3/65	8/60*	8/58*	2/66

†Statistically significant negative dose-related trend ($p<0.01$).
††Statistically significant positive dose-related trend ($p<0/05$).
†††Statistically significant positive dose-related trend ($p<0/01$).
*Statistically significantly different from controls ($p<0.05$).

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a. Non-neoplastic observations (continued)

Hardarian gland: The investigators noted that a subclinical epidemic of a sialodacryoadenitis virus infection was present in test animals at the time of the interim sacrifice and affected the test groups unevenly (see Addendum below for additional information). An increased incidence of male rats with mononuclear cell infiltration of the hardarian gland in the 400 ppm group observed at that time was attributed to the infection since animals dying in the latter portion of the study as well as animals sacrificed at termination of the study did not exhibit the effect.

Spleen: The degree of pigment deposition was affected in a statistically significant manner (a positive dose-related trends in female rats). The incidence of those lesions is summarized as follows:

Observation	Dose level (ppm) 0	10	40	400
Interim sacrifice				
Minimal	5/20	2/20	0/20	0/20
Slight	6/20	11/20	4/20	6/20
Moderate	8/20	7/20	11/20	11/20
Marked	1/20	0/20	5/20	3/20
Intercurrent deaths*				
Minimal	7/30	10/31	5/30	2/27
Slight	10/30	9/31	9/30	10/27
Moderate	10/30	12/31	12/30	13/27
Marked	3/30	0/31	3/30	2/27
Terminal sacrifice				
Minimal	10/20	0/19	14/20	0/23
Slight	9/20	9/19	3/20	7/23
Moderate	1/20	9/19	2/20	12/23
Marked	0/20	1/19	1/20	4/23

*Statistically significant positive dose-related trend when severity of lesion is considered ($p<0.01$).
**Statistically significant positive dose-related trend when severity of lesion is considered ($p<0.05$).

When sections were stained with Perl's stain and examined, only a slightly significant ($p<0.1$) increase in pigment deposition was noted in females at the interim sacrifice. The reported incidences are as follows:

Observation	Dose level (ppm) 0	10	40	400
Minimal	0/20	0/20	1/20	0/20
Slight	1/20	0/20	2/20	1/20
Moderate	13/20	11/20	6/20	6/20
Marked	6/20	8/20	10/20	12/20
Severe	0/20	1/20	0/20	1/20

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a. Non-neoplastic observations (continued)

The report indicated that these lesions were unlikely to be associated with treatment.

ii) Organs affected by test substance

Liver: Males exhibited dose-related effects, and incidences of some of some of the most frequently observed lesions are summarized in Table 2 below. According to the report female rats were not affected in a manner that could be associated with administration of the test substance.

The incidence of male rats with focal cystic hepatocyte degeneration was not statistically significantly different for treatment groups when compared with controls, and a significant dose-related trend ($p<0.05$) was found only when severity of the lesion was considered for animals dying during the study. The incidence of cystic hepatocyte degeneration in those animals is summarized as follows:

Observation	Dose level (ppm) 0	10	40	400
Number examined	26	26	23	29
Focal cystic degeneration of hepatocytes* (minimal)	5	4	2	9
(slight)	2	1	1	1
(moderate)	1	0	0	0

*Statistically significant trend when severity of lesion was taken into account.

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a. Non-neoplastic observations

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ii) Organs affected by test substance (continued)

Table 2

Summary of non-neoplastic lesions reported to exhibit statistically significant differences between groups or a dose-related trend in the liver of male rats.

Observation	Dose level (ppm) 0	10	40	400
Fat deposits in non-specific distribution				
Intercurrent deaths	4/26	1/26	3/23	8/29
Terminal sacrifice	7/24	5/24	6/27	11/21
Total†	11/70	6/70	9/70	19/70
Groups of finely vacuolated hepatocytes				
Terminal sacrifice††	10/24	11/24	8/27	15/21
Prominant areas of vacuolated hepatocytes				
Intercurrent deaths	0/26	0/26	0/27	4/29
Terminal sacrifice	1/24	7/24	1/27	3/21
Overall***	1/70	7/70	1/70	7/70
Centrilobular hepatocyte enlargement				
Interim sacrifice	0/20	0/20	0/20	18/20*
Intercurrent deaths	1/26	0/26	0/23	3/29
Terminal sacrifice	0/24	0/24	0/27	10/21*
Overall	1/70	0/70	0/70	31/70*
Centrilobular hepatocyte vacuolation				
Interim sacrifice†	3/20	6/20	1/20	10/20**
Intercurrent deaths	0/26	2/26	1/23	0/29
Terminal sacrifice	0/24	0/24	0/27	1/21
Overall†	3/70	8/70	2/70	11/70**
Focal hepatocyte necrosis				
Interim sacrifice†	2/20	0/20	0/20	5/20
Intercurrent deaths	3/26	1/26	0/23	3/29
Terminal sacrifice	0/24	1/24	1/27	0/21
Overall	5/70	2/70	1/70	8/70

*Statistically significantly different form controls ($p<0.001$).
**Statistically significantly different form controls ($p<0.001$).
***Statistically significant between groups variation ($p<0.05$).
†Statistically significant positive dose-related trend ($p<0.01$)/
††Statistically significant positive dose-related trend ($p<0.05$).

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a. Non-neoplastic observations 006232

ii) Organs affected by test substance (continued)

Thyroid: The incidence of agglomeration of colloid in males was affected in a dose-related manner at terminal sacrifice. The incidence of that effect is summarized as follows:

Observation	Dose level (ppm) 0	10	40	400
Interim sacrifice				
Minimal	7/20	3/20	5/20	4/19
Slight	1/20	1/20	1/20	3/19
Moderate	1/20	4/20	2/20	6/19
Marked	1/20	0/20	0/20	1/19
Total*	10/20	8/20	8/20	14/20
Intercurrent deaths				
Minimal	4/26	1/21	1/20	6/22
Slight	9/26	1/21	3/20	6/22
Moderate	4/26	0/21	1/20	1/22
Marked	0/26	0/21	0/20	0/22
Total	17/26	2/21	5/20	8/22
Terminal sacrifice				
Minimal	4/24	4/23	9/27	4/21
Slight	6/24	5/23	4/27	11/21
Moderate	2/24	1/23	1/27	3/21
Marked	0/24	0/23	1/27	0/21
Total**	12/24	10/23	15/27	18/21***

*Statistically significant positive dose-related trend ($p<0/01$).
**Statistically significant positive dose-related trend ($p<0.001$).
***Statistically significantly different from controls ($p<0.05$)

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Neoplastic lesions

Adrenal medulla: The authors noted that 8 of the 12 pheochromocytomas found in female rats were observed in the 10 ppm dose group. The incidence of pheochromocytomas are summarized as follows:

Observation	Dose level (ppm) 0	10	40	400
Pheochromocytomas				
Interim sacrifice	0/20	0/20	0/20	0/20
Intercurrent deaths				
Benign	0/25	5/31	0/28	1/27
Malignant	0/25	0/31	2/28	0/27
Terminal sacrifice				
Benign	0/20	3/19	1/20	0/23
Malignant	0/20	0/19	0/20	0/23
Overall*	0/65	8/70**	1/68	1/70

*Statisticallly significant variation between groups ($p<0.01$).
**Statistically significantly different from controls ($p<0.05$).

These tumors were not considered to be related to the administration of the test substance because they did not occur in a dose-related manner.

. Neoplastic lesions (continued) 006232

Mammary tumors: The incidence of mammary tumors in female rats exhibited a statistically significant negative dose-related trend. Incidences of those tumors were reported as follows:

Observation	Dose level (ppm) 0	10	40	400
Fibroadenomas (one or more/animal)				
Interim sacrifice	0/20	1/20	3/20	0/20
Intercurrent deaths	13/30	12/31	10/30	6/27
Terminal sacrifice	1/20	2/19	2/20	2/23
Overall	14/70	15/70	15/70	8/70
Adenomas (one or more/animal)				
Intercurrent deaths	3/30	5/31	2/30	0/27
Terminal sacrifice	1/20	0/19	1/20	1/23
Overall	4/70	5/70	3/70	1/70
Adenocarcinomas (one or more/animal)				
Intercurrent deaths	2/30	4/31	4/30	2/27
Terminal sacrifice	4/20	1/19	4/20	2/23
Overall	6/70	5/70	8/70	4/70
Mammary tumors combined*				
Grand total†	28/70	23/70	21/70	16/70**

*Includes animals with one or more mammary tumors of any type.
**Statistically significantly different from controls ($p<0.05$).
†Statistically significant negative dose-related trend ($p<0.05$).

Thyroid: The investigators noted a statistically significant positive trend with respect to the incidence of follicular cell tumors in males ($p<0.05$). The incidence of the tumor in males was reported as follows:

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b. Neoplastic lesions (continued)

Observation	Dose level (ppm) 0	10	40	400
Follicular cell hyperplasia				
Interim sacrifice	0/20	0/20	2/20	1/20
Intercurrent deaths	1/26	0/26	1/23	1/29
Terminal sacrifice	1/24	2/24	5/27	3/21
Follicular cell tumor				
Interim sacrifice				
Benign	0/20	0/20	0/20	0/20
Probably malignant	0/20	0/20	0/20	0/20
Malignant	0/20	0/20	0/20	0/20
Total	0/20	0/20	0/20	0/20
Intercurrent deaths				
Benign	1/26	0/26	0/23	0/29
Probably malignant	0/26	0/26	0/23	0/29
Malignant	1/26	0/26	1/23	0/29
Total	2/26	0/26	1/23	0/29
Terminal sacrifice				
Benign	0/24	1/24	0/27	3/21
Probably malignant	0/24	0/24	0/27	2/21
Malignant	0/24	1/24	1/27	3/21
Total*	0/24	2/24	1/27	8/21
Overall				
Benign	1/70	1/70	0/70	3/70
Probably malignant	0/70	0/70	0/70	2/70
Malignant	1/70	1/70	1/70	3/70
Total*	2/70	2/70	1/70	8/70

*Statistically significant positive dose-related trend ($p<0.05$).

III. DISCUSSION

Authors' conclusions: According to the authors, there were no marked or persistant treatment-related effects on behavior or condition, body weight, food and water consumption, hematology, urine analysis, or ophthalmological observations.

Effects the investigators associated with the test substance included a slight increase in free thyroxine levels in blood taken from males at 27 months.

Liver weights and weight ratios for male and female rats given the 400 ppm diet were slightly elevated above that for control group animals, but no statistically significant increase in absolute liver weights were noted in those groups.

III. DISCUSSION (continued)

Histological changes occurred in the liver and thyroid of males in a dose-related manner, and they included agglomeration of colloid in the thyroid, centrilobular hepatocyte enlargement and vacuolation, focal cystic degeneration of hepatocytes, and diffuse distribution of fat deposits in the livers of high dose group males. Based on these observations, the investigators concluded that a no-effect level was established in the experiment at 40 ppm (1.95 mg/kg/day).

The authors noted that the incidence of mammary tumors in female rats decreased with dose, and the incidence of thyroid follicular cell tumors in male rats exhibited a statistically significant positive dose-related trend (primarily because of the highest dose).

B. Reviewer's Conclusions:

1. Non-neoplastic effects: There were adequate data presented to support the author's conclusions that clofentezine had no effects on behavior or condition, body weight, food and water consumption, hematology, urine analysis, or ophthalmological observations. Based on the liver and thyroid effects, a noobserved-effect level of 40 ppm and a lowest-effect level of 400 ppm are established by the experiment.

2. Neoplastic effects:

a. Thyroid tumors: The first follicular cell tumor was observed in a control group male that died during week 88 of the study. The other thyroid tumor in that group was found during week 114. The only other follicular cell tumor observed during the study was in one male of the mid dose group at week 114. The remainder of the follicular cell tumors were observed in rats sacrificed at the end of the study, including all eight tumors observed in the high dose group.

If reported incidences are adjusted by censoring those animals sacrificed at 12 months and those dying before the first diagnosis was made in the control group (week 88), the incidences would be 2 of 47, 2 of 41, 2 of 40, and 8 of 40 for the control, low, mid, and high dose groups, respectively. A Fisher's Exact test on the control and high dose group incidences shows that the difference between the groups is statistically significant ($p = 0.024$). The adjusted results also show a significant positive trend ($p < 0.005$; Cochran-Armitage test).

There was no statistically significant increase in the incidence of follicular cell hyperplasia (see page 16 above), and no follicular cell tumors were noted in those animals with hyperplasia.

b. Other neoplastic lesions: There were adequate data presented in the report to support the conclusions of the authors. Administration of Clofentezine in the diet of male and female rats for over two years did not increase the incidence of tumors other than those observed in the thyroid of treated males.

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IV. References

Ginnocchio, A. V., and B. A. Mallyon. December, 1983. The Oncogenicity and Chronic Toxicity of Technical NC 21 314 (Clofentezine) in the Diet of the Rat. Interim Report: At 18 Months) Unpublished report prepared by FBC Limited. Report No. TOX/ 83/167-62. Submitted by BFC Chemicals, Inc. Wilmington, DE. EPA Acc. No. 257993.

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ADDENDUM

Data Evaluation Record of the Interim Report

nnocchio, A. V., and B. A. Mallyon. December, 1983. The Oncogenicity and ronic Toxicity of Technical NC 21 314 (Clofentezine) in the Diet of the Rat. terim Report: At 18 Months) Unpublished report prepared by FBC Limited. port No. TOX/ 83/167-62. Submitted by BFC Chemicals, Inc. Wilmington, DE. A Acc. No. 257993.

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DATA EVALUATION RECORD

CHEMICAL: Clofentezine; NC 21314; APOLLO®; 3,6-bis(2-chlorophenyl)-1,2,4,5-tetrazine

TEST MATERIAL: Technical grade Clofentezine (CF 20099/12, purity unspecified) was used.

STUDY/ACTION TYPE: Chronic - rats (Interim Report)

STUDY IDENTIFICATION: Ginnochio, A. V., and E. A. Mallyon. December, 1983. The Oncogenicity and Chronic Toxicity of Technical NC 21 314 (Clofentezine) in the Diet of the Rat. Interim Report: At 18 Months) Unpublished report prepared by FBC Limited. Report No. TOX/ 83/167-62. Submitted by BFC Chemicals, Inc. Wilmington, DE. EPA Acc. No. 257993.

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CONCLUSION: Results from an interim sacrifice of 20 rats of each sex from each group suggest that a no-observed-effect level (NOEL) for liver effects (slightly increased absolute and relative weight and hepatocellular hypertrophy) is 40 ppm in the diet. Test diets were fed for 18 months, and the effects were more evident in male rats than in females. The lowest-effect level (LEL) was 400 ppm (highest dose tested).

Core classification: Supplementary. The report is an 18-month interim report on a long-term feeding study. Many of the effects observed are present in the group of animals that were sacrificed at 18 months (diet cell hyperplasia in the pancreas and elevated serum glucose) or in those continued on test diets (an increase in mortality during the 19th through the 24th months of the study). Those effects do not consistently appear in both groups, and they are not consistently statistically significant. Final conclusions regarding the toxicological significance of those results can not be made until the final report is made available.

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B. MATERIALS AND METHODS

Test species: Male and female 4-week old Charles River Crl:CD Sprague-Dawley BR strain rats were used. The males weighed from 57 to 79 g, and the females weighed from 53 to 73 g on receipt at the laboratory. The animals were approximately 6 weeks of age when placed on test diets.

Experimental procedure: Rats were assigned to four groups each containing 80 individuals of each sex. Immediately prior to the start of the test, 10 animals of each sex from each group were used for collection of pre-test blood samples. The remaining 70 male and 70 female rats in each group were then given diets containing 0, 10, 40, or 400 ppm test substance for up to 25 months.

All test animals were observed twice a day for signs of toxicity and mortality. The report stated that observations were made only once each day on weekends and holidays. The signs, their severity, time of onset, and duration were recorded.

Body weights for each animal were obtained on the day of their arrival at the laboratory, at pre-test during randomized group assignment, at weekly intervals through the first 13 weeks of the study, and biweekly thereafter. Animals were also weighed on the day of necropsy. Food and water consumption were determined weekly for each cage of 5 animals.

As mentioned above, blood samples were taken from 10 male and 10 female rats from each group before the test started. These animals were then discarded from the study. During the feeding period, blood and urine samples were collected from 10 animals of each sex from each group at weeks 25, 51, and 77. Blood was collected from the retro-orbital sinus, and the animals were fasted overnight prior to sampling.

Hematological observations included hematocrit, hemoglobin, red blood cell and platelet counts, and total and differential white blood cell counts. Indices calculated from some of these observations included mean corpuscular hemoglobin concentration, mean cell volume, and mean corpuscular hemoglobin. Blood chemistry observations included total protein, albumin, total globulins, albumin/globulin ratio, blood urea nitrogen, electrolytes, creatinine, uric acid, glucose, total cholesterol, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), lactate dehydrogenase (LDH), and triglycerides. Urinalysis included volume, pH, protein, glucose, ketones, bilirubin, occult blood, urobilinogen, specific gravity, and microscopic examination of centrifuged deposits.

Ophthalmological examinations were conducted at 12 months on all surviving rats with the exception of those scheduled for sacrifice at that time.

Twenty animals of each sex were sacrificed after 12 months on test diets. Animals found in extremis during the feeding period were also sacrificed. These rats and any that were found dead were necropsied; gross lesions were noted; and the liver, kidneys, spleen, heart, adrenals, gonads, brain, and

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8. MATERIALS AND METHODS (continued)

pituitary were removed and weighed. The following tissues were processed for microscopic examination:

Adrenals	Heart	Rectum	Thymus
Aorta	Ileum	Salivary gland	Thyroid with para-thyroid
Bone and marrow	Jejunum	Seminal vesicles	
Brain	Liver	Sciatic nerve	Trachea
Cecum	Lungs	Skeletal muscle	Urinary bladder
Colon	Lymph nodes	Skin and subcutis	Uterus with cervix
Duodenum	Mammary glands	Kidneys	Vagina
Ears	Esophagus	Spleen	All macroscopic abnormalities
Epididymides	Pancreas	Spinal cord	
Eyes with Harderian gland	Pituitary	Stomach	
	Prostate	Testes/ovaries	

9. REPORTED RESULTS

The report stated that there were no treatment-related effects on mortality (see Table 1 and Section 10. DISCUSSION). There were also no treatment-related effects on body weight, food or water consumption, behavior, condition, or occurrence of clinical signs according to the report. No consistent or dose-related effects on the hematological, clinical chemistry (with the exception of phosphate, sodium, and glucose levels discussed below), or urine analysis parameters monitored during the study. Isolated statistically significant differences between treatment groups and the controls with respect to those parameters were described as within normal limits for the age and strain of the test animals.

Table 1

Mortality in the main study

Dose (ppm)	Mortalities* during weeks: Males 1-26	Males 27-52	Males 52-78	Females 1-26	Females 27-52	Females 52-78
0	0	3	7	0	1	1
25	1	0	3	0	0	4
75	0	0	5	1	0	3
225	1	1	5	0	1	1

*Includes those animals sacrificed at 52 months.

The investigators reported a statistically significant increase in relative liver weight for high dose group rats (see Table 2). The increase was associated with an increased incidence of centrilobular hepatocyte hypertrophy in males (see Table 3).

9. RESULTS REPORTED (continued)

Table 2

Group mean body and liver weights and weight ratios for rats sacrificed at 12 months

Parameter	Males		Females	
	0 ppm	400 ppm	0 ppm	400 ppm
Body weight (g)	609	601	340	332
Liver weight (g)	15.89	17.68	8.16	8.45
Liver/body weight ratio (%)	2.61	2.94**	2.41	2.56*

*Statistically significantly different ($p<0.05$)

**Statistically significantly different ($p<0.01$)

Other effects noted by the investigators included an increased group mean serum glucose and decreased phosphate and sodium levels for the high dose males (see Table 4). There was also an increased incidence of islet cell hyperplasia in the high dose group males above controls (see Table 3 and section 10. DISCUSSION below).

The tumor incidence observed during the first 18 months of the study is summarized in Tables 5 and 6. There was no dose-related incidence observed according to the authors. The first tumor was diagnosed in a mid-dose group female that died during week 18 of the study; it was a lymphosarcoma according to the report. The earliest tumor observed in male rats was found in a high-dose group animal that died during week 41 of the study. The tumor was also reported to be a lymphosarcoma.

10. DISCUSSION

Although the authors concluded that there were no effects on mortality, Table 1 (page 3, above) suggests that there might be a dose-related trend in males during the last half of the study. Another trend, which did not appear to be statistically significant, was the increased incidence in islet cell hyperplasia (Table 3) in male rats sacrificed at 18 months (Chi square = 3.89, $p = 0.0211$ for high dose group males when compared with controls). In addition, the effects on serum glucose levels in males is apparently associated with dose, but significant differences between treated and control group males noted during the first half of the study disappear during the last half (at 77 weeks). This pattern of response emphasizes the need for the final report before conclusions can be made regarding mortality, pancreatic effects, and serum glucose results.

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Table 3

Most frequent histopathological observations (non-neoplastic) in animals sacrificed after 12 months

Observation	Dose level (ppm) 0	10	40	400
Males				
Liver				
Number examined	20	20	20	20
Focal vacuolation	3	3	8	5
Centrilobular hepatocyte vacuolation	3	6	1	10*
Centrilobular hepatocyte hypertrophy	0	0	0	15**
Bile duct proliferation	1	1	2	3
Focal hepatocyte necrosis	2	2	0	5
Pancreas				
Number examined	20	18	19	19
Pigment deposits	7	3	8	12
Islet cell hyperplasia	5	4	6	9
Harderian gland				
Number examined	20	20	20	20
Interstitial mononuclear cells	3	2	5	10*
Females				
Liver				
Number examined				
Centrilobular hepatocyte hypertrophy	0	0	0	2
Focal hepatocyte necrosis	2	2	0	5

*Statistically significant difference from controls ($p<0.05$)

**Statistically significant difference from controls ($p<0.001$)

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Table 4

Selected group mean clinical chemistry results for male rats

Dose (ppm)	Phosphate (mmol/l) Pre-test	Week 25	Week 51	Week 77	Sodium (mmol/l) Pre-test	Week 25	Week 51	Week 77	Glucose (mmol/l) Pre-test	Week 25	Week 51	Week 77
0	3.70	2.56	2.64	2.02	145.6	150.0	155.3	144.8	3.4	6.9	6.5	6.9
10	3.76	2.32	2.27**	2.09	144.9	144.9†	151.6	142.9**	3.9*	7.3	6.9	7.1
40	4.02*	2.54	2.46	2.00	145.2	145.5**	151.3	144.4	3.7	6.8	7.1**	6.6
400	4.00*	2.15†	2.20†	1.97	143.4**	142.9†	145.1**	144.6	3.4	7.5**	7.5**	6.7

*Statistically significantly different from controls ($p<0.05$)

**Statistically significantly different from controls ($p<0.01$)

†Statistically significantly different from controls ($p<0.001$)

Table 5

Incidence of tumors (number of animals with a tumor/20 animals) in rats sacrificed at 12 months

Observation	Dose level (ppm) 0	10	40	400
Males				
Pituitary, pars anterior adenoma	0	2	1	0
Thyroid, follicular cell adenoma	0	1	0	0
Subcutaneous fibroma	0	1	0	0
Squamous papilloma	0	0	1	0
Thymus, squamous cell carcinoma and fibrosarcoma	0	1	0	0
Females				
Pituitary, pars anterior adenoma	3	2	2	2

Table 6

Incidence of tumors (number of animals with a tumor/number bearing tumors) in rats dying during the first 18 months (as reported in Table 17b of the original report)

Observation	Dose level (ppm) 0	10	40	400
Males				
Myxofibroma	1/3	0/0	0/1	0/1
Fibrosarcoma	1/3	0/1	0/3	0/1
Leiomyosarcoma	1/3	0/0	0/1	1/1
Lymphosarcoma	1/3	0/0	0/1	1/1
Females				
Lymphosarcoma	0/1	0/0	1/1	0/1

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10. DISCUSSION (continued)

On the other hand, elevated absolute and relative liver weights were also slightly increased (see Table 2) for the high dose group males. These weight changes and the increased incidence of hepatocellular hypertrophy were consistent with results from subchronic studies (Ginnochio and Brooks, 1981 and 1982; Brooks and Turnbull, 1983; and FBC Limited, 1983). Therefore, a no-observed-effect level (NOEL) on the basis of liver effects is established at 40 ppm, and the lowest-effect level (LEL) is 400 ppm (highest dose tested).

Since there were no deaths during the first year of the study among the animals scheduled for the 12-month interim sacrifice and the diffences between the mid and low-dose groups were not significant, the NOEL's and LEL's for the other effects are likely to be the same as those for the liver effects.

11. REFERENCES

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FBC Limited. June, 1983. Histopathological report on livers from FBC Study No. TOX 81019 report titled NC 21314: 90-day dietary toxicity study in the rat. Unpublished report prepared by Huntingdon Research Centre for FBC Limited. Submitted by BFC Chemicals, Inc. EPA Acc. No. 071659.

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R.H. 9-21-87

Reviewed by: Roger Gardner
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Secondary Reviewer: Judith Hauswirth, Ph. D. JHH 8/21/87
Section 6, Toxicology Branch (TS 769C)

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DATA EVALUATION RECORD

STUDY TYPE: Oncogenicity (Guideline §83-2)

ACCESSION NUMBER: 262261, 262262

TEST MATERIAL: Technical grade Clofentezine (CR 20099/12, purity of 98.7%) was used. It was described as a magenta colored crystaline substance.

SYNONYMS: Clofentezine; NC 21314; APOLLO®; 3,6-bis(2chlorophenyl)-1,2,4, 5-tetrazine

STUDY NUMBER(S): TOX/ 85/167-80

SPONSOR: FBC Limited

TESTING FACILITY. Huntingdon Research Center, Huntingdon, Cambridgeshire, UK

TITLE OF REPORT: Technical NC 21314: Oncogenicity in the Diet to the Mouse (Final Report).

AUTHOR(S): Lloyd, G. K., D. J. Spencer-Briggs, R. Heywood, C. Gopinath, and C. P. Cherry.

REPORT ISSUED: November, 1985

CONCLUSIONS: Clofentezine was administered in the diet to male and female CD-1 Swiss mice for up to 105 weeks at levels of 0, 50, 500, or 5000 ppm. Clofentezine treatment was associated with statistically significant reductions in group mean body weight (approximately 7% below control means) and body weight gains (approximately 15 to 22% less than that for control group) for males at 5000 ppm. These differences were observed during the first three months of the feeding period (see Addendum below). A statistically significant increase in mortality was observed in the high dose group females when compared to the control group for the last six months of the study (statistically significant dose-related decrease in survival indicated by the Cox and generalized K/W tests; draft analysis provided by the Toxicology Branch Biostatistics Team). The investigators characterized amyloidosis as a contributing factor in those deaths. The highest dose group males showed a statistically significantly increased incidence of altered hepatocytes (areas or foci of eosinophilic hepatocytes). In female mice given the 5000 ppm diet for up to two years, there were statistically significant increases in liver weights and the incidence of altered hepatocytes (eosinophilic and/or basophilic areas or foci).

The investigators noted that benign and malignant liver cell tumors were a contributing factor to deaths of some treated male mice during the study. However, the incidence of those tumors was not dose-related (see Section III. B. 1. below).

CONCLUSIONS (continued)

The only tumors that were apparently increased by clofentezine in the diet of mice were liver cell adenomas and carcinoma in females (see Section III. B. below).

Core classification: Minimum

I. PROTOCOL

A. MATERIALS

1. Test compound: The test compound is described as a magenta crystaline substance with a purity of 98.7%.

2. Test species: Male and female 4-week old Charles River CD-1 mice of Swiss origin were used. The male weights were within a 10 g range, and the female weights were within an 8 g range at the start of the study. The animals were approximately 6 weeks of age when placed on test diets.

3. Diet preparation: Basal diet consisted of Spratt's Laboratory Animal Diet No. 2 (source unspecified). Test diets were prepared weekly and stored at room temperature. Diets were analyzed for concentration of test substance prior to the start of the study and at approximately 3 month intervals during the study.

B. STUDY DESIGN

1. Animal assignment: Animals were randomly assigned to test groups as follows:

Test groups		Dose	Animals per sex		
No.	Designation	(ppm)	Main study*	Pre-test	Interim Sacrifice**
1	Control	0	52	--	--
2	Low (LDT)	50	52	--	--
3	Mid	500	52	--	--
4	High (HDT)	5000	52	--	--
5	Health check	--	--	10	--

*105 weeks.

2. Observations schedule

Type of observation	Number of animals per sex per group	Frequency
Mortality	All	Twice a day*
Signs of toxicity	All	Twice a day*
Body weight	All	On day of arrival at laboratory, at pre-test during randomized group assignment, at weekly intervals thereafter.

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B. STUDY DESIGN (continued)

2. Observations schedule (continued)

Type of observation	Number of animals per sex per group	Frequency
Food consumption	All	Weekly**
Water consumption	All	Weekly**
Blood smears	All	For those animals sacrificed during or at termination ot the study.
Blood samples	8	At week 52.
	All	At 105 weeks.
Necropsy	Animals found dead or moribund	When found.
	Survivors	At 105 weeks

*The report stated that observations were made only once each day on weekends and holidays. The signs, their severity, time of onset, and duration were recorded.
**For each cage of 4 animals.

C. METHODS

1. Observation of blood samples: Blood was collected from the retro-orbital sinus, and the animals were fasted overnight prior to sampling.

Hematology

X Hematocrit	X Differential white cell counts
X Hemoglobin	X Mean corpuscular hemoglobin concentration
X Red cell count	X Mean cell volume
X Platelet count	X Mean corpuscular hemoglobin
X Total white cell count	

3. Necropsy Gross lesions were noted.

a. Weighed organs

X Adrenals	X Heart	X Lungs	X Thymus
X Brain	X Kidneys	X Pituitary	X Thyroid
X Gonads	X Liver	X Spleen	X Uterus

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3. Necropsy (continued)

b. Tissues examined microscopically

X	Adrenals	X	Jejunum	X	Skin and subcutis
	Aorta	X	Kidneys	X	Spleen
X	Bone and marrow	X	Liver	X	Spinal cord
X	Brain	X	Lungs	X	Stomach
X	Cecum	X	Lymph nodes	X	Testes/ovaries
X	Colon	X	Mammary glands	X	Thymus
X	Duodenum	X	Pancreas	X	Thyroid with parathyroid
	Ears	X	Pituitary		
X	Epididymides	X	Prostate	X	Trachea
X	Esophagus	X	Rectum	X	Urinary bladder
X	Eyes with Hardarian gland	X	Salivary gland	X	Uterus with cervix
		X	Seminal vessicles	X	Vagina
X	Heart	X	Sciatic nerve	X	All macroscopic abnormalities
X	Ileum	X	Skeletal muscle		

D. STATISTICAL ANALYSIS

1. Continuous variables: (body weight, food consumption, hematology, and organ weights.

Statistical procedure	Purpose
Bartlett's Test	Determine homogeneity of variance.*
One-way analysis of variance	Determine significance of variability among all groups.**
Students "t" test	Determine significance of differences between the control and each treatment group.**
Kruskal-Wallis Test	Detect any significant group differences.***

*If variances are not homogeneous ($p>0.05$), the data are transformed (log transformation is used)

**Used on transformed or untransformed data only when variances are shown to be homogeneous or equal.

***Nonparametric test performed on data with heterogenous variances.

D. STATISTICAL ANALYSIS (continued)

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2. Frequency data (gross and microscopic observations at necropsy)

( ) One-tailed or (X) two-tailed probability values were calculated.

Statistical procedure	Purpose
Fisher's Exact Test Chi-square Tests with Yates' correction	Determine significance of differences between individual groups, overall variations, or trends
Kruskal-Wallis Test	Determine significance of differences for graded responses.

3. Other methods: (data adjustments or transformations, criteria of significance, etc)

II. REPORTED RESULTS

A 52-week interim report (Wood, Heywood and Gopinath, 1983) on this study was reviewed previously (see Addendum I below). Therefore, the results described in this section are primarily from observations made during the 12th through 24th months of the study.

A. Mortality and Signs of Toxicity: No treatment-related effects were observed according to the report. However, the investigators noted that the mortality rate during the last 26 weeks of the study was significantly increased in all test groups with respect to earlier periods in the study. The authors specifically noted an increase in mortality for the high dose group females in comparison to the control group female mice during the last six months of the study. Those results are summarized as follows:

	Mortalities during weeks							
	Males				Females			
Dose (ppm)	0-52	52-78	78-104	Termination	0-52	52-78	78-104	Termination
0	4	13	22	13	6	9	12	25
50	7	9	19	17	1	8	15	28
500	7	8	21	16	1	6	18	27
5000	6	12	22	11	7	13	22	10

B. Body Weight, Food and Water Consumption: These observations in treated groups were described as comparable to those in the control groups throughout the last half of the study.

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C. Test Substance Intake: The average daily intake for treated animals was reported as follows:

Dose (ppm)	Daily dose (mg/kg/day) Males	Females
50	5.0	5.3
500	50.7	56.9
5000	543.4	557.1

D. Clinical Pathology - Hematology: The investigators noted that there were slightly decreased red cell and platelet counts for mice in the high dose group at 52 weeks when compared to those observations for the control groups. These decreases were not observed at the end of the study, and the differences were not treatment related. Group means for those parameters at 52 weeks are summarized as follows:

	Dose level (ppm)			
	Males		Females	
Observation	0	5000	0	5000
Red cells (10^6/cmm)	6.5	5.7*	6.4	5.8
Platelets (10^3/cmm)	670	616*	510	494

*Statistically significant difference, Williams' test (0.05>p>0.01).

Total white cell counts were slightly lower in male mice given the 5000 ppm diet when compared to controls at the end of the study, and the lymphocyte counts for all treated groups of males were lower than controls at the same observation. Those values are summarized as follows:

	Dose level (ppm)			
Observation	0	50	500	5000
Total white cells (10^3/cmm)	8.1	4.7	5.5	4.0*
Lymphocytes (10^3/cmm)	6.30	5.55*	3.77*	2.96*

*Statistically significant difference, Williams' test (0.05>p>0.01).

E. Necropsy

1. Organ weights: The only treatment related effect on organ weight was observed for the liver in female mice of the high dose group. Group mean values for those animals are summarized as follows:

	Dose level (ppm)	
Observation	0	5000
Body weight (g)	40	39
Liver weight (g)	2.01	2.38**

**Statistically significantly different from control (p<0.01).

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1. Organ weights (continued)

The authors considered the increase in liver weight toxicologically significant because at necropsy the incidence of livers with masses in the high dose group females was increased above controls (4 of 25 and 6 of 10 examined at the end of the study in the control and high dose groups, respectively).

Liver weights in high dose group males were also greater than those for the control group, but the difference was not statistically significant.

Other organ weights were affected also, but the investigators did not consider these weight changes to be toxicologically significant. These observations included a slight increase in heart weights for the high dose group females and increased testes weights in high dose group males. There were no macroscopic or microscopic changes associated with these weight changes that indicated a toxicologically significant effect.

2. Gross observations: In addition to the increased number of liver masses observed in the high dose group females (see section E. 1., above), males from the mid and high dose groups also had an increased incidence of liver masses. The results for males are summarized as follows:

Dose (ppm)	Incidence (no. affected/no. examined) Those dying during the study	Terminal sacrifice	Overall
	Males		
0	12/39	9/13	21/52
50	13/35	10/17	23/52
500	21/36	14/16	35/52
5000	20/41	8/11	28/52
	Females		
0	1/27	4/25	5/52
50	6/24	3/28	9/52
500	3/25	5/27	8/52
5000	4/42	6/10	10/52

3. Histopathology

a. Non-neoplastic observations: The incidence of mice with areas or foci of altered hepatocytes was increased at the highest dose in males and females. The results are summarized below:

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a. Non-neoplastic observations (continued)

Hepatocellular alteration	Dose level (ppm) 0	50	500	5000
Males				
Eosinophilic cells only				
Intercurrent deaths	2/39†	3/35	4/36	8/41*
Terminal sacrifice	1/13	4/17	4/16	2/11
Overall	3/52††	7/52	8/52	10/52*
Basophilic cells only				
Intercurrent deaths	2/39	6/35	5/36	1/41
Terminal sacrifice	2/13	1/17	1/16	0/11
Overall	4/52	7/52	6/52	1/52
Eosinophilic and basophilic				
Intercurrent deaths	1/39	1/35	1/36	0/41
Terminal sacrifice	1/13	1/17	0/16	0/11
Overall	2/52	2/52	1/52	0/52
Eosinophilic and/or basophilic				
Intercurrent deaths	5/39	10/35	10/36	9/41
Terminal sacrifice	4/13	6/17	5/16	2/11
Overall	9/52	16/52	15/52	11/52
Females				
Eosinophilic cells only				
Intercurrent deaths	1/27	1/24	5/25	4/42
Terminal sacrifice	2/25	2/28	4/27	5/10*
Overall	3/52	3/52	9/52	9/52
Basophilic cells only				
Intercurrent deaths	0/27	0/24	0/25	0/42
Terminal sacrifice	0/25	1/28	1/27	1/10
Overall	0/52	1/52	1/52	1/52
Eosinophilic and basophilic				
Intercurrent deaths	0/27	1/24	2/25	1/42
Terminal sacrifice	0/25	0/28	0/27	0/10
Overall	0/52	1/52	2/52	1/52
Eosinophilic and/or basophilic				
Intercurrent deaths	1/27	2/24	7/25	5/42
Terminal sa rifice	2/25	3/28	5/27	8/20
Overall	3/52	5/52	12/52	13/52**

*Statistically significantly different from controls ($p<0.05$ by Fisher's Exact test; analysis performed by reviewer).
**Statistically significantly different from controls ($p<0.01$ by Fisher's Exact test; analysis performed by reviewer).
†Statistically significant trend (Cochran-Armitage test; $p<0.05$)
††Statistically significant difference between control and pooled treated groups (Fisher's Exact test; $p = 0.00566$)

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a. Non-neoplastic observatins (continued)

The authors noted that other lesions observed microscopically were spontaneous or age-related, and they occurred with incidences that were within historical ranges.

b. Neoplastic lesions: The report stated that there was no statistically significant increase in the incidence of benign liver cell tumors in male mice or malignant liver cell tumors in either sex.

The investigators noted that there was a statistically significant dose-related trend ($p<0.01$) with respect to the incidence of benign liver cell tumors in female mice. However, the investigators noted that the trend was not significant when the high dose group incidence was omitted, and there were no statistically significant differences for pair-wise (treated vs. control) or overall analyses (treatment-related effects, 2 X 4 contingency table, Chi square tests).

Male mice in the mid and high dose groups exhibited a statistically significant increase in the incidence of benign and malignant hepatocellular tumors combined, but no statistically significant positive dose-related trend was observed. The incidence of female mice with benign and/or malignant liver cell tumors was statistically significantly increased above controls in the highest dosed group, and a positive dose-related trend was noted. The positive trend was not significant when the high dose group incidence was omitted according to the authors.

The incidence of benign and malignant hepatocellular tumors in control and treated groups of this study was described as outside historical ranges.

The reported incidence of liver tumors is summarized as follows:

Observation	Dose level (ppm) 0	50	500	5000
Males				
Benign	6/52	7/52	13/52	8/52
Malignant	14/52	16/52	24/52	19/52
Benign and/or malignant combined	19/52	20/52	33/52*	25/52**
Females				
Benign †	4/52	3/52	3/52	7/52
Malignant	0/52	0/52	0/52	1/52
Benign and/or malignant combined††	4/52	3/52	3/52	7/52**

*Statistically significantly different from control ($p<0.01$ by Chi square test).
**Statistically significantly different from control ($p<0.05$ by Chi square test).
†Statistically significant positive trend ($p<0.01$), but the trend was not significant when the high dose group was omitted.
††Statistically significant positive trend ($p<0.001$), but the trend was not significant when the high dose group was omitted.

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b. Neoplastic lesions (continued)

A statistically significant increase in the number of female mice with lung tumors was also reported in the mid dose group, but there was no significant dose or treatment-related trend found after statistical analysis. The results are summarized as follows:

Observation	Dose level (ppm) 0	50	500	5000
	Females			
Pulmonary adenocarcinoma				
Intercurrent deaths	0/27	3/24	5/25	2/42
Terminal sacrifice	4/25	4/28	6/27	3/10
Overall	4/52	7/52	11/52*	5/52

*Statistically significantly different from control ($p<0.05$).

No other types of tumors were observed to occur at significantly increased incidences according to the report.

III. DISCUSSION

A. Non-neoplastic Effects

Survival up to 18 months in the experiment was greater than 50% in all test groups. After 18 months, the investigators noted increased mortality in the high dose group females (see Section II. A. above). According to the investigators, amyloidosis was a major contributing factor to the death of female mice. The reported incidence of that lesion is summarized as follows:

Observation	Dose level (ppm) 0	50	500	5000
Intercurrent deaths	6/27	10/24	13/25	19/42
Terminal sacrifice	12/25	13/28	8/27	3/10
Overall	18/52	23/52	21/52	22/52

Review of the individual animal data for those animals dying during the study showed the following incidences with respect to the week of death:

Dose (ppm)	0-52*	53-78*	79-106*	Terminal sacrifice*	Total*
0	0/6	2/9	3/12	12/25	17/52
50	0/1	2/8	2/15	11/28	15/52
500	0/6	4/6	5/12	4/25	13/52
5000	1/1	5/6	12/18	3/27	21/52

*These results are based on individual animal data for specific tissues such as the liver, lungs, and lymph nodes. Therefore, discrepancies exist between reported incidences of amyloidosis and the incidences independently compiled from individual animal reports.

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A. Non-neoplastic Effects (continued)

These results suggest that amyloidosis appears earlier in the study for female mice given the 500 and 5000 ppm diets. On the basis of the incidence of these effects, a no-observed effect level for female mice appears to be 50 ppm, and the lowest effect level is 500 ppm.

Group mean body weight for the high dose group males at 26 weeks was 7% less than that for controls, and after the first year of the study the high dose group mean was 6% less than controls. The differences for mean body weights of treated and control groups were not statistically significant according to the report.

The report stated that body weight gain was statistically significantly lower in the high dose group males than in the controls for weeks 0-26 and 0-52. Weight gains for the first period were 60 and 45% for the control and high dose groups, respectively, and those values for the first year were 67% for the control group and 55% for the high dose group.

There were no statistically significant differences between the high dose group and control group males with respect to food consumption, but food conversion (weight of food consumed per unit body weight gain) was affected according to the report. Selected group mean food conversion ratios are summarized as follows:

Week of study	Dose level (ppm) 0	50	500	5000
1	11.5	13.9	10.8	10.5
3	19.8	29.8	16.9	22.2
4	38.6	21.7	33.8	27.3
8	70.2	103.1	79.0	n/a*
9	94.2	221.9	233.6	n/a*
10	98.8	40.4	76.0	52.3
11	27.3	33.1	22.7	29.9
12	40.3	55.4	145.8	217.4
13	32.1	43.4	116.6	31.8

* n/a = insufficient body weight gain to give a meaningful result.

As noted above for male rats, there were no dose-related clinical signs observed; liver weights in the high dose group were not statistically significantly different from those in the control group; and there was an increase in the incidence of mice with basophilic and/or eosinophilic hepatocytes in all treated groups when compared with controls. The incidence of male mice with altered hepatocytes was not dose-related unless those with eosinophilic hepatocytes only were considered (see page 8 above).

In addition, there were slightly decreased red cell and platelet counts for mice in the high dose group at 52 weeks when compared to those observations for the control groups, but these decreases were not observed at the end of the study, and the differences were not treatment related. Total white cell counts were also slightly lower in male mice given the 5000 ppm diet when compared to

A. Non-neoplastic Effects (continued)

controls at the end of the study, and the lymphocyte counts for all treated groups of males were lower than controls at the same observation.

These results suggest minimally toxic effects at the highest dose in male mice (NOEL = 500 ppm).

B. Neoplastic Lesions

1. Male mice

Based on an independent review of the individual animal reports for male mice in the study, the incidence of liver tumors (malignant and benign combined) can be summarized as follows:

Dose (ppm)	No. with tumor/no. examined 1-52	53-106†	Termination†	Total†
0	1/4	10/35	8/13	19/52
50	0/6	12/29	8/17	20/52
500	1/7	19/29**	12/16	33/52*
5000	0/6	17/35*	8/11	25/52

*Statistically significantly different from controls ($p<0.05$, Fisher's Exact test).
**Statistically significantly different from controls ($p<0.01$, Fisher's Exact test).
†No significant trend (Cochran Armitage test).

Diagnoses of the first liver tumor in the control, low, mid, and high dose groups of males were made during the 48th, 72nd, 43rd, and 67th weeks, respectively. However, most of the tumors in each test group were diagnosed in the last 6 months or at terminal sacrifice. When animals dying before the week of the first diagnosis are eliminated from consideration, the incidence of liver tumors in male mice can be summarized as follows:

Dose (ppm)	No. with tumor/no. examined 43-74	75-106	Termination	Total
0	4/13	7/24†	8/13	19/50
50	1/8	11/21	8/17	20/46
500	3/9	17/23**	13/16	33/48**
5000	1/11	16/26*	8/11	25/48

*Statistically significantly different from control ($p < 0.05$ by Fisher's Exact test).
*Statistically significantly different from control ($p < 0.01$ by Fisher's Exact test).
†No statistically significant trend (Cochran-Armitage test $0.1 < p < 0.5$).

B. Neoplastic Lesions (continued) 006232

The distribution of type of liver cell tumors is summarized as follows:

Type of Tumor	Dose level (ppm) 0	50	500	5000
Benign only	5	4	9	6
Malignant only	13	13	20	17
Benign and malignant	1	3	4	2
Benign and/or malignant	19	20	33	25

These results do not indicate a dose-related increase in the malignant tumors in male mice bearing hepatocellular tumors.

Historical control data for male Charles River CD-1 mice from six other studies indicated the following mean incidence and range (expressed as %) for liver cell tumors:

	Intercurrent deaths		Terminal Sacrifice	
Tumor type	Mean %	Range	Mean %	Range
Benign	10.0	3.4 - 21.2	20.8	10.0 - 28.0
Malignant	15.7	6.1 - 25.8	13.2	4.8 - 28.0
		Benign Tumors		
Control	15.4	----	7.7	----
50 ppm	11.4	----	17.6	----
500 ppm	22.9	----	29.4	----
5000 ppm	12.2	----	27.3	----
		Malignant Tumors		
Control	17.9	----	53.8	----
50 ppm	0	----	29.4	----
500 ppm	37.1	----	47.1	----
5000 ppm	34.0	----	45.5	----

2. Female mice

According to the report, the incidence of liver tumors in female mice was statistically significantly increased based on reported results of a trend test. An overall Chi square analysis as well as a pairwise comparison (Chi square test) of the control and high dose group incidences showed no statistically significant differences between groups.

Diagnoses of the first liver tumor in the control, low, mid, and high dose groups of female mice were made during terminal sacrifice, the 98th, 95th, and 93rd weeks, respectively. When animals dying before the week of the first diagnosis are eliminated from consideration, the incidence of liver tumors in female mice can be summarized as follows:

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B. Neoplastic Lesions (continued)

Dose (ppm)	No. with tumor/no. examined 93-106*	Termination*	Total**
0	0/7	4/25	4/32
50	1/7	2/29	3/36
500	1/11	2/27	3/38
5000	4/13†	3/10†	7/23†

*Marginally significant trend ($0.05 < p < 0.1$; Cochran-Armitage trend test).
**Statistically significant trend ($p < 0.01$; Cochran-Armitage trend test).
†Not significantly different from control ($p > 0.05$; Fisher's Exact test).

Only one of the 17 liver tumors found in the female mice was classified by the investigators as malignant, and that was diagnosed in a high dose group female at the terminal sacrifice.

The incidence (expressed as %) of liver tumors in female mice from the Apollo study is presented along with historical control results from 6 studies of comparable duration as follows:

	Intercurrent deaths		Terminal Sacrifice	
Tumor type	Mean %	Range	Mean %	Range
Benign	2.0	0.0 - 6.7	3.1	0.0 - 8.0
Malignant	2.2	0.0 - 8.0	1.5	0.0 - 4.7
		Benign Tumors		
Control	0.0	----	0.0	----
50 ppm	4.2	----	7.1	----
500 ppm	4.0	----	7.4	----
5000 ppm	7.1	----	30.0	----
		Malignant Tumors		
Control	0.0	----	0.0	----
50 ppm	0.0	----	0.0	----
500 ppm	0.0	----	0.0	----
5000 ppm	2.3	----	0.0	----

The incidence of liver tumors in females of the study is comparable to the historical control data provided for animals dying during the studies, but the incidence reported for those animals sacrificed at the end of the study appears to be higher than historical controls.

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ADDENDUM

Data Evaluation Record for the Interim Report
of a Mouse Long-Term Feeding Study with Clofentezine

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DATA EVALUATION RECORD

1. CHEMICAL: Clofentezine; NC 21314; APOLLO; 3,6-bis(2chlorophenyl)-1,2,4,5-tetrazine

2. TEST MATERIAL: Technical grade Clofentezine (CR 20099/12, purity 98.7 +/- 1.3%) was used.

3. STUDY/ACTION TYPE: Oncogenicity - mice (Interim Report)

4. STUDY IDENTIFICATION: Wood, J., R. Heywood, and C. Gopinath. December, 1983. The Oncogenicity of Technical NC 21 314 (Clofentezine) in the Diet to the Mouse. (Interim Report: At 52 Weeks) Unpublished report prepared by FBC Limited. Report No. TOX/ 83/167-64. Submitted by BFC Chemicals, Inc. Wilmington, DE. EPA Acc. No. 257995.

5. REVIEWED BY:

Name: Roger Gardner
Title: Toxicologist
Organization: Review Section 6
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Signature: Roger Gardner
Date: 8-29-85

6. APPROVED BY:

Name: Jane Harris, Ph. D.
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Signature: Jane E. Harris
Date: 8/28/85

7. CONCLUSION: The body weight and food consumption results do not conclusively indicate that the highest dose (5000 ppm) has a toxic effect in male mice. There were inadequate histological observations available during the first 52 weeks of the study from which to determine toxic effects. Under these circumstances the interim report is considered to provide supplementary information.

Core classification: Supplementary. The report describes observations in a study that has not been completed (see Section 10. DISCUSSION).

8. MATERIALS AND METHODS

Test species: Male and female 4-week old Charles River CD-1 Swiss mice were used. The males weighed an average of 20 g, and the females averaged 8 g on the day they were randomly assigned to test groups. The animals were approximately 6 weeks of age when placed on test diets.

Experimental procedure: Mice were assigned to four groups each containing 52 individuals of each sex, and each group was given a diet containing 0, 50, 500, or 5000 ppm test substance.

All test animals were observed twice a day for signs of toxicity and mortality. The report stated that observations were made only once each day on weekends and holidays. The signs, their severity, time of onset, and duration were recorded.

Body weights for each animal were obtained on the day of their arrival at the laboratory, during randomized group assignment, and at weekly intervals throughout the study. Animals were also weighed on the day of necropsy. Food consumption was determined weekly for each cage of 4 animals.

Blood smears were made from all male and female mice killed during the study. At week 52 blood samples were collected from 5 animals of each sex from each group. Blood was drawn from the retro-orbital sinus of unfasted animals. Hematological observations included hematocrit, hemoglobin, red blood cell and platelet counts, and total and differential white blood cell counts. Indices calculated from some of these observations included mean corpuscular hemoglobin concentration, mean cell volume, and mean corpuscular hemoglobin.

Animals found in extremis during the feeding period were sacrificed. Those mice and any that were found dead were necropsied; gross lesions were noted; and the liver, kidneys, spleen, heart, adrenals, gonads, brain, and pituitary were removed and weighed at the discretion of the pathologist. The following tissues were processed for microscopic examination:

Adrenals	Ileum	Prostate gland	Thymus
Brain	Jejunum	Rectum	Thyroid with parathyroid
Cecum	Kidneys	Salivary glands	Trachea
Duodenum	Lacrimal gland	Sciatic nerve	Urinary bladder
Esophagus	Liver	Seminal vesicles	Uterus with cervix
Eyes	Lungs	Skeletal Muscle	Vagina
Femur	Lymph nodes	Skin	Gonads
Gall bladder	Large intestine	Spleen	All macroscopic abnormalities
Harderian gland	Mammary glands	Spinal cord	
Head	Pancreas	Sternum	
Heart	Pituitary	Stomach	

9. REPORTED RESULTS

The only effects noted in the interim report were on body weight, food consumption, and food conversion ratio for the 5000 ppm group males (see Table 2).

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8. REPORTED RESULTS (continued)

In addition to these effects, the authors noted lower red cell counts in the high dose group males (5.7×10^6/cmm in the high dose group males compared with 6.5×10^6/cmm in the controls).

The authors stated that there were no histological changes that could be associated with treatment at the 52-week interval of the study.

Table 1

Selected group mean body weights (g), food consumption (g), and food conversion ratios for control and high dose group males

	Body weight (g)		Food consumption*		Conversion ratio**	
		5000		5000		5000
Week	0 ppm	ppm	0 ppm	ppm	0 ppm	ppm
1	34	34	32	31	--	--
13	45	44	32	31	--	--
26	48	45	33	35	46.1	60.1
52	51	48	31	35	--	--

*Grams/mouse/week

**Grams food consumed/g body weight gain for weeks 1 to 26

10. DISCUSSION

The body weight and food consumption results shown in Table 1 above suggest that the apparent decrease in food efficiency in the high dose group compared to the control groups for male mice may be attributable to the small decrease (6%) in body weight combined with the increase (6 to 13%) in food consumption. No conclusion can be made regarding the oncogenic potential of NC 21314 (APOLLO®) in mice on the basis of the interim report at the present time.

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DATA EVALUATION RECORD

1. CHEMICAL NAME: NC 21314, Clofentezine, APOLLO® 3,6-bis[2-chlorophenyl]-1,2,4,5-tetrazine

2. TEST MATERIALS: Technical grade clofentezine (Batch no. 20099/12, 98.8 ± 1.4% purity were used.

   Reference mutagens: Ethylmethane sulfonate (EMS) and dimethylbenzanthracene (DMBA) were used as positive control substances.

   Vehicle: Dimethyl sulfoxide (DMSO) was used as the vehicle for DMBA; distilled water was used for EMS; and acetone was used for the test substance.

3. STUDY/ACTION TYPE: Mutagenicity assay with mouse L5178Y cells in vitro.

4. STUDY IDENTIFICATION: Bootman, O., and R. Rees. October 14, 1982. Technical NC 21314: Investigation of Mutagenic Activity in the Tk +/- Cell Mutation System. Unpublished report no. TOX/82/167-38 prepared by Life Science Research, Stock, Essex, England. FBC Limited. Submitted by Nor-Am Chemical Co.; EPA Acc. No. 262268.

5. REVIEWED BY:

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   Date: 8-21-87

6. APPROVED BY:

   Name: Judith Hauswirth, Ph. D.
   Title: Section Head
   Organization: Review Section 6, Toxicology Branch
   Signature: Judith W Hauswirth
   Date: 8/25/87

7. DISCUSSION AND CONCLUSION: Concentrations of 15, 30, 70, 100, and 128 ug/ml without metabolic activation or 2, 10, 30, 80, 100, and 128 ug/ml with metabolic activation were tested for mutagenic activity at the Tk locus of L5178Y mouse lymphoma cells. Although the investigators stated that the highest mutation frequency observed in treated cultures was within the control range for the laboratory, no tabulated data were included in the report to support that statement. Without these results, the conclusion that clofentezine did not significantly increase the frequency of mutations at the TK locus in L5178Y cells under the test conditions is only partially supported by the report.

   Core classification: Provisionally acceptable. A final determination will be made when historical control data are submitted to support conclusions of the investigators.

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8. MATERIALS AND METHODS

Test species: L5178Y mouse lymphoma cells ($Tk^{+/-}$) were used.

Cell culture conditions: Cultures were grown in 450 ml of medium designated ROP with 50 ml horse serum. The ROP medium contained 450 ml RPMI 1640, 7.5 ml sodium bicarbonate (7.5%), 10 ml pluronic F68 (5%), 2.5 ml sodium pyruvate (200 mM), and 5 ml penicilin streptomycin solution (10,000 units/ml). This medium also contained 30 mg thymidine, 50 mg hypoxanthine, and 75 mg glycine per 100 ml ROP. Cell densities were maintained from 2 X 10^4 to 1 X 10^6 cells/ml.

Every 72 hours, 10 ug methotrexate per 100 ml medium was added to eliminate Tk (-/-) revertants. Cells were washed and returned to medium without methotrexate 24 hours after it was added.

Cloning medium consisted of 450 ml ROP medium supplemented with 7.5 ml sodium bicarbonate (7.5%), 5 ml sodium pyruvate (200 mM), 5 ml Fungizone, 5 ml penicilin streptomycin solution (10,000 units/ml), and 100 ml horse serum.

The selection medium was made with cloning medium and bromodeoxyuridine (BUdR) (50 ug/ml).

Microsomal enzyme (S-9) preparation: Liver microsomal preparations were obtained from Aroclor 1254 induced rats. The reported composition of the S-9 mix is as follows:

Component	Amount (ml)
0.1 M NADP, sodium salt in aqueous solution	0.4
0.1 M glucose-6-phosphate, sodium salt in aqueous solution	0.5
0.4 M $MgCl_2.6H_2O$/1.65 M KCl aqueous solution	0.2
Supernatant from liver homogenate	1.5
0.1 M KH_2PO_4-Na_2HPO_4 buffer (pH 7.4) to final volume of	10.0

Experimental procedures---Preliminary toxicity assay: The report stated that ten concentrations from 0.25 to 128 ug test substance per ml of medium were evaluated for their toxicity to the lymphoma cell cultures. A vehicle control (acetone) was also evaluated. The 128 ug/ml concentration was the maximum solubility for the test substance.

Exponentially growing culutres were adjusted so that cell density was 6 X 10^5 per ml, and 2.5 ml of the suspension was added to 2.5 ml of the ROP medium for assays without S-9 mixture and 2.25 ml of the suspension for assays with S-9. Fifty ul aliquots of the test solution was added to each 5 ml culture suspension, and 0.25 ml S-9 mixture was added when metabolic activation was being evaluated. Duplicate cultures were prepared in this manner for each test concentration.

These cultures were then incubated at 37° C in a shaking water bath for four hours. After treatment cells were centrifuged, washed, and resuspended in RIOP medium for incubation at 37° C in a 5% CO_2 atmosphere. Cell densities were determined at 24-hour intervals after the initiation of treatment with a hemocytometer, and the results were used as a measure of the effects of test solutions on growth.

8. MATERIALS AND METHODS (continued)

Experimental procedure---Main study: Cultures were maintained and treated in this part of the assay as described above for the preliminary assay. For cultures without the S-9 mixture, the test doses were calculated to be 0, 15, 30, 70, 100, and 128 ug test substance per ml medium, and the concentrations for EMS and DMBA were 300 or 5 ug/ml, respectively. Cultures with the S-9 mixture contained 0, 2, 10, 30, 80, 100, and 128 ug/ml test substance, and the positive control concentrations tested were the same as those used in the assay without S-9. The period following treatment was designated the expression time and lasted for 3 days.

After the expression period, cell suspensions were drawn from each culture and diluted for addition to selective and non-selective media. The report stated that 1.5×10^6 cells were added to a flask containing 70 ml cloning medium containing trifluorthymidine (TFT) (selective agent) at a concentration of 4 ug/ml. Another 6×10^2 cells was added to 70 ml cloning medium without the selective agent. Agar was then added to both of these cultures (5.25 ml of 3.5% agar at 70° C), and the volume of each was divided equally among 3 culture plates. The plates were then incubated for two to three weeks.

Colonies were counted on each plate after the incubation period and growth and mutation frequency data were calculated.

Calculations: The following calculations were used to maintain constant cell concentrations during the study:

$$\text{Volume of cells to retain} = \frac{(3 \times 10^5 \text{ cells/ml})(10 \text{ ml})}{(\text{Number of cells/ml})}$$

$$\text{Volume of media to add} = 10 - \text{Volume of cells to be retained}$$

Toxicity of the test substance was determined with the following formulas:

$$\text{Total suspension growth} = \frac{\text{Day 1 cell concentration}}{3 \times 10^5 \text{ cells/ml}} \times \frac{\text{Day 2 cell concentration}}{3 \times 10^5 \text{ cells/ml}}$$

$$\% \text{ solvent control suspension growth} = \frac{\text{Total suspension growth}}{\text{Average solvent control total suspension growth}} \times 100$$

Cloning results were calculated as follows:

$$\text{Mutant frequency per } 10^5 \text{ survivors} = \frac{\text{No. of survival cells plated}}{\text{Av. No. of colonies}} \times \frac{\text{Av. No. of TFT colonies}}{5}$$

$$\text{Induced mutation frequency} = \text{Mutation frequency of test material} - \text{Av. mutation frequency of solvent controls}$$

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8. MATERIALS AND METHODS (continued)

$$\text{\% of control cloning growth} = \frac{\text{Average number of plate colonies from treated cultures}}{\text{Average number of plate colonies from solvent controls}} \times 100$$

Total growth was determined as follows:

$$\text{Total growth (\%)} = \frac{\text{(\% suspension growth) (\% cloning growth)}}{100}$$

9. REPORTED RESULTS

Results from the preliminary toxicity test are presented as reported in Addendum I below. The report noted that metabolic activation with the S-9 mixture increased the toxicity of the test substance at the highest dose (128 ug/ml). Growth in cultures at the highest dose level with and without S-9 was reported to be 25.1 and 42.7% of the vehicle control, respectively.

Total growth results for the main study are reproduced in Addendum II below. The range observed for cultures without S-9 mixture was reported to be 79.7% at the lowest dose level and 30.6% at the highest dose level. The range for cultures with S-9 was from 74.5% at the lowest dose level to 30.6% at the highest.

According to the investigators, the mutation frequencies observed in treated cultures (see Addendum II below) were higher than those observed in the concurrent control cultures, and the increases appeared to be dose-related. However, they noted that the highest frequency (11.1 per 10^5 cells at 128 ug/ml) was not twice that of the control cultures (6.6 per 10^5 cells) and was within the range historically observed at the laboratory (4.4 to 15.3 per 10^5 cells). Based on these two criteria, the investigators concluded that the apparent increase in mutation frequency was not biologically significant.

Mutation frequencies of the cultures to EMS with and without metabolic activation (S-9) were 26.2 and 45.1 per 10^5 cells, respectively. The respective responses for cultures treated with DMBA were 40.6 and 8.5 per 10^5 cells.

APPENDIX I

Results of the Preliminary Toxicity Test
(reproduced from the original report)

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CLOFENTEZINE TOXICOLOGY REVIEW

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