

KB-315  
TXR-7503



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

SEP 27 1989

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OFFICE OF  
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

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*Budd  
9/13/89  
KB 9/14/89*

SUBJECT: Clofentezine (Apollo™ SC): Review of Additional Rat Studies  
Relating to Thyroid Neoplasia and Other Outstanding Issues  
(EPA Reg. No. 45639-RGL; Tox. Chem. No. 593A; HED Project No.  
9-0755).

Actions Requested

1. Response to Registrant's comments on reviews on previous reviews of mutagenicity, metabolism, and acute inhalation toxicity studies.
2. Review of three new rat studies and Registrant's comments on the issue of thyroid neoplasia in rats.

Recommendations and Conclusions

1. The mouse lymphoma study indicated that clofentezine is not a mutagen according to the criteria described in the original report, and the additional historical control data indicated that the assay is acceptable.
2. There is no need for additional metabolism data on clofentezine since a study identifying fecal metabolites in the rat was previously submitted and accepted by the Agency (memorandum dated July 10, 1989).
3. The physical properties of APOLLO™ 50 SC, low toxicity of another clofentezine formulation (80 WP) the difficulties encountered in attempts to generate a test atmosphere with the 50 SC formulation, and the size distribution of particles generated by orchard sprayers (99.9% with a diameter greater than 20 um) indicate that an acute inhalation toxicity study with the 50 SC formulation is not necessary to support registration.

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4. All of the special thyroid studies showed:
  - a. that a dietary level of 30,000 ppm clofentezine induced a temporary pituitary-thyroid hormone imbalance in male rats.
  - b. that a shift in the metabolism and excretion pattern for thyroid hormones occurs in rats at the 30,000 ppm level, but no significantly increased clearance of thyroxine and its metabolites was clearly indicated.
  - c. that thyroid follicular cell hyperplasia is part of the thyroid gland's enlargement as an adaptation to the effects of clofentezine at dietary concentrations of 400 ppm or less.
5. The compensatory response suggested by the special studies is contradictory to a consistent progression of events that leads to the development of thyroid tumors in laboratory animals treated with clofentezine.
6. The thyroid data did not establish the conditions (dose level and duration of treatment) under which the thyroid gland becomes incapable of compensating for the effects of clofentezine on glandular structure or function. The special studies also provided no clear indication of the mechanism for the thyroid tumors observed in the 400-ppm dose group in the rat chronic feeding study.

#### I. Background

In comments dated January 6, 1989 (MRID No. 40958301), the Registrant responded to requests for additional information on the following:

1. Historical control data for a mouse lymphoma mutagenicity assay.
2. Identification of fecal metabolites of clofentezine in treated rats.
3. An acute inhalation toxicity study with APOLLO™ 50 SC.
4. An additional rat feeding study to more substantially support the proposed mechanism of thyroid tumor induction observed in the chronic feeding study.

#### A. General Considerations

##### 1. Mutagenicity

In the discussion of additional mutagenicity data, the Registrant indicated that a request was made of the testing facility where the mouse lymphoma assay was conducted to provide appropriate historical control data for

comparison with the results from the Clofentezine study. Data from six studies conducted prior to September of 1982 are summarized as follows:

Study no.	Solvent	Mutants per 10 <sup>5</sup> survivors in control cultures (individual values)	
		With S 9 mix	Without S-9 mix
1	Dist. H <sub>2</sub> O	9.0, 8.9	9.6, 8.9
2		5.4, 5.1	6.1, 5.9
3		5.8, 4.5	5.4, 6.1
4		5.5, 5.8	4.9, 4.4
5		7.6, 8.1	6.6, 5.3
6	DMSO	5.7, 5.2	5.7, 6.0

The Registrant further commented:

The top value in the range came from control data generated during the initial validation of the Tk +/- assay in the laboratory but since they do not form part of sponsored studies they have not been retained and no reconstruction is now possible.

...the above range may understate the normal control range in the Tk +/- assay. The range quoted by Myhr, *et al.* (ref. cited on page 8 of the report) agrees well with the range which we stated at the time.

## 2. Metabolism

The Registrant noted that the fecal metabolites were fully identified in a report cited as follows:

FBC Report No. M36 - The Metabolism of Clofentezine in the Rat dated November, 1985.

This study was reviewed in a Data Evaluation Record dated July 7, 1989 and discussed in a memorandum dated July 10, 1989. The citation of the report in that memorandum was as follows:

Challis, I. R., and Needham, D. November 4, 1985. The Metabolism of Clofentezine in the Rat. Unpublished report no. METAB/85/5 prepared by FBC Limited, Chesterford Park Research Station, Saffron, Walden, Essex CB10 1XL, England. Submitted by Nor-Am Chemical Co., Wilmington, DE. MRID No. 409420-01

The study was accepted, and a recommendation was made that no additional metabolism data should be required in support of registration of clofentezine.

### 3. Inhalation Toxicity of APOLLO™ 50 SC

According to the Registrant, clofentezine was described by the Registrant as a solid with a melting point of 179 to 182° C and a vapor pressure of  $9.76 \times 10^{-17}$  mm Hg at 25° C.

The 50 SC formulation has the following physical properties:

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

The distribution of particle sizes from orchard sprayers was described in two references appended to the Registrant's discussion document (Retter, et al., 1978, and Reichard, et al., 1977). The discussion summarized, "...99.9% by volume of the droplets produced will have a particle size diameter of greater than 20 um."

In addition, the Registrant stated:

For final reassurance we have looked at the LC<sub>50</sub> study on the 80 WP where the compound was classified in Toxicity Category III, which is relatively non-toxic by this route. The data show a maximum absorption of clofentezine over the 6 hour exposure period of 1195 mg/kg (assuming 100% absorption)... In contrast even if all of the SC formulation was respirable and inhaled by an orchard sprayer at the use spray dilution over an 8 hour working day, and was 100% absorbed, this would only result in a body burden of 0.037 mg/kg, i.e., a 32,000 fold safety factor compared to the rat LC<sub>50</sub> study described above.

Appendix I below contains the calculations used to determine the values used in the discussion quoted above.

### 4. Thyroid Effects of Clofentezine

#### a. Previous Agency Assessments

On September 16, 1987, the Toxicology Branch Peer Review Committee considered the available toxicity data in a weight-of-evidence analysis and classified clofentezine as a Group C oncogen (possible human carcinogen) based on the following:

1. Clofentezine was associated with an increase in tumors (benign and malignant thyroid follicular cell tumors combined) in the male rat.

2. The observed response exceeded the historical control range, and was observed at the highest dose tested which was well below the Maximum Tolerated Dose (MTD) predicted by subchronic feeding studies.

A quantitative risk assessment was conducted based on the adjusted incidences of combined benign and malignant thyroid tumors in male rats in the 27-month feeding study. The unit risk in human equivalents (multi-stage model  $Q_1^*$ ) based on those data was  $0.053 \text{ (mg/kg/day)}^{-1}$ .

On March 2, 1988, the Federal Insecticide, Fungicide, and Rodenticide Act Scientific Advisory Panel (SAP) considered the Peer Review Committee's weight-of-the-evidence analysis and classification of the oncogenic potential of clofentezine in a public meeting. Additional data provided by the Registrant were also briefly considered by the Panel.

The Panel pointed out that there were no compound-related effects on tumor incidence in the mouse oncogenicity study and that clofentezine is not mutagenic. They did not believe that the thyroid tumors in male rats provide evidence of human risk for carcinogenicity. The Panel further concluded:

...(clofentezine) belongs in category D. This interpretation is based primarily on the data demonstrating increased TSH, hyperplasia, and decreased half-life of  $T_4$  and  $T_3$ . It is well known that this sequence leads to thyroid tumors in rats. Exposure to agents that cause this sequence in rats has not resulted in increased TSH, hyperplasia, and thyroid tumors in humans. Therefore, there is inadequate data for suggesting human carcinogenicity.

The Panel also concluded that the data were not adequate for a quantitative risk assessment.

On May 18, 1988, the Peer Review Committee met again to assess the SAP review and data on thyroid effects. The thyroid data were evaluated according to several indicators of pituitary-thyroid hormone imbalance which are described in a draft document entitled Thyroid Follicular Cell Carcinogenesis: Mechanistic and Science Policy Considerations prepared by the Technical Panel of the Agency's Risk Assessment Forum (dated December 15, 1987).

The Peer Review Committee concluded that an additional study of greater than 13 weeks' duration with a broader dose range ( $>400 \text{ ppm}$ ) and interim thyroid biochemistry and necropsy observations would be needed to more completely define the mechanism of clofentezine's thyroid tumor induction in terms of the criteria in the proposed policy mentioned above. The Committee further concluded that the new evidence was not sufficient to change the classification of clofentezine from Category C.

A quantitative risk assessment based on the thyroid tumor incidence was considered inappropriate by the Peer Review Committee for the following reasons:

1. The increased tumor incidence was marginally greater than the control incidence ( $p = 0.048$ ) only at the highest dose tested (400 ppm) in the chronic feeding study.
2. The increased tumor incidence was observed only in male rats.
3. The thyroid tumor incidence in the chronic feeding study's highest dose group (20%) was slightly greater than the historical range provided by limited data (7.5 to 15%; from 2 studies). (Data were adjusted for animals dying prior to appearance of the first thyroid tumor.)
4. The additional thyroid function studies suggest the possibility of an indirect mechanism for follicular cell tumor induction that may be associated with clofentezine's liver toxicity.

b. Previously Submitted Thyroid Studies

Because of the increased incidence of thyroid tumors in male rats in the chronic feeding study, re-evaluations of thyroid slides and additional studies of clofentezine's effects on the thyroid were conducted. These and more recently submitted studies are discussed in more detail in Section II. B. below.

1. Goitrogenic Effects

Microscopic observation of thyroids from rats in chronic and subchronic studies indicated increased activity in the glands of treated male rats. There was a dose-related increase in the severity of colloid depletion along with dose-related increases in the incidence and severity of follicular cell changes (hypertrophy and hyperplasia). In short-term studies at high doses (3,000 to 30,000 ppm), follicular cell hypertrophy was reversed in the four week period on control diets that followed a 9-week treatment period. Observation of hyperplasia was limited to the chronic feeding study, and its incidence was not dose-related. Thyroid weights were increased along with the microscopic changes but not in a statistically significant manner.

2. Effects on Thyroid Clinical Chemistry Parameters

In male rats given diets containing 30,000 ppm clofentezine for 6 weeks, elevated TSH levels were observed, but thyroxine levels were also increased. In addition, the thyroxine levels were elevated in male rats at the end of the chronic study at the 400 ppm dose level without an increase in their TSH levels. No thyroid blood chemistry tests were conducted during earlier portions of the chronic feeding study, and the hormone changes were not consistent with an imbalance in the thyroid-pituitary hormone feedback mechanism.

### 3. Effects of Clofentezine on Thyroxine Clearance

Two experiments demonstrated that clofentezine does not selectively accumulate in the thyroid gland, and iodine uptake is increased in the gland of treated male rats. These observations and the histological changes (follicular cell hypertrophy and hyperplasia as well as colloid depletion) indicating increased activity in the thyroid gland suggested that clofentezine treatment might affect clearance of thyroid hormones. Additional studies appeared to confirm this conclusion by showing that short-term administration of high doses of clofentezine (3000 to 30,000 ppm in the diet) caused increased liver enzyme activity and increased bile flow in male rats. Results of two other subchronic experiments at 30,000 ppm suggested that these effects increased the metabolism and enhanced excretion of thyroid hormone.

## II. Discussion

### A. Mutagenicity

The mutation frequencies observed in treated cultures were higher than those observed in the concurrent control cultures (from 7.2 to 11.1 per  $10^5$  cells in treated groups compared with 6.6 per  $10^5$  cells for the acetone control group). The criteria used in the original report to classify a test substance as a mutagen were:

1. at least a doubling of the mutation rate observed in the solvent control group, and
2. a dose-related increase in the mutation frequency.

The highest frequency (11.1 per  $10^5$  cells at 128 ug/ml; highest dose tested) was not twice that of the control cultures, but the mutation rates appeared to be dose related since the highest values (10.6 to 11.1 per  $10^5$  cells).

The original report also noted that the increased frequencies were within the range historically observed at the laboratory (4.4 to 15.3 per  $10^5$  cells), and the historical control range proposed by Myhr, et al. (1982), was 1.0 to 15 per  $10^5$  cells according to the original report.

As mentioned above, the top value in the range originally reported came from control data generated during the initial validation of the TK +/- assay in the laboratory, and it can not be recovered. The reference for historical control information (Myhr, et al., 1982) cited in the original report is an abstract and was apparently described an acceptable range for mouse lymphoma assay control values. The available information confirms that the mutation frequency at the two highest doses tested (100 and 128 ug/ml) slightly exceeded the historical range, but they also indicated that the assays with clofentezine were within acceptable ranges as proposed by Myhr, et al. There is no further need for additional information, and the study indicated that clofentezine is not a mutagen according to the criteria described in the original report.

## B. Metabolism

As noted above, there is no need for additional metabolism data on clofentzine since a study identifying fecal metabolites in the rat has been submitted and accepted by the Agency.

## C. Inhalation Toxicity of the 50 SC Formulation

A Data Evaluation Record for the inhalation study with the 50 SC formulation described the difficulties in generating a test atmosphere as follows:

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

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.

The physical properties and low toxicity described in Section I. A. 3. above, support the conclusion that a test atmosphere containing the active ingredient from the 50 SC formulation would be difficult to achieve for an acute inhalation toxicity study. Therefore, requiring an acute inhalation toxicity study on the 50 SC formulation is probably unreasonable.

## D. Thyroid Effects

### 1. New Data

Data Evaluation Records for the new studies are included in Appendix II, and the new studies are discussed in more detail in Section II. B. below.

Slides from thyroid glands of male rats given diets containing 0 or 30,000 ppm clofentzine for 6 weeks were examined morphometrically as an extension of histopathology examinations done previously. The results indicated that the thyroid glands were enlarged in treated rats, and the number of follicular cells in thyroid glands from treated rats was increased.

In a new feeding study, diets containing 0, 10, 400, 3,000, or 30,000 ppm clofentzine were fed to groups of 80 male Sprague-Dawley strain rats for 28 days. At 400 ppm or higher, increased liver enzyme activity (UDPGT) and thyroid activity (hypertrophy, hyperplasia and mitotic activity of follicular cells, colloid depletion and an increase in thyroid weight)

were noted. The hypertrophy, hyperplasia and mitotic activity seen at the 400 ppm level appeared to decrease in incidence and severity during the study. The thyroid follicular cell effects appeared to be reversed in the 400 ppm dose group by the end of the 28-day study, and a no-effect level for those changes was established at 10 ppm.

In another new feeding study, diets containing 0 or 30,000 ppm clofentazine were fed to groups of 50 male Sprague-Dawley strain rats for up to 14 days. The results of the study indicated that the 30,000 ppm diet caused liver enlargement, decreased serum T<sub>3</sub> levels, increased TSH levels, and increased incidence of proliferative changes in the thyroid gland (follicular cell hypertrophy and hyperplasia).

## 2. Interpretation of Thyroid Studies

In a discussion document (MRID 40958303), the Registrant interpreted all of the special thyroid studies described above. Exerpts from that document are discussed in detail below.

### a. Effects on Thyroid Clinical Chemistry Parameters

Clofentazine's effects on thyroid hormone levels were generally described in the discussion document as follows:

The data...show a time related response with very early reductions in the thyroid hormones..., which occur after only 48 hours treatment. Feedback results in increased TSH, which is observed after 4 and 7 days although T<sub>3</sub> remained reduced until day 14, by which time normal thyroid hormone values indicate restoration by increased thyroid gland activity and hence secretion. After 4 weeks a similar situation is seen, and by 6 weeks a small "overshoot" in the thyroid hormones T<sub>3</sub> and T<sub>4</sub> is apparent...

In treated rats from the 14-day experiment, T<sub>3</sub> levels were initially low, rose slightly on day 2, and fell until after day 7 when they rose again to normal levels by the end of the experiment. The T<sub>4</sub> levels in the treated group were high initially (days 1 and 2) and they fell and rose again by the end of the study. When both T<sub>3</sub> and T<sub>4</sub> levels were at their lowest values on day 7 of the study, TSH in the treated rats was at its highest level. These results indicated a response to an imbalance in thyroid and pituitary hormone levels.

T<sub>3</sub> and TSH levels for the control group in the 14-day study remained consistent except at the day 2 observation when the results were greater than the other measurements. T<sub>4</sub> values changed in a manner similar to those values for treated rats in that they fell and rose after day 4 of the study.

Table 1 also indicates that T<sub>3</sub> values were generally slightly lower in treated rats, and T<sub>4</sub> and TSH levels were generally higher at the 30,000 ppm level than in control animals. These results suggest that a pituitary-thyroid hormone imbalance may have been induced by the 30,000 ppm dose level.

Increased TSH levels were also observed in rats given 30,000 ppm clofentazine after 4 or 6 weeks of treatment (see Tables 2 and 3). T<sub>4</sub> levels were not affected at the end of the 4-week feeding study, and they were statistically significantly increased after 6 weeks. The T<sub>3</sub> levels were comparable to control levels at the end of the 4-week studies, and statistically significantly elevated after 6 weeks on the 30,000 ppm diet (see Tables 1, 2, and 3).

Table 1

Group mean values for TSH, T<sub>3</sub>, and T<sub>4</sub> in male rats given a diet containing 30,000 ppm for 14 days.

Study day	Thyrotrophin (TSH) (ng/ml)		Total T <sub>4</sub> (nmol/l)		Total T <sub>3</sub> (nmol/l)	
	Control	Treated	Control	Treated	Control	Treated
1	5.8	5.9	83	92	1.5	1.4
2	8.4	7.2	85	92	2.0	1.6*
4	5.3	7.5**	70	73	1.6	1.4*
7	5.2	7.6***	77	70	1.6	1.2**
14	4.9	6.7*	79	79	1.6	1.6

\* Statistically significantly different from controls, p<0.05.

\*\* Statistically significantly different from controls, p<0.01.

\*\*\* Statistically significantly different from controls, p<0.001.

Hormone data from the 400-ppm dose group are less detailed than those from the 30,000 ppm dose group, but a consistent pattern is noted for the 4- and 6-week studies as well as the 27-month study (see Tables 2, 3, and 4); thyroxine is the only hormone that is statistically significantly different from control values (significantly increased).

Table 2

Effects of Clofentazine on Thyroid Function in Male Rats Treated with Clofentazine in the Diet for 4 Weeks

Observation	Dose level (ppm)		
	0	400	30,000
Tri-iodothyroxine (nmol/l)	2.0	2.0	2.0
Thyroxine (nmol/l)	79	87	84
Thyrotrophin (ng/ml)	6.0	7.1	12.4*

\* Statistically significantly different from control (p<0.001; t test).

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Table 3

Effects of Clofentezine on Thyroid Function in Male Rats Treated with Clofentezine in the Diet for 6 Weeks

Observation	Dose level (ppm)		
	0	400	30,000
Tri-iodothyroxine (nmol/l)	0.9	1.0	1.1*
Thyroxine (nmol/l)	54	62*	69**
Thyrotrophin (ng/ml)	5.4	5.7	9.0*

\* Statistically significantly different from control (p<0.05; t test).

\*\* Statistically significantly different from control (p<0.001; t test).

Table 4

Group Mean Values (and Standard Deviations) for Thyroid Blood Chemistry Tests in Male Rats Treated with Clofentezine for 27 Months.

Observation	0	Dose level (ppm)		
		10	40	400
Total tri-iodothyroxine (nmol/l)	2.0 (0.6)	2.1 (0.5)	1.9 (0.3)	1.9 (0.3)
Thyroxine (nmol/l)	34 (8)	32 (5)	34 (6)	35 (6)
Free tri-iodothyroxine (pmol/l)	1.4 (0.5)	1.6 (1.0)	1.4 (0.5)	<1.4 ( - )
Free thyroxine (pmol/l)	13.4 (6.3)	14.7 (7.6)	17.2 (5.6)	19.9 † (6.6)
Thyrotrophin (ng/ml)	5.2 (1.5)	5.3 (2.3)	4.9 (2.2)	5.3 (1.6)

† Statistically significantly different from control (p<0.01; t test).

Uncertainties regarding these results were described in the discussion document as follows:

While increased TSH is commonly seen with direct or indirectly acting goitrogens, the observed increase in thyroid hormone is less common,...such effects are far more typical of direct acting thyroid gland inhibitors, than indirectly acting compounds such as clofentezine.

In this regard it should be noted that such isolated "snap shots" of hormone levels fail to provide the "complete picture" of the time and dose related endocrine response to clofentezine treatment and are therefore misleading.

Based on these uncertainties an interpretation of the hormone imbalances observed in clofentezine treated animals should be considered with evidence from the other thyroid studies.

b. Effects on Thyroxine Clearance

The discussion document summarized appropriate studies as follows:

A number of studies...show a lack of accumulation of clofentezine in the thyroid, increased thyroid iodine uptake, increased bile flow with correspondingly increased fecal excretion of radiolabeled iodine, reduced blood levels of radioactivity in treated rats 72 hours after administration, reduced  $^{125}\text{I}$  half life, increased conjugation (measuring UDPGT), and increased excretion of thyroxine metabolites. This picture is entirely consistent with increased thyroidal uptake of iodine and hormone output to compensate for increased "systemic drainage"...

Seventy-two hours after administration of a radiolabeled dose of thyroxine approximately 40% of the radiolabel was recovered in the feces from rats pretreated with clofentezine (30,000 ppm in the diet); 26% was recovered in the feces of control rats during the same period. The total amount of radioactivity recovered from urine and feces combined over the 72-hour period in control and clofentezine-pretreated rats was 54% and 55%, respectively.

Four hours after intravenous injection of radiolabeled thyroxine to two groups of bile duct cannulated rats, the total amount of the  $^{125}\text{I}$  dose recovered from bile samples was only slightly higher in clofentezine pretreated rats (30,000 ppm) than in control animals (10.2% from pretreated and 6.4% from control rats) despite nearly a two-fold increase in the bile flow rate. The concentration of  $^{125}\text{I}$  in bile samples from the clofentezine pretreated rats ranged from 21.6 to 43.5% of that reported for the control group.

Thyroxine half-life values (time required for half of a given concentration of  $^{125}\text{I}$ -thyroxine to be eliminated from the body) were measured in two groups of male rats before and after they were given diets containing 0 or 30,000 ppm clofentezine for 29 days. Table 5 summarizes the results.

Table 5

Mean Half-life (hours) for Thyroxine in the Blood of Clofentezine-Treated Male Rats.

	Dose (ppm)*	
	0	30,000
Before treatment with clofentezine	16.70 <u>+1.09</u>	17.05 <u>+1.08</u>
After treatment with clofentezine	17.61 <u>+1.62</u>	16.42 <u>+1.44</u>
Mean change (hours) during 1 month test period	+ 0.91 <u>+1.91</u>	- 0.64 ** <u>+1.18</u>

\* Test diets were administered to rats for one month.

\*\* P < 0.05, Mann Whitney test.

No statistically significant differences between the treated and control groups were noted after clofentezine administration, but there was a statistically significant difference with respect to the mean change in values before and after clofentezine treatment. This statistically significant change is not likely to be biologically significant without statistically significant differences in the half-life values at each time point.

The results from the thyroxine metabolism and bile flow studies suggest that a shift in the metabolism and excretion pattern for the hormone occurs, but no indication of significantly increased clearance of thyroxine and its metabolites is evident from these data.

c. Goitrogenic Effects

The discussion document stated:

...clofentezine does cause thyroid follicular cell hyperplasia and reductions in thyroid hormones in male rats within one week of initiation of treatment. This early response is associated with statistically significant increases in liver weight, thyroid weight and microsomal UDPGT activity. Subsequently there is clear endocrine and morphological evidence for the stabilization of the thyroid gland at a new steady state of increased activity to compensate for the increased thyroid hormone turnover and clearance.

In subchronic feeding studies, hyperplasia appeared early for rats given the 30,000 ppm diet and persisted as the study progressed (see Table 6). At the 400 ppm level, mitotic activity decreased by the end of the 28-day feeding period suggesting that the thyroid gland may be capable of compensating for the effects of some dietary levels of clofentezine.

Table 6

Incidence of follicular cell observations in male rats given diets containing clofentezine for up to four weeks.

Observation *	Study days				Study days				Study days							
	4	7	14	28	4	7	14	28	4	7	14	28				
Number examined	20	20	20	20	20	20	20	20	20	20	20	20				
	Control				400 ppm				3,000 ppm				30,000 ppm			
Hypertrophy	5	3	1	5	2	6	4	7	2	5	5	1	2	2	3	2
minimal	4	4	4	3	7	4	4	2	11	0	4	4	10	6	7	5
slight	3	2	2	1	3	4	1	4	4	7	8	8	4	9	7	11
moderate	0	0	0	1	1	2	1	1	1	2	1	6	0	2	1	2
severe																
Increased mitotic activity	1	0	1	0	6	2	0	0	7	7	6	5	3	9	7	8
minimal	0	0	0	0	0	0	0	0	2	0	1	0	1	6	3	4
slight	0	0	0	0	0	0	0	0	3	0	0	0	4	0	2	0
moderate	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0
severe																
Hyperplasia	0	0	0	0	0	0	1	0	1	1	2	2	0	2	1	2
minimal	0	0	2	0	0	0	1	0	2	2	1	2	0	4	4	5
slight	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
moderate	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
severe																

\* No "very severe" scores were given for any of these observations.

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The results of the morphometric evaluation of slides were interpreted in the discussion document as follows:

The results clearly showed that in comparison with untreated controls, the glands were enlarged and that this was associated with a very significant increase ( $p < 0.01$ ) in the total numbers of follicular cells, clearly representing thyroid follicular cell hyperplasia.

The area ( $\text{mm}^2$ ) of each thyroid gland section was increased in treated rats and the number of follicular cells and follicles in each gland was increased by treatment (see Table 7).

Table 7

Results from Morphometric Evaluations of Thyroid Slides from Rats Given Diets Containing 0 or 30,000 ppm Clofentezine for Six Weeks.

Observation	Control	Treated
Mean thyroid area ( $\text{mm}^2$ )	2.523	3.978*
Mean total number of follicles	730.3	909.7
Mean number of follicular cells per thyroid	15,781	23,132**

\* Reported to be statistically significantly different from the control group value,  $p < 0.05$ .

\*\* Reported to be statistically significantly different from the control group value,  $p < 0.05$ .

At low doses (400 ppm or less) in the chronic feeding study, the thyroid glands of treated male rats weighed slightly more than those from the control group (see Table 8). The thyroid weights and thyroid-to-body weight ratios were also statistically significantly increased after 29 days on test diets containing 400 or 30,000 ppm (see Table 9).

Table 8

Summary of Group Mean Thyroid Weights (g) and Organ-to-Body-Weight Ratios (%) in Clofentezine Treated Male Rats

Observation	Dose level (ppm)			
	0	10	40	400
At 12 months				
Body weight	609	585	615	601
Thyroid weight	0.027	0.026	0.024	0.026
Thyroid weight ratio	0.005	0.004	0.004	0.004
At 27 months				
Body weight	563	623	630**	589
Thyroid weight	0.035	0.045	0.044	0.050
Thyroid weight ratio	0.006	0.007	0.007	0.008

Table 9

Group Mean Organ Weight Results from Male Rats Given Clofentezine in Their Diets for up to 29 Days

Dose (ppm)	Study day of observation			
	5	8	15	29
Thyroid weight (g)				
0	0.017	0.016	0.017	0.024
10	0.018	0.016	0.017	0.021
400	0.020*	0.016	0.019**	0.022
3,000	0.019	0.021**	0.022**	0.026
30,000	0.018	0.020**	0.024**	0.028**
Thyroid-to-body weight ratio (%)				
0	0.0044	0.0040	0.0042	0.0053
10	0.0044	0.0040	0.0042	0.0047
400	0.0049	0.0041	0.0047*	0.0049
3,000	0.0050	0.0052**	0.0051**	0.0058
30,000	0.0047	0.0051**	0.0057**	0.0066**

\* Statistically significantly different from control,  $p < 0.05$ .

\*\* Statistically significantly different from control,  $p < 0.01$ .

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The thyroid follicular cell hyperplasia observed in short-term studies may be part of the gland's growth process since normal structure is re-established after 28 days and thyroid weights remained higher than control values in subchronic and chronic studies.

d. Progression of Lesions

The discussion document provided the following interpretation:

...Following the initial thyroid stimulation, a marked proliferative response occurs with clear evidence of hypertrophy and hyperplasia. Subsequently, the picture of high glandular activity reduces, indicating physiological compensation or adjustment to maintain a new steady-state...This 'compensated' picture is manifest by follicular cell hypertrophy, colloid depletion, and...hyperplasia...

A dose-response relationship between follicular cell hypertrophy and clofentezine treatment is clearly defined (see Table 10) in the chronic feeding study.

For the incidence of follicular cell hyperplasia in that study, the pair-wise statistical comparisons between the control and treated groups did not suggest a significant dose-related increase, but a statistically significantly positive dose-related trend was observed in one of the re-evaluations. These statistical results suggest a possible effect on thyroid follicular cell morphology, but a study with a broader dose range probably would more clearly established a relationship between dose and follicular cell hyperplasia.

Table 9

Summary of Microscopic Lesions Observed  
in Male Rats Fed Clofentezine for up to 27 Months.

Observation	Dose level (ppm)			
	0	10	40	400
Follicular cell hypertrophy				
Original Report **	4/69	3/65	3/67	5/63
Re-evaluation 1 **	16/70 *	19/67	26/67 †	32/67 ††
Re-evaluation 2 ***	-	-	-	-
Follicular cell hyperplasia				
Originally reported	2/70	2/70	8/70†††	5/70
Re-evaluation 1 **	3/70 *	2/67	3/68	7/69
Re-evaluation 2	5/70	3/70	10/70	6/69

† Statistically significantly different from control (p = 0.033; Fisher's Exact test).

†† Statistically significantly different from control (p = 0.002; Fisher's Exact test).

††† Statistically significantly different from control (p = 0.048; Fisher's Exact test).

\* Statistically significant positive dose-related trend (p<0.05; Cochran-Armitage trend test).

\*\* Follicular cell size was not assessable in some animals according to these reports, and the lesion was graded as follows: 1 = minimum, 2 = slight, 3 = moderate, 4 = severe.

\*\*\* Not assessed in this re-evaluation.

The Agency's proposed threshold policy for thyroid follicular cell neoplasia (cited in Section I. A. above) states:

...A consistent progression of events is noted: reduction in thyroid hormone concentrations, elevation in TSH levels, cellular hypertrophy and hyperplasia,...and neoplasia. Hyperplasia and sometimes neoplasia in the pituitary can also be seen. A block in any of the early steps acts as a block for subsequent steps including tumor development, and cessation of treatment at an early stage in the progression results in regression toward normal thyroid structure and function.

If follicular cell hyperplasia and neoplasia are accepted as manifestations of clofentezine's indirect effect on the thyroid, the combined incidence

of these lesions as reported in the chronic rat feeding study suggests the kind of progression described above (see Table 10 below).

Table 10

Incidence of Thyroid Follicular Cell Lesions Reported in the Chronic Feeding/Oncogenicity Study with Clofentezine in Rats.

Observation	0	Dose level (ppm)		
		10	40	400
Follicular cell hyperplasia	2/70	2/70	8/70 *	5/70
Benign follicular cell tumor	1/70	1/70	1/70	3/70
Malignant follicular cell tumor	1/70	1/70	1/70	5/70
Follicular cell tumors combined	2/70 †	2/70	2/70	8/70 *
Tumors and hyperplasia combined	4/70 †	4/70	10/70	13/70 **

\* Statistically significantly different from control; P = 0.048, Fisher's Exact test.

\*\* Statistically significantly different from control; P = 0.036, Fisher's Exact test.

† Statistically significant trend; p < 0.05; Cochran-Armitage test.

Data from the short-term studies with clofentezine over a broad dose range (10 to 30,000 ppm) suggested that the thyroid gland is capable of adapting to the test diets. The extent and duration of the pituitary-thyroid hormone imbalances induced in treated rats may be dependent on dose, but even at a dose level that is 75-fold greater than the 400-ppm level associated with tumor induction, the disruption of the hormone balance is temporary and of questionable biological significance. The absence of clinical signs such as weight loss, changes in food consumption, or behavioral signs in animals given diets containing large amounts of clofentezine (30,000 ppm) is consistent with this interpretation.

The thyroid gland appears to respond to clofentezine administration by enlarging, but there are no data available to establish the dose level or conditions under which the thyroid gland becomes incapable of compensating for the effects of clofentezine on thyroid hormone physiology. The compensatory response described in the discussion document is contradictory to the expected "consistent progression of events" described in the Agency's proposed policy for assessing compounds that induce thyroid tumors in laboratory animals. Therefore, a mechanism for the induction of thyroid follicular cell tumors in the chronic feeding study in rats has not been completely defined.

III. References

Myhr, B., A. Mitchell, W. Caspary, Y. Lee, S. Poulton. 1982. Acceptability criteria for the evaluation of data in the mouse lymphoma 5178Y forward mutation assay at the TK locus. Abstract from the Thirteenth Annual Meeting of the Environmental Mutagen Society held at Boston, Mass. Environmental Mutagenesis 4(3):309.

Reichard, D. L., Retzer, H. J., Liljedahl, L. A., and Hall, F. R. 1977. Spray Droplet Size Distributions Delivered by Air Blast Orchard Sprayers. Transactions of ASAE. 20(2):232-242.

Retzer, H. J., Hall, F. R., and Reichard, D. L. 1978. Distributions of Droplet Size Delivered by Orchard Sprayers. J. Econ. Ent. 71(1):53.

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APPENDIX I

Exposure calculations submitted to support  
arguments against requiring an acute inhalation  
toxicity study with APOLLO™ 50 SC

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## APPENDIX II

## Data Evaluation Records for

Markham, L. P., and Mallyon, B. A. December 22, 1988. Technical Clofentezine: investigations on the indirect effects of Clofentezine on the thyroid of the male rat. Unpublished report no. TOX/88/167-110 prepared by Schering Agrochemicals Limited, Chesterford Park Research Station, Saffron, Walden, Essex CB10 1XL, England. Submitted by Nor-Am Chemical Co., Wilmington, DE. MRID No. 409755-01

Yarwood, A., and C. Gopinath January 4, 1989. Technical NC 21314: 6 Week Dietary Investigation of Thyroid Function in the Rat. A Morphometric Study on the Thyroid Glands. Unpublished report no. TOX/88/167-113 prepared by Huntingdon Research Centre, Huntingdon, Cambridgeshire, England. Submitted by Nor-Am Chemical Co., Wilmington, DE. MRID No. 409755-02

Mallyon, B. A. January 6, 1989. Technical Clofentezine: Further investigations on the indirect effects of Clofentezine in the male rat. Unpublished report no. TOX/88/167-112 prepared by Schering Agrochemicals Limited, Chesterford Park Research Station, Saffron, Walden, Essex CB10 1XL, England. Submitted by Nor-Am Chemical Co., Wilmington, DE. MRID No. 409755-03

007503

Reviewed by: Roger Gardner *Roger Gardner 9-21-89*  
Section 1, Toxicology Branch 1  
Insecticides/Rodenticides (H7509C)  
Secondary Reviewer: *Ed Budd 9/11/89*  
Section 1, Toxicology Branch 1  
Insecticides/Rodenticides (H7509C)

DATA EVALUATION RECORD

STUDY TYPE: Subchronic feeding study supplement

MRID NUMBER: 409755-01

TEST MATERIAL: Technical grade Clofentezine (Batch no. CR 20099/15, unspecified purity) was used.

SYNONYMS: NC 21314; Apollo; 3,6-bis[2-chlorophenyl]-1,2,4,5-tetrazine; clofentezine

STUDY NUMBER(S): TOX/88/167-110

SPONSOR: Nor-Am Chemical Co., Wilmington, DE

TESTING FACILITY: Schering Agrochemicals Limited, Chesterford Park Research Station, Saffron, Walden, Essex CB10 1XL, England

TITLE OF REPORT: Technical Clofentezine: investigations on the indirect effects of Clofentezine on the thyroid of the male rat.

AUTHOR(S): Markham, L. P., and Mallyon, B. A.

REPORT ISSUED: December 22, 1988.

CONCLUSIONS: Diets containing 0 or 30,000 ppm clofentezine were fed to groups of 50 male Sprague-Dawley strain rats for up to 14 days. The results of the study indicated that the 30,000 ppm diet caused liver enlargement, decreased serum T<sub>3</sub> levels, increased TSH levels in the blood, and increased the incidence of proliferative changes in the thyroid gland (follicular cell hypertrophy and hyperplasia). Based on colloid depletion observations and the incidence of follicular cell changes, the thyroid glands in treated rats appeared to begin compensating for an increased thyroid hormone demand resulting from effects of clofentezine on the liver.

Core Classification: Not applicable.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test species: Male Charles River Cr1: COBS CD(SD)BR Sprague-Dawley rats were used. They were 12 weeks of age on arrival at the laboratory, and they were acclimated to laboratory conditions for 5 days before the start of the experiment. The body weights of test rats at the beginning of the test ranged from 346 to 405 g.

2. 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. They contained 0 or 30,000 ppm clofentezine.

B. STUDY DESIGN

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

<u>Test groups</u>		<u>Dose</u>	<u>Animals/sex *</u>
<u>No.</u>	<u>Designation</u>	<u>(ppm)</u>	
1	Control	0	50
5	High (HDT)	30,000	50

\* Males only.

2. Observations schedule

<u>Type of observation</u>	<u>Number of animals per sex per group</u>	<u>Frequency</u>
Mortality	Survivors	Twice a day*
Signs of toxicity	Survivors	Twice a day*
Body weights	Survivors	On receipt, at randomization and assignment; on the day before start of test; after days 1, 2, 4, 7, and 14; at necropsy
Food consumption	Survivors	At weekly intervals during the study
Blood samples	10	At days 1, 2, 4, 7, and 14
Necropsy	10	At days 1, 2, 4, 7, and 14

\* The study lasted for 14 days.

C. METHODS

1. Observation of blood samples: Blood was collected from the abdominal aorta of anesthetized animals after 1, 2, 4, 7, or 14 days on the test diets.

Blood samples were assayed for thyrotrophin (TSH), total triiodothyroxine (TT3), and total thyroxine (TT4) concentrations.

2. Necropsy:

- a. Weighed organs: These included the liver and thyroid. Thyroids were weighed after fixation.

## 2. Necropsy (continued)

- b. Tissues examined microscopically: The thyroid gland and liver were prepared for microscopic examination.

The report noted that the sections were scored according to the following:

1. Colloid depletion - a reduction in the overall luminal size and staining intensity (degree of eosinophilia) of the colloid.
2. Follicular cell size - cells lining the lumen of the follicle were either squamous in the resting follicles, low cuboidal, cuboidal or columnar in highly active follicles. Lobes with a greater proportion of cuboidal and columnar cells were given a higher score for this condition.
3. Follicular cell hyperplasia - normally follicles are lined by a single layer of cells all the way round the lumen. Any sign of stratification or papillary formation was regarded as abnormal and scored as hyperplasia.
4. Increased mitotic activity of follicular cells - mitotic figures are rarely seen in the lining of thyroid follicles of adult laboratory rats. An occasional cell undergoing division may be observed in either lobe but the presence of several dividing cells is an indication of a proliferative condition which will give rise to hyperplasia in the absence of compensating cell loss.
5. Central resting follicles: - islands of follicles were readily distinguished by their large size, eosinophilia of the colloid and squamous appearance of the lining cells from the surrounding, more uniform follicular tissues in some glands.

For the first four conditions, numerical scores were given for these conditions as follows: 0 = absent, 1 = minimal, 2 = slight, 3 = moderate, 4 = severe, and 5 = very severe. The fifth condition was only rated as present or absent.

4. Statistical analyses: According to the report, parametric statistical tests based on the assumption of normally distributed results were used. For results not normally distributed, nonparametric tests such as the Mann Whitney U test were used. Significant differences between treated and control groups were determined by two-tailed procedures, and a probability value of less than 5% ( $p < 0.05$ ) was considered statistically significant.

## II. REPORTED RESULTS

A. Mortality and Clinical Signs: There were no deaths or clinical signs attributable to administration of the test substance according to the report.

B. Body Weight and Food Consumption: The report indicated that rats given the 30,000 ppm diet had a body weight gain that was 25% less than that reported for the control group. Group mean body weight for the 30,000 ppm group at the end of the 2-week study was 416.3 g compared with 424.3 g for the control group. With the exception of group mean body weight on day 2 of the study, there were no statistically significant differences reported between treatment and control group means with respect to body weights. On day 2 the group mean body weight for controls was reported to be 383.9 g. compared with 371.4 g. for the 30,000 ppm group.

The report stated that food consumption for the 30,000-ppm dose group was 25% less than that for the control group during the first two days of the study, and thereafter the treated group consumed similar amounts of food as the control group rats. Group mean food consumption values for the 30,000-ppm dose group were statistically significantly less than that for the control group at days 2 and 3 in the study.

C. Test Substance Intake: Based on the nominal dietary test substance concentrations and food consumption results, the test substance intake was estimated to be 1915 mg/kg/day for the 30,000 ppm dose group.

D. Clinical Chemistry: The report indicated that triiodothyroxine (T3) levels fell early in the two week feeding period and returned to normal by the end of the study. Thyroxine (T4) levels showed a similar pattern, but no statistically significant differences were observed between the control and treated groups. Thyrotrophin (TSH) was statistically significantly increased from day 4 to the end of the study. Group mean values for TSH, T3, and T4 are summarized from the report as follows:

Study day	Thyrotrophin (ng/ml)		Total T4 (nmol/l)		Total T3 (nmol/l)	
	Control	Treated	Control	Treated	Control	Treated
1	5.8	5.9	83	92	1.5	1.4
2	8.4	7.2	85	92	2.0	1.6*
4	5.3	7.5**	70	73	1.6	1.4*
7	5.2	7.6***	77	70	1.6	1.2**
14	4.9	6.7*	79	79	1.6	1.6

\* Statistically significantly different from controls,  $p < 0.05$ .

\*\* Statistically significantly different from controls,  $p < 0.01$ .

\*\*\* Statistically significantly different from controls,  $p < 0.001$ .

E. Organ Weights: Group mean organ weights (g) were not statistically significantly different from control values for the 30,000 ppm dose group with the exception of day 3. These values are summarized from the report as follows:

Dose (ppm)	2	Study day of observation			15
		3	5	8	
Liver weight (g)					
0	13.90	11.40	13.25	14.25	14.41
30,000	12.38*	13.19**	20.59**	20.12**	22.47**
Thyroid weight (g)					
0	0.020	0.019	0.020	0.020	0.022
30,000	0.022	0.022*	0.022	0.021	0.024

\* Statistically significantly different from control, p<0.05.

\*\* Statistically significantly different from control, p<0.01.

Group mean relative organ-to-body weight ratios (% body weight) for the liver and thyroid of treated male rats are summarized from the report as follows:

Dose (ppm)	2	Study day of observation			15
		3	5	8	
Liver weight (g)					
0	3.579	3.179	3.562	3.536	3.390
30,000	3.347	3.705**	3.378*	20.12**	22.47**
Thyroid weight (g)					
0	0.020	0.019	0.020	0.020	0.022
30,000	0.022	0.022*	0.022	0.021	0.024

\* Statistically significantly different from control, p<0.05.

\*\* Statistically significantly different from control, p<0.01.

F. Necropsy Results: The authors noted that there were no treatment-related macroscopic changes observed in test animals.

Microscopic observations were described in the report as follows:

No changes were observed between the treated and control thyroid glands from rats which had been exposed for up to 2

F. Necropsy Results (continued)

days. After 4 days, although there was still no observable decrease in stored colloid or hypertrophy of the lining cells and the gland looked the same as that of the controls in most respects, 4/10 rats displayed increased mitotic activity in the cells lining the follicles.

After 7 days a greater proportion of the rats given 30000 ppm were displaying an increased mitotic activity of the follicular cells and a frank hyperplasia had developed in some of the animals. There was a pronounced hypertrophy of the lining cells and colloid was severely depleted in 3 animals and moderately so in a further 3, with luminal volume reduced to a minimum so that the follicles appeared to have collapsed.

Cellular division was not so apparent at 14 days..., although it was still significantly greater than controls, but both hypertrophy and hyperplasia of the follicular cells were more severe in a greater proportion of the treated rats. In addition, colloid was depleted in all the dosed rats, although the level of depletion was not as severe as it had been after 7 days.

The incidence of microscopic observations in the thyroid gland are summarized from the report as follows:

Observation	Study days					Study days				
	1	2	4	7	14	1	2	4	7	14
	Control					30,000 ppm				
Number examined	10	10	10	10	10	10	10	10	10	10
Hypertrophy										
minimal	1	1	0	2	1	1	1	1	2	0
slight	3	4	5	4	5	5	4	3	3	1
moderate	2	2	3	2	1	1	2	4	4	7
severe *	0	0	0	0	0	0	0	1	1	2
Increased mitosis										
minimal	0	0	1	0	0	0	1	1	4	5
slight	0	0	0	0	0	0	0	3	1	0
moderate *	0	0	0	0	0	0	0	1	2	0
Hyperplasia										
minimal	0	0	0	0	0	0	0	0	2	1
slight	0	0	1	0	0	0	1	1	0	3
moderate *	0	0	0	0	0	0	0	1	2	4

\* Highest grade with an incidence of 1 or more.

F. Necropsy Results (continued)

Observation	Study days					Study days				
	1	2	4	7	14	1	2	4	7	14
	Control					30,000 ppm				
Number examined	10	10	10	10	10	10	10	10	10	10
Colloid depletion										
minimal	3	0	3	3	3	6	1	2	1	0
slight	2	3	0	2	1	0	3	2	2	2
moderate	0	1	4	1	0	0	2	3	3	8
severe *	0	0	0	0	0	0	0	1	3	0
Central resting follicles										
absent	7	6	10	6	3	7	4	4	3	3
present	3	4	0	4	7	3	6	6	7	7

\* Highest grade with an incidence of 1 or more.

## III. DISCUSSION

A. Authors' Conclusions: The authors' concluded:

The data provide convincing evidence that the initial sequence of events in the male rat following clofentezine administration is liver enlargement (induction), increased T<sub>3</sub> disposal, triggering TSH increase, and thyroid gland stimulation and proliferation (hypertrophy and hyperplasia). After approximately 10 days, compensation leading to a new steady state between thyroid supply and disposal occurs. TSH remains elevated to maintain the necessary drive on the thyroid to meet the increased hormone demands of the new steady state.

Over the longer term it can be anticipated that this effect of clofentezine on the liver, leading to altered thyroid homeostasis is sufficient to cause the small increase in thyroid follicular cell tumors observed in the high dose level male rats in the combined chronic and oncogenicity study.

B. Reviewer's Discussion: The data support the conclusion of the authors that the thyroid gland appears to compensate for initial effects of clofentezine treatment on the liver (increased liver weight) and thyroid gland proliferation (hypertrophy and hyperplasia of follicular cells). However, the dose tested was approximately 75 times the highest dose tested in the chronic/oncogenicity study in rats, and treatment was administered for a much shorter period of time (2 weeks as compared to 118 to 119 weeks). In view of the proliferative

**B. Reviewer's Discussion (continued)**

changes indicated in the short-term high dose study, it is possible that the thyroid gland could be enlarged sufficiently to maintain a homeostasis between thyroid hormone supply and a greater hormone disposal rate without continuous TSH stimulation. If such compensation occurs at the 400-ppm dose level tested in the chronic feeding study, then continuous TSH stimulation may not be an appropriate explanation for the increased follicular cell tumors observed in the chronic/oncogenicity study.

Reviewed by: Roger Gardner *Roger Gardner 8-21-89*  
Section 1, Toxicology Branch 1  
Insecticides/Rodenticides (H7509C)  
Secondary Reviewer: *Ed Budd* *Ed Budd*  
Section 1, Toxicology Branch 1  
Insecticides/Rodenticides (H7509C)

007503

DATA EVALUATION RECORD

STUDY TYPE: Subchronic feeding study supplement

MRID NUMBER: 409755-02

TEST MATERIAL: Technical grade Clofentezine

SYNONYMS: NC 21314; Apollo; 3,6-bis[2-chlorophenyl]-1,2,4,5-tetrazine;  
clofentezine

STUDY NUMBER(S): TOX/88/167-113

SPONSOR: Nor-Am Chemical Co., Wilmington, DE

TESTING FACILITY: Huntingdon Research Centre, Huntingdon, Cambridgeshire,  
England

TITLE OF REPORT: Technical NC 21314: 6 Week Dietary Investigation of  
Thyroid Function in the Rat. A Morphometric Study on the Thyroid Glands

AUTHOR(S): Yarwood, A., and C. Gopinath

REPORT ISSUED: January 4, 1989

CONCLUSIONS: Slides from thyroid glands of male rats given diets containing  
0 or 30,000 ppm clofentezine for 6 weeks were examined morphometrically  
as an extension of histopathology examinations done previously. The  
results indicated that the thyroid glands were enlarged in treated rats.  
The results also suggested that follicular size was increased.

Core Classification: Not applicable

I. BACKGROUND

The report describes a re-evaluation of thyroid tissue sections from a  
6-week study designed to further investigate effects of clofentezine at  
dietary levels of 0, 400, or 30,000 ppm on the thyroid gland in male  
rats.

II. MATERIALS AND METHODS

The thyroid sections originally stained with hematoxylin and eosin were  
re-examined using morphometric methods. The tissue sections were taken  
from groups of 5 male rats given diets containing 0 or 30,000 ppm clofen-  
tezine for six weeks.

## II. MATERIALS AND METHODS (continued)

The parameters noted by the investigators were:

1. The area on the slide occupied by the thyroid glands which was considered a representation of thyroid size.
2. The total number of follicles in the glands.
3. The total number of follicular cells.

Methods were described in the report as follows:

Using a camera lucida, an outline of each thyroid was drawn, at x29 magnification, onto a suitable card. Parathyroids were excluded from the outline. 2 thyroids were drawn for each animal. From each thyroid, 4 selected areas were chosen from the apices, edge and centre. Each of these selected areas was also drawn, at x229.2 magnification, using the camera lucida. In each selected area a count of follicle numbers and follicular cell numbers was made.

In order to obtain the areas of the drawn outlines, a BBC Master 128 K microcomputer, Hegotron Robotics Grafpad 2+ and DIGIT image analysis software were used. Each outline was digitised on the Grafpad, and the area of each was obtained with the DIGIT software. The total of the 4 selected areas for each thyroid was obtained, and expressed as a percentage of the area of the whole thyroid. Using the total number of follicles and thyroid follicular cells counted in the 4 selected areas of each thyroid, and the percentage area in which the counts were performed, a figure for total number of follicles and total number of thyroid follicular cells was obtained for each thyroid. Statistical analyses were performed on the results obtained to test for any treatment-related changes in the parameters examined.

No description of statistical methods was provided in the report.

## III. REPORTED RESULTS

The addendum below includes the results as reported. The investigators noted that the area of thyroid sections from rats given the 30,000-ppm diet were significantly larger than those from control rats, and the number of follicles counted were greater in the treated group than in the control group. They also indicated that the number of follicular cells counted was significantly greater in the 30,000-ppm group than in control animals.

## IV. DISCUSSION

A. Investigators' Conclusions:

Morphometric studies on the thyroid glands of rats treated with 30,000 ppm clofentezine showed that, in comparison with untreated controls, the glands were enlarged and that this was accompanied by an increase in the number of follicular cells, representing follicular cell hyperplasia. These data extend the original findings of colloid depletion and follicular cell hypertrophy observed during routine histopathological examination.

B. Reviewer's Discussion: Since no information on the statistical methods was included in the report, a determination of whether they are appropriate for such small group sizes can not be made.

The measurements of thyroid gland size suggest that the gland is enlarged as the result of clofentezine treatment. However, the results do not clearly support the conclusion that thyroid hyperplasia is occurring. To illustrate this point, a comparison of the number of follicles per unit area and the number of follicular cells per follicle are considered as follows:

<u>Observation</u>	<u>Control</u>	<u>Treated</u>
Mean thyroid area (mm <sup>2</sup> )	2.523	3.978*
Mean total number of follicles	730.3	909.7
Mean number of follicular cells per thyroid	15,781	23,132**
Follicles per mm <sup>2</sup> of thyroid	289.5	228.7
Follicular cells/follicle	21.6	25.4

\* Reported to be statistically significantly different from the control group value,  $p < 0.05$ .

\*\* Reported to be statistically significantly different from the control group value,  $p < 0.05$ .

The increase in thyroid area observed in treated rats can be attributed to an increase in both size and number of follicles, and the increased number of follicular cells may also be associated with enlargement of the thyroid gland in response to clofentezine treatment.

ADDENDUM

Individual Data on Morphometric Observations  
of Thyroid Glands from Male Rats Given Diets Containing  
0 or 30,000 ppm Clofentezine

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Reviewed by: Roger Gardner *Roger Gardner 1-21-89*  
Section 1, Toxicology Branch 1  
Insecticides/Rodenticides (H7509C)  
Secondary Reviewer: *Ed Budd 9/11/89*  
Section 1, Toxicology Branch 1  
Insecticides/Rodenticides (H7509C)

DATA EVALUATION RECORD

STUDY TYPE: Subchronic feeding study supplement

MRID NUMBER: 409755-03

TEST MATERIAL: Technical grade Clofentezine (Batch no. CR 20099/15, unspecified purity) was used.

SYNONYMS: NC 21314; Apollo; 3,6-bis[2-chlorophenyl]-1,2,4,5-tetrazine; clofentezine

STUDY NUMBER(S): TOX/88/167-112

SPONSOR: Nor-Am Chemical Co., Wilmington, DE

TESTING FACILITY: Schering Agrochemicals Limited, Chesterford Park Research Station, Saffron, Walden, Essex CB10 1XL, England

TITLE OF REPORT: Technical Clofentezine: Further investigations on the indirect effects of Clofentezine in the male rat.

AUTHOR(S): Mallyon, B. A.

REPORT ISSUED: January 6, 1989

CONCLUSIONS: Diets containing 0, 10, 400, 3,000, or 30,000 ppm clofentezine were fed to groups of 80 male Sprague-Dawley strain rats for 28 days. At 400 ppm or higher, increased liver enzyme activity (UDPGT) and thyroid activity (hypertrophy, hyperplasia and mitotic activity of follicular cells, colloid depletion and an increase in thyroid weight) were noted. The thyroid changes appeared to diminish in frequency and severity during the study based on microscopic examination of serially sacrificed rats. A no effect level was established at 10 ppm.

Core Classification: Not applicable.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test species: Male Charles River Crl: COBS CD(SD)BR Sprague-Dawley rats were used. They were 28 days of age on arrival at the laboratory, and they were acclimated to laboratory conditions for 56 days before being placed on the study. The body weights of test rats ranged from 298 to 470 g.

2. 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. They contained 0, 10, 400, 3000, or 30,000 ppm clofentezine.

B. STUDY DESIGN

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

<u>Test groups</u>		<u>Dose</u>	<u>Animals/sex</u> *
<u>No.</u>	<u>Designation</u>	<u>(ppm)</u>	
1	Control	0	80
2	Low (LDT)	10	80
3	Low mid	400	80
4	High mid	3,000	80
5	High (HDT)	30,000	80

\* Males only.

2. Observations schedule

<u>Type of observation</u>	<u>Number of animals per sex per group</u>	<u>Frequency</u>
Mortality	All	Twice a day*
Signs of toxicity	All	Twice a day*
Body weights	All	On receipt, at randomization and assignment; at weekly intervals pre-test; study days 1 and 8 and weekly thereafter; at necropsy
	20	Only for animals sacrificed on day 5.
Food consumption	All	At weekly intervals during the study and on day 5 for sacrificed animals only.
Blood samples	20	At days 4, 7, 14, and 28
Necropsy	20	At days 4, 7, 14, and 28

\* The study lasted for 28 days.

C. METHODS

1. Observation of blood samples: Blood was collected from the abdominal aorta of anesthetized animals after 4, 7, 14, or 28 days on the test diets. According to the report, samples were kept frozen for possible hormonal analysis at a later time.

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2. Other special observations: Microsomal uridine diphosphoglucuronate glucuronyl transferase activity was measured in liver samples prepared at necropsy. The method used was described in the report as follows:

..., the livers were removed from the first five animals in each group (except group 4)...and approximately 3 gram quantities were homogenized into ice-cold buffer...Microsomes were prepared from these samples for UDPGT analysis.

Microsomal uridine diphosphate glucuronosyl transferase was estimated by measuring the decrease in absorbance at 405 nm of nitrophenol. Results were expressed as the change in absorbance per minute per miligram of microsomeal protein.

3. Necropsy Gross lesions were noted.
- a. Weighed organs: These included the liver and thyroid. Thyroids were weighed after fixation.
- b. Tissues examined microscopically: The thyroid gland and liver were prepared for microscopic examination.

The report noted that the sections were scored according to the following:

1. Colloid depletion - a reduction in the overall luminal size and staining intensity (degree of eosinophilia) of the colloid.
2. Follicular cell size - cells lining the lumen of the follicle were either squamous in the resting follicles, low cuboidal, cuboidal or columnar in highly active follicles, and lobes with a greater proportion of cuboidal and columnar cells were given a higher score for this condition.
3. Follicular cell hyperplasia - normally follicles are lined by a single layer of cells all the way round the lumen. Any sign of stratification or papillary formation was regarded as abnormal and scored as hyperplasia.
4. Increased mitotic activity of follicular cells - mitotic figures are rarely seen in the lining of thyroid follicles of adult laboratory rats. An occasional cell undergoing division may be observed in either lobe but the presence of several dividing cells is an indication of a proliferative condition which will give rise to hyperplasia in the absence of compensating cell loss.
5. Central resting follicles: - islands of follicles were readily distinguished by their large size, eosinophilia of the colloid and squamous appearance of the lining cells from the surrounding, more uniform follicular tissues in some glands.

b. Tissues examined microscopically (continued)

For the first four conditions, numerical scores were given for these conditions as follows: 0 = absent, 1 = minimal, 2 = slight, 3 = moderate, 4 = severe, and 5 = very severe. The fifth condition was only rated as present or absent.

4. Statistical analyses: According to the report, parametric statistical tests based on the assumption of normally distributed results were used. For results not normally distributed, nonparametric tests such as the Mann Whitney U test were used. Significant differences between treated and control groups were determined by two-tailed procedures, and a probability value of less than 5% ( $p < 0.05$ ) was considered statistically significant.

## II. REPORTED RESULTS

- A. Mortality and Clinical Signs: There were no deaths or clinical signs attributable to administration of the test substance according to the report.
- B. Body Weight and Food Consumption: The report indicated that rats given the 30,000 ppm diet had a body weight gain that was 25% less than that reported for the control group. Group mean body weight for the 30,000 ppm group at the end of the 4-week study was 428.3 g compared with 442.9 g for the control group. There were no statistically significant differences reported between treatment and control group means with respect to body weights.

The report stated that food consumption for the 30,000-ppm dose group was 17% less than that for the control group at day 4 of the study, and thereafter the highest dosed group consumed approximately 6% less food than the control group rats. Group mean food consumption values for the 30,000-ppm dose group were statistically significantly less than that for the control group at days 4 and 7 in the study, and no other significant differences were noted in other treatment groups or at study days 14 and 28 in the 30,000-ppm dose group.

- C. Test Substance Intake: Based on the nominal dietary test substance concentrations and food consumption results, the test substance intake was estimated to be 0.58, 22.69, 169.4, and 1635 mg/kg/day for the 10, 400, 3,000, and 30,000 ppm dose groups, respectively.
- D. Special Observations (UDPGT Activity): According to the report, the 400 and 30,000 ppm dose groups were the only groups that had consistently increased UDPGT activity (change in absorbance/min/mg protein). Mean values for that observation are summarized as follows:

D. Special Observations (UDPGT Activity) (continued)

Dose (ppm) †	Study day of observation			
	5	8	15	29
0	0.019	0.034	0.019	0.018
10	0.031*	0.039	0.025*	0.028
400	0.032*	0.065***	0.045***	0.041***
30,000	0.098***	0.125***	0.101***	0.098***

† No measurements were made for the 3,000 ppm group.

\* Statistically significantly different from control,  $p < 0.05$ .

\*\*\* Statistically significantly different from control,  $p < 0.001$ .

E. Organ Weights: Group mean organ weights were statistically significantly increased above control values for the 3,000 and 30,000 ppm dose groups throughout the study. These values are summarized from the report as follows:

Dose (ppm)	Study day of observation			
	5	8	15	29
Liver weight (g)				
0	13.59	13.46	13.05	14.46
10	14.00	13.60	13.73	14.69
400	14.87	14.06	15.40**	15.90
3,000	17.48**	17.04**	19.34**	20.51**
30,000	18.44**	19.69**	21.90**	22.40**
Thyroid weight (g)				
0	0.017	0.016	0.017	0.024
10	0.018	0.016	0.017	0.021
400	0.020*	0.016	0.019**	0.022
3,000	0.019	0.021**	0.022**	0.026
30,000	0.018	0.020**	0.024**	0.028**

\* Statistically significantly different from control,  $p < 0.05$ .

\*\* Statistically significantly different from control,  $p < 0.01$ .

Group mean relative organ-to-body weight ratios (% body weight) for the liver and thyroid of treated male rats are summarized from the report as follows:

E. Organ Weights (continued)

Dose (ppm)	Study day of observation			
	5	8	15	29
Liver-to-body weight ratio (%)				
0	3.508	3.350	3.259	3.262
10	3.562	3.409	3.365	3.286
400	3.692	3.466	3.763**	3.578**
3,000	4.555**	4.284**	4.466**	4.569**
30,000	4.852**	5.091**	5.265**	5.228**
Thyroid-to-body weight ratio (%)				
0	0.0044	0.0040	0.0042	0.0053
10	0.0044	0.0040	0.0042	0.0047
400	0.0049	0.0041	0.0047*	0.0049
3,000	0.0050	0.0052**	0.0051**	0.0058
30,000	0.0047	0.0051**	0.0057**	0.0066**

\* Statistically significantly different from control,  $p < 0.05$ .

\*\* Statistically significantly different from control,  $p < 0.01$ .

F. Necropsy Results: According to the report, there were no treatment-related macroscopic findings observed at necropsy.

The incidence of microscopic lesions in the thyroid were described in the report as follows:

After 4 days the level of mitotic activity in follicular cells of the thyroid gland was increased at a statistically significant level in the animals receiving 30000, 3000 and 400 ppm. There was a mild depletion of luminal colloid and the size of follicular cells appeared to have increased in many of these animals, but significance was achieved only in the 3000 ppm group in comparison with the controls. Three of the rats on this level also showed mild hyperplastic changes in the follicular cells.

After 7 days there was a less active level of mitotic activity in the follicular cells of animals receiving 30000, 3000, and 400 ppm, although it was seen in a greater proportion of animals at the highest dose than had been observed after only 4 days of administration. There was a marked depletion of colloid with 16/20 animals showing at least a slight response compared with only 4 controls. Follicular cell hypertrophy was also clearly demonstrated, with 17/20 animals showing at least a slight response compared with only 6 controls. There was a clear dose response relationship for all these three conditions with

#### F. Necropsy Results (continued)

no effect at 10 ppm and an increase in frequency and severity with ascending level of dose to 30000 ppm where the pairwise comparison against the control distribution was highly statistically significant for each of these conditions. Follicular cell hyperplasia was observed at 30000 and 3000 ppm.

After 14 days fewer animals were showing increased mitotic activity of the follicular cells, although this condition was still significantly greater than normal in the 30000 and 3000 ppm groups. In the 400 ppm group no animals had an increase in mitotic activity of the follicular cells. Colloid depletion and hypertrophy of the follicular cells were still showing a dose response relationship with the most severe effect at 30000 ppm and no change at 10 ppm. The hyperplasia scored at 400, 3000, and 30000 ppm indicated a dose response relationship which would be expected from the preceding levels of mitotic activity in these cells at those doses.

After 28 days mitotic activity of the follicular cells was seen in even fewer animals to a lesser degree. Conversely colloid depletion, cellular hypertrophy and cellular hyperplasia were increased in both severity and incidence. Pairwise comparisons of all three conditions were highly statistically significant for both 30000 and 3000 ppm against controls and colloid depletion was also statistically significant at 400 ppm. One animal in the 3000 ppm group had a focus of hyperplasia which consisted of five follicles, none of which displayed cystic distention, lined by follicular cells with hyperchromatic nuclei. The cells were of moderate size, with stratification and one cell was undergoing mitotic division.

### III. DISCUSSION

#### A. Investigators' Conclusions: The investigators concluded:

...dietary administration of clofentezine to the male rat at dose levels of 400 ppm and above resulted in early and profound histological changes in the thyroid gland. Events were characterized by an initial increase in mitotic activity of follicular cells followed by colloid depletion, hypertrophy, hyperplasia of the follicular lining cells and an increase in thyroid weight. These changes were concurrent with marked effects on the liver i. e. increased liver weight and levels of microsomal UDPGT.

The no effect level was between 10 and 400 ppm.

- B. Reviewer's Discussion and Conclusions: Trends in the incidence and severity of microscopic changes in the thyroid glands of treated male rats suggest that the animals may be adapting to clofentezine in their diet.

Results in the 400-ppm dose group indicated that the rats could be establishing a new steady state with respect to thyroid function by the end of the 28-day treatment period. The frequency and degree of increased mitotic activity, hypertrophy, and hyperplasia in the follicular cells (see Table 1) are consistent with increased thyroid gland weights observed at 400-ppm or higher levels. These changes suggest that the animals are adapting to continuous clofentezine treatment by thyroid gland enlargement which increases the animal's capacity to maintain a higher level of thyroid activity in response to the test substance. The follicular cell changes appear to diminish in frequency and severity during the 28-day test at the 400-ppm dose level further suggesting that the observed thyroid gland enlargement is sufficient to support a higher level of thyroid activity without toxicologically significant histopathological changes.

Colloid depletion and central resting follicle observations (see Table 2) do not strongly suggest adaptation of thyroid function at 400, 3,000, or 30,000 ppm. However, a longer treatment period may show that these changes could also reverse as did the follicular cell hypertrophy, mitotic activity, and hyperplasia at the 400-ppm level.

The results as presented support the conclusions of the investigators that a no-observed-effect level with respect to increased liver enzyme activity and thyroid activity is established at 10 ppm.

Table 1

Incidence of follicular cell observations in male rats given diets containing clofentezine for up to four weeks.

Observation *	Study days				Study days				Study days			
	4	7	14	28	4	7	14	28	4	7	14	28
Number examined	20	20	20	20	20	20	20	20	20	20	20	20
	Control				400 ppm				3,000 ppm			
	5	3	1	5	2	6	4	7	2	5	5	1
Hypertrophy	4	4	4	3	7	4	4	2	11	0	4	4
minimal	3	2	2	1	3	4	1	4	4	7	8	8
slight	0	0	0	1	1	2	1	1	1	2	1	6
moderate												
severe												
	1	0	1	0	6	2	0	0	7	7	6	5
Increased mitotic activity	0	0	0	0	0	0	0	0	2	0	1	0
minimal	0	0	0	0	0	0	0	0	3	0	0	0
slight	0	0	0	0	0	0	0	0	1	6	3	4
moderate	0	0	0	0	0	0	0	0	4	0	2	0
severe	0	0	0	0	0	0	0	0	1	0	0	0
	0	0	0	0	0	0	1	0	1	1	2	2
Hyperplasia	0	0	2	0	0	0	1	0	2	2	1	2
minimal	0	0	0	0	0	0	1	0	0	0	0	0
slight	0	0	0	0	0	0	1	0	0	0	0	1
moderate	0	0	0	0	0	0	1	0	0	0	0	0
severe	0	0	0	0	0	0	0	0	0	0	0	0

\* No "very severe" scores were given for any of these observations.

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Table 2

Incidence of colloid and follicular observations in male rats given diets containing clofentazine for up to four weeks.

Observation *	Study days															
	4	7	14	28	4	7	14	28	4	7	14	28	4	7	14	28
Number examined	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
	Control															
	400 ppm															
	3,000 ppm															
	30,000 ppm															
Colloid depletion																
minimal	5	3	2	2	4	10	6	9	6	4	3	3	7	3	5	5
slight	1	4	3	2	3	2	1	2	10	7	5	7	6	7	8	5
moderate	3	0	0	0	3	1	2	1	1	1	5	8	2	9	4	8
severe	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	2
Central resting follicles																
absent	12	12	14	16	9	11	17	13	11	6	14	11	9	8	13	13
present	8	8	6	4	11	9	3	7	9	14	6	9	11	12	7	7

\* No "very severe" scores were given for any of these observations.

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