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Toxicology and Carcinogenesis Studies of Microencapsulated Citral in Rats and Mice

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Citral, a widely used natural ingredient, is added to foods and cosmetics as a flavoring and fragrance agent. Male and female F344/N rats and B6C3F1 mice were exposed to microencapsulated citral in the feed for 14 weeks or two years. All studies included untreated and vehicle control groups. In the 14-week studies, rats and mice were given diets containing 3900, 7800, 15,600, or 31,300 ppm citral. In rats, food consumption was reduced in the two highest dose groups. In mice an apparent increase in food consumption was observed, but was due to mice scattering the feed. Body weights of all treated animals were less than controls. All rats and four male mice were killed moribund in the high dose groups. In rats, forestomach and kidney lesions were observed. At the higher doses, lesions observed in the bone marrow, testes, and thymus in rats and in the ovary in mice were considered related to inanition and resultant moribundity. In the two-year studies, rats were exposed to 1000, 2000, or 4000 ppm citral. Body weights were reduced in the 4000 ppm rats. Mice were exposed to 500, 1000, or 2000 ppm citral. Body weights in the 1000 and 2000 ppm groups were reduced. No neoplasms were attributed to citral in rats or mice. Malignant lymphoma occurred with a positive trend and was significantly greater than controls in female mice in the 2000 ppm group. However, the incidences were within the NTP historical control range and could not be clearly related to citral administration.

Key Words: **food additives; fragrance additives; GRAS list; microencapsulation; toxicity; malignant lymphoma; vinyl aldehyde; rats; mice; nephropathy.**

Citral is a β-substituted vinyl aldehyde that occurs naturally in the leaves and fruit of several plant species including myrtle trees, African basil, lemons, limes, oranges, and tomatoes (Furia and Bellanca, 1975; Opdyke, 1979). Because of its strong lemon flavor and odor, citral is used as a flavoring and fragrance agent in foods and cosmetics and is a Generally Recognized as Safe (GRAS) list chemical. The average daily intake of citral in humans was estimated to be 5 mg/kg (Council of Europe, 1974). It is present in chewing gum, baked goods, candy, ice cream, and in beverages at concentrations ranging from 9 to 170 ppm (Opdyke, 1979). It also is used to impart lemon or verbena scents in soaps, detergents, creams, lotions, and perfumes (Opdyke, 1979). In addition, citral is used as a chemical intermediate in the synthesis of vitamin A, ionone, and methylionone (Budavari, 1989).

Citral was selected for carcinogenicity studies because of its widespread use as a flavoring and fragrance ingredient. Human exposure can be anticipated to occur primarily through the oral route, but also through contact with skin. Citral administered dermally has previously been shown in multiple species to be a sensitizing agent. Citral was found to be severely irritating to albino angora rabbits, male Hartley guinea pigs, and humans (Basketter and Scholes, 1992; Cardullo *et al.,* 1989; Motoyoshi *et al.,* 1979). It was positive in the local lymph node assay (Basketter and Scholes, 1992). Citral also has been shown to induce benign and atypical prostatic hyperplasia in rats when applied dermally for one or more months (Engelstein *et al.,* 1996, Kessler *et al.,* 1998, Scolnik *et al.,* 1994; Servadio *et al.,* 1986).

Because the most widespread exposure to citral likely occurs from consumption of foods, administration through the diet was preferable. However, because citral volatilizes rapidly and binds to reactive moieties in the diet, traditional feeding studies were not possible (Kuhn *et al.,* 1991). A microencapsulation technique was developed to allow for administration of citral in the diet with minimal loss (Kuhn *et al.,* 1991). A comparative study in F344/N rats and B6C3F1 mice exposed to citral for 14 days by oral gavage or through microencapsulated citral in the diet showed that microencapsulation was an acceptable alternative to gavage administration (Dieter *et al.,* 1993). Previous work also has shown that the bioavailability and toxicity of a similar compound, cinnamaldehyde, administered in microcapsules was not altered compared to corn oil gavage (Hébert *et al.,* 1994; Yuan *et al.,* 1993).

The present studies were performed to characterize the toxicity of citral when administered in the diet to F344/N rats and B6C3F1 mice. The details of these studies have been reported

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in a Technical Report (NTP, 2001). Major findings from these studies are presented here.

MATERIALS AND METHODS

Chemical. Citral (CAS# 5392-40-5; geometric isomer ratio of 2:1 geranial:neral) was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI) in two lots. Lot A (~ 97.6% pure) was used during the 14-week studies, and lot B (~ 94% pure) was used during the two-year studies. The neat chemical was stable when stored at temperatures up to 60°C and protected from light.

Microcapsule formulation and analyses. Citral was microencapsulated by Midwest Research Institute (MRI, Kansas City, MO). Microcapsules loaded with neat citral and placebos (empty microcapsules) were prepared in several batches by a proprietary process using food-grade sugar and starch to produce dry microspheres. The batches were homogenized and passed through 40- over 140-mesh sieves and were stored in amber glass bottles at room temperature before shipping to the testing laboratory, Battelle (Columbus, OH). The citral load of the microcapsules, determined by HPLC, was 32.9%. The microcapsules were stored in amber glass bottles, protected from light, at approximately 5°C during the studies. The stability of the microcapsules was monitored during the 14-week and two-year studies; no loss of citral from the microcapsules was detected.

Preparation and analysis of dose formulations. The dose formulations were prepared with NTP-2000 feed (Zeigler Brothers, Inc., Gardners, PA) every two to four weeks during the 14-week studies and approximately every four weeks during the two-year studies. Dose formulations were analyzed at three timepoints (14-week studies) and every 9 to 12 weeks (two-year studies) and were within 10% of the target concentrations.

Fourteen-week studies. Four-week-old male and female F344/N rats and B6C3F1 mice were obtained from Taconic Laboratory Animals and Services (Germantown, NY) and were quarantined for 11 to 15 days. Animals were approximately six weeks old on the first day of the studies. Before the studies began, five male and five female rats and mice were randomly selected for parasite evaluation and gross observation for evidence of disease. All tests for viral titers from rats and mice were negative.

Groups of 20 male and female rats and 10 male and female mice were fed the following diets: control (without microcapsules), placebo control (with empty microcapsules), 3900, 7800, 15,600, or 31,300 mg microencapsulated citral/kg diet (ppm) for 14 weeks. Placebo and/or loaded microcapsules were combined with feed to a concentration of 10% microcapsules. Feed and water were available *ad libitum*. Rats and female mice were housed five per cage, and male mice were housed individually. Clinical findings were recorded weekly for rats and mice. Feed consumption was recorded twice weekly or once weekly (male mice). The animals were weighed initially, weekly thereafter, and at the end of the studies.

Blood was collected from the retroorbital sinus of 10 designated rats from each group under carbon dioxide anesthesia on days 4 and 22 for hematology and clinical pathology and then euthanized with CO_2. Using the same method, blood was collected from all core study rats and mice surviving to the end of the studies for hematology (both species) and clinical chemistry (rats) analyses. Blood samples for hematology analyses were placed in microcollection tubes containing potassium EDTA. Erythrocyte, platelet, leukocyte counts, hematocrit values, hemoglobin concentration, mean cell volume, mean cell hemoglobin, and mean cell hemoglobin concentration were determined using a Serono-Baker System 9000 hematology analyzer (Serono-Baker Diagnostics, Allentown, PA) with reagents supplied by the manufacturer. Differential leukocyte counts and erythrocyte and platelet morphologies were determined microscopically from blood smears stained with a modified Wright-Giemsa stain on a Hema-Tek® slide stainer (Miles Laboratory, Ames Division, Elkhart, IN). A Miller disc was used to determine reticulocyte counts from smears prepared with blood stained with new methylene blue. For clinical chemistry analyses, blood samples from rats were placed into microcollection serum separator tubes and centrifuged. The serum samples were analyzed using a Hitachi 704® chemistry analyzer (Boehringer Mannheim, Indianapolis, IN) using commercially available reagents. Clinical chemistry endpoints included: urea nitrogen, creatinine, total protein, albumin, alanine aminotransferase, alkaline phosphatase, creatine kinase, sorbitol dehydrogenase, and bile acids.

Necropsies were performed on all core study animals. The heart, right kidney, liver, lung, right testis, and thymus were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 μm, and stained with hematoxylin and eosin. A complete histopathologic examination was performed on all core study untreated control and vehicle control rats and mice, 15,600 ppm rats, and 31,300 ppm rats and mice.

Two-year studies. Groups of 50 male and 50 female rats and mice were fed the following diets: control (without microcapsules), placebo control (with microcapsules), 500 (mice only), 1000, 2000, or 4000 (rats only) ppm microencapsulated citral for up to two years. Placebo and/or loaded microcapsules were combined with feed to a concentration of 1.25% microcapsules. Male rats were housed three per cage, female rats and mice were housed five per cage, and male mice were housed individually. Feed and water were available *ad libitum*. Feed consumption was measured over a one-week period approximately every four weeks by cage. Cages were changed once (male mice) or twice weekly; cages and racks were rotated every two weeks.

All animals were observed twice daily for moribundity and mortality. Clinical findings and body weights were recorded initially (body weights only), on week 2, week 6, every four weeks thereafter, and at the end of the studies. Complete necropsies and microscopic examinations were performed on all rats and mice. At necropsy, all organs and tissues were examined for grossly visible lesions, and all major tissues were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 μm, and stained with hematoxylin and eosin for microscopic examination.

All rodent studies were conducted at Battelle Columbus Laboratories, Columbus, Ohio, accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC, Rockville, MD); Institutional Animal Use and Care Committees approved the experimental protocols. Animal use was in accordance with the United States Public Health Service policy on humane care and use of laboratory animals and the Guide for the Care and Use of Laboratory Animals. Developmental chemistry efforts were conducted at Midwest Research Institute, Kansas City, MO.

Statistical methods. The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958). Statistical analyses for possible dose-related effects on survival used Cox's (1972) method for testing two groups for equality and Tarone's (1975) life table test to identify dose-related trends. Organ and body weight data were analyzed with the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Hematology and clinical chemistry data were analyzed using the nonparametric multiple comparison methods of Shirley (1977) and Dunn (1964). Extreme values were identified by the outlier test of Dixon and Massey (1951). Average severity values were analyzed for significance with the Mann-Whitney *U*-test (Hollander and Wolfe, 1973). The Poly-k test (Bailer and Portier, 1988; Portier and Bailer, 1989) was used to assess neoplasm and nonneoplastic lesion prevalence.

RESULTS

Rats

Fourteen-week studies. In the second week of the study, all rats in the 31,300 ppm groups were killed moribund (Table 1). Final mean body weights and body weight gains of males and females that survived to the end of the study were generally significantly less than those of the vehicle controls. Feed con-

TABLE 1
Survival, Body Weights, and Feed Consumption of Rats in the 14-Week Feed Study of Citral

		Mean body weight (g)				Feed consumption	
Concentration (ppm)	Survival	Initial	Final	Change	Final weight (%)	Week 1	Week 14
Male							
Vehicle control	10/10	81 ± 2	336 ± 6	255 ± 6	—	15.4	18.7
3900	10/10	80 ± 2	318 ± 6*	238 ± 5**	95	15.9	19.9
7800	10/10	84 ± 3	292 ± 4**	208 ± 3**	87	15.1	20.1
15,600	10/10	84 ± 2	247 ± 4**	163 ± 4**	73	8.4	15.6
31,300	0/10[a]	80 ± 2	—	—	—	4.0	—
Female							
Vehicle control	10/10	82 ± 3	190 ± 4	108 ± 4	—	12.8	10.7
3900	10/10	79 ± 3	180 ± 4*	101 ± 4	95	11.6	9.6
7800	10/10	84 ± 3	181 ± 2*	97 ± 2*	96	11.8	10.8
15,600	10/10	84 ± 3	166 ± 2**	82 ± 2**	88	6.5	10.2
31,300	0/10[a]	84 ± 2	—	—	—	4.7	—

Note. Survival values, number of animals surviving at 14 weeks/number initially in group. Weights and weight changes are given as mean ± SE. Final mean body weights were not calculated for groups with 100% mortality. Final weight value is relative to vehicle controls. Feed consumption is expressed as grams per animal per day.

[a]Week of moribund sacrifice: 2.

*Significantly different ($p \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test.

**$p \leq 0.01$.

sumption by 15,600 and 31,300 ppm males and females was less than that of the vehicle controls during the first week of the study, possibly due to poor palatability. Dietary concentrations of 3900, 7800, 15,600, and 31,300 ppm resulted in average daily doses of approximately 345, 820, 1785, and 1586 mg citral/kg body weight to males and 335, 675, 1330, and 2127 mg/kg to females.

There were several transient treatment-related hematological

TABLE 2
Incidences of Selected Nonneoplastic Lesions in Rats in the 14-Week Feed Study of Citral

		Concentration (ppm)			
	Vehicle control	3900	7800	15,600	31,300
Male					
Stomach, forestomach	10[a]	0	0	10	10
Hyperkeratosis	0[b]	0	0	0	2 (1.0)[c]
Epithelium, hyperplasia	0	0	0	0	2 (2.0)
Bone marrow	10	0	10	10	10
Atrophy	0	0	0	7** (1.0)	10** (1.9)
Hemorrhage	0	0	0	0	10** (1.9)
Kidney	10	10	10	10	10
Nephropathy	0	3 (1.0)	10** (1.0)	8** (1.0)	0
Female					
Stomach, forestomach	10	0	0	10	10
Hyperkeratosis	0	0	0	0	4* (1.5)
Epithelium, hyperplasia	0	0	0	0	4* (1.3)
Bone marrow	10	0	10	10	10
Atrophy	0	0	0	8** (1.0)	4* (1.5)
Hemorrhage	0	0	0	0	9** (2.1)

[a]Number of animals with tissue examined microscopically.

[b]Number of animals with lesion.

[c]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

*Significantly different ($p \leq 0.05$) from the vehicle control group by the Fisher exact test.

**$p \leq 0.01$.

and serum biochemical effects (data not shown; NTP, 2001). Increases in hematocrit values, hemoglobin concentrations, and erythrocyte and platelet counts, and decreases in mean cell volumes, mean cell hemoglobin, and reticulocyte counts were observed. These effects were transient, were not observed after day 22, and were consistent with physiologic responses related to decreased feed and water consumption.

No gross lesions were observed that could be attributed to citral exposure. Microscopically, exposure of rats to citral was associated with forestomach epithelial hyperplasia and hyperkeratosis (Table 2). Characterized by minimal to mild thickening of the stratified squamous epithelium and of the cornified superficial layer of the mucosa, forestomach epithelial hyperplasia and hyperkeratosis were observed in several 31,300 ppm males and females, with a greater incidence in females.

In male rats, nephropathy was generally a minimal to mild change characterized by foci of regenerative epithelium, occasional eosinophilic casts, peritubular mononuclear infiltrates and dilated tubules (Table 2). Granular casts were few and scattered within the outer strip of the outer medulla. They were characterized by dilated tubules filled with granular eosinophilic material presumed to be proteinacious material and cellular debris.

The incidences of bone marrow atrophy were significantly increased in 15,600 and 31,300 ppm males and females (Table 2). In the groups receiving 31,300 ppm, atrophy was of mild severity and was characterized by decreased myelopoietic cells with a relative increase in the adipose cells in the marrow spaces. Hemorrhage was also present in all males and nine females exposed to 31,300 ppm and was attributed to loss of vascular sinus integrity and extravasation of erythrocytes throughout the marrow spaces. Minimal atrophy, without accompanying hemorrhage, was considered a borderline lesion in the 15,600 ppm groups. It was not clear if the bone marrow lesions were a direct effect of citral toxicity or were due to inanition, but it was probable that inanition contributed to the lesions.

Also in the 31,300 ppm group, thymic atrophy was observed in males and females (data not shown). Aspermia was observed in the testes of all 31,300 males (data not shown). These lesions only occurred in the highest exposure group and were likely related to inanition and the moribund condition of the animals.

Because of an apparent early palatability problem that resulted in a reduction in body weight gain in males exposed to 7800 ppm and the fact that they never recovered from this initial effect, 4000 ppm was selected as the high dose for the two-year study. For females, the reduction in body weight gain at the end of the study was approximately the same for the 3900 and 7800 ppm groups, however, females in the 7800 ppm group were slightly more adversely affected by treatment in the first two weeks. Therefore, 4000 ppm was selected as the high dose for the two-year studies. Lower doses of 1000 and 2000 ppm also were used in the two-year study to evaluate dose-response relationships.

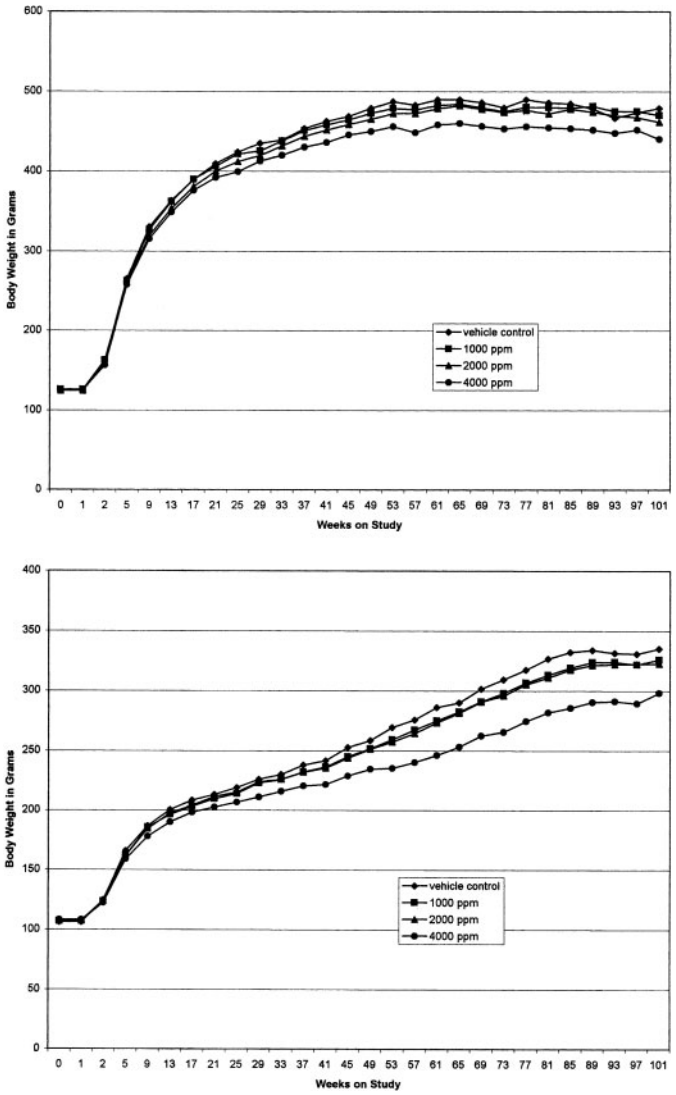

FIG. 1. Growth curves for male (top) and female (bottom) rats exposed to citral in feed for two years.

Two-year study. In the two-year rat study, survival of all exposed groups of males (control 22/50, low dose 32/50, mid dose 35/50, and high dose 34/50) was significantly greater than that of the vehicle control group, and survival of females was similar to that of the vehicle control group (control 40/50, low dose 36/50, mid dose 36/50, and high dose 36/50). Mean body weights of rats exposed to 4000 ppm were generally less than those of the vehicle controls from week 49 (males) or 25 (females) to the end of the study (Fig. 1). Feed consumption by exposed groups was similar to that by vehicle controls (data not shown). Dietary concentrations of 1000, 2000, and 4000 ppm delivered average daily doses of approximately 50, 100, and 210 mg citral/kg body weight to males and females. There were no clinical findings attributed to citral exposure.

There were dose related increases in the incidence (42/50, 45/50, 48/50, 50/50) and severity (1.0, 1.0, 1.4, 2.4) of kidney

TABLE 3
Survival, Body Weights, and Feed Consumption of Mice in the 14-Week Feed Study of Citral

		Mean body weight (g)				Feed consumption	
Concentration (ppm)	Survival	Initial	Final	Change	Final weight (%)	Week 1	Week 14
Male							
Vehicle control	10/10	20.6 ± 0.3	33.2 ± 0.8	12.6 ± 0.7		4.4	4.5
3900	10/10	20.3 ± 0.3	28.1 ± 0.6**	7.9 ± 0.6**	85	4.6	5.0
7800	10/10	20.3 ± 0.4	25.6 ± 0.6**	5.2 ± 0.4**	77	4.3	5.9
15,600	10/10	20.0 ± 0.3	21.3 ± 0.6**	1.3 ± 0.5**	64	4.0	6.2
31,300	6/10[a]	19.8 ± 0.3	17.1 ± 0.4**	–2.9 ± 0.4**	52	4.1	6.2
Female							
Vehicle control	10/10	16.4 ± 0.1	29.8 ± 0.7	13.4 ± 0.8		3.4	3.6
3900	10/10	16.6 ± 0.2	26.1 ± 0.4**	9.5 ± 0.4**	88	3.3	5.1
7800	10/10	17.0 ± 0.3	21.2 ± 0.4**	4.2 ± 0.4**	71	2.3	6.4
15,600	10/10	16.8 ± 0.2	18.2 ± 0.2**	1.4 ± 0.3**	61	2.3	6.5
31,300	10/10	16.5 ± 0.2	16.2 ± 0.2**	–0.2 ± 0.2**	55	2.1	5.3

Note. Survival values, number of animals surviving at 14 weeks/number initially in group. Weights and weight changes are given as mean ± SE. Subsequent calculations are based on animals surviving to the end of the study. Final weight value is relative to vehicle controls. Feed consumption is expressed as grams per animal per day.

[a]Week of moribund sacrifice: 2.

**Significantly different ($p \leq 0.01$) from the vehicle control group by Williams' or Dunnett's test.

mineralization in males exposed to 0, 1000, 2000, or 4000 ppm citral, respectively. The lesion was characterized by the presence of minute to focally extensive mineralization of stromal tissue between collecting ducts. Because the vehicle control incidence of renal mineralization was 84%, the increased incidences observed in the exposed groups were believed to reflect an exacerbation of this spontaneously occurring lesion. These renal changes were considered to have minimal toxicologic significance. A reexamination of kidneys from the 14-week study did not reveal a treatment-related increase in the incidence of renal mineralization. There were no other significant neoplastic or nonneoplastic findings attributed to citral administration in the 2-year rat study.

Mice

Fourteen-week study. In the second week of the study, four males in the 31,300 ppm group were killed moribund, all other animals survived to the end of the study (Table 3). Surviving animals exposed to 31,300 ppm lost weight during the study. Final mean body weights and body weight gains were significantly decreased in all exposed groups of males and females. Food consumption by females exposed to citral at 7800 ppm or greater was less than the vehicle controls during the first week of the study but by the end of the study measured consumption was greater than that of the vehicle controls. The increased feed consumption was due to the mice scattering feed, an indication of poor palatability. Thus, intake calculations for the 3900, 7800, 15,600, and 31,300 ppm groups were possibly slightly inflated. Based on estimated consumption, mice received average daily doses of approximately 745, 1840, 3915, and 8110 mg citral/kg body weight to males and 790, 1820, 3870, and 7550 mg/kg to females.

Treatment-related decreases in lymphocyte counts were observed (data not shown) but were associated with the marked suppression in mean body weights suggesting a physiological response consistent with a stress-related (corticosteroid-induced) lymphopenia.

The wall of the forestomach of many male and female mice exposed to 15,600 and 31,300 ppm of citral was variably thickened (2–5 times normal) and the mucosa (squamous epithelium) and submucosa often rugose (data not shown). Although thickened, all three main components (mucosa, submucosa, and muscle) appeared proportional to each other and to those of control animals. Therefore, this alteration was considered the result of a contracted stomach rather than a pathological alteration. There did, however, appear to be an excessive amount of keratin (hyperkeratosis) on the surface of the epithelium of these animals, but it was minimal. Keratin is normally produced by the forestomach epithelium and is naturally removed by physical contact with feed. Reduced feed intake by these animals may have contributed to this condition.

The incidences of ovarian atrophy were significantly increased in females exposed to 15,600 or 31,300 ppm; the atrophy was moderate in the 15,600 ppm females, and marked in the 31,300 ppm females (data not shown). This lesion was likely a secondary effect due to the poor condition of mice exposed to 15,600 or 31,300 ppm.

Reduced body weights were observed in all exposed mice. Typically, in the absence of other information, high exposure concentrations for two-year studies are chosen based on the

concentration that causes less than a 10% reduction in body weight. Because male and female mice exposed to the lowest dose tested (3900 ppm) in the 14-week study exceeded this percentage, the high exposure concentration chosen for the two-year studies was lowered to 2000 ppm. Lower doses of 1000 and 500 ppm also were used in the two-year study to evaluate dose-response relationships.

Two-year study. In the two-year mouse study, survival of exposed groups of males and females was similar to that of the vehicle control groups (controls, 43/50, low dose 40/50, mid dose 42/50, high dose, 40/50 for males; and 41/50, 45/50, 43/50, and 40/50, respectively, for females). Mean body weights of mice exposed to 1000 or 2000 ppm were generally less than vehicle controls and mean body weights of 500 ppm females were less from week 30 until the end of the study (Fig. 2). Feed consumption by the exposed groups was similar to that by the vehicle controls (data not shown). Dietary concentrations of 500, 1000, and 2000 ppm delivered estimated average daily doses of approximately 60, 120, and 260 mg citral/kg body weight to males and females, respectively. There were no clinical findings attributed to citral exposure.

The incidence of malignant lymphoma in females occurred with a positive trend (Table 4). The incidence in 2000 ppm females was significantly greater than that in the vehicle control group but was within the historical ranges in controls given NTP-2000 diet or controls in older dosed feed studies with the NIH-07 diet. Tissues most commonly affected by malignant lymphoma were the spleen, mesenteric lymph node, thymus, and, to a lesser extent, the ovary. The extent of tissue involvement was not noticeably different between treated and control groups. The three earliest cases of malignant lymphoma were observed in moribund animals in the 1000 (469 days on study) and 2000 (491 and 523 days on study) ppm groups; all remaining malignant lymphomas were observed at the end of the study. To further characterize the nature of the lymphomas in vehicle control and exposed mice, all cases of lymphoma were sectioned and immunostained using CD-3 to identify T cells and CD-45R (B220 clone) to identify B cells. Special stains did not reveal any differences in the origin of lymphomas between vehicle controls and females exposed to citral. The majority of lymphomas were immunopositive for CD-45R.

Inflammation and ulceration of the oral mucosa were present in all groups of mice (Table 5). The incidences of inflammation in 2000 ppm males and inflammation and ulceration in all groups of exposed females were significantly increased. With rare exception, the areas of inflammation and ulceration were directly medial to the molar teeth. The inflammation was minimal to mild and was characterized by an accumulation of mixed inflammatory cells within and just beneath the oral mucosa adjacent to the medial aspect of the teeth. In a majority of the cases, hair shafts were present in the inflamed areas and appear to have penetrated the tooth socket. Ulcers were minimal to mild in severity and consisted of focal areas with loss of

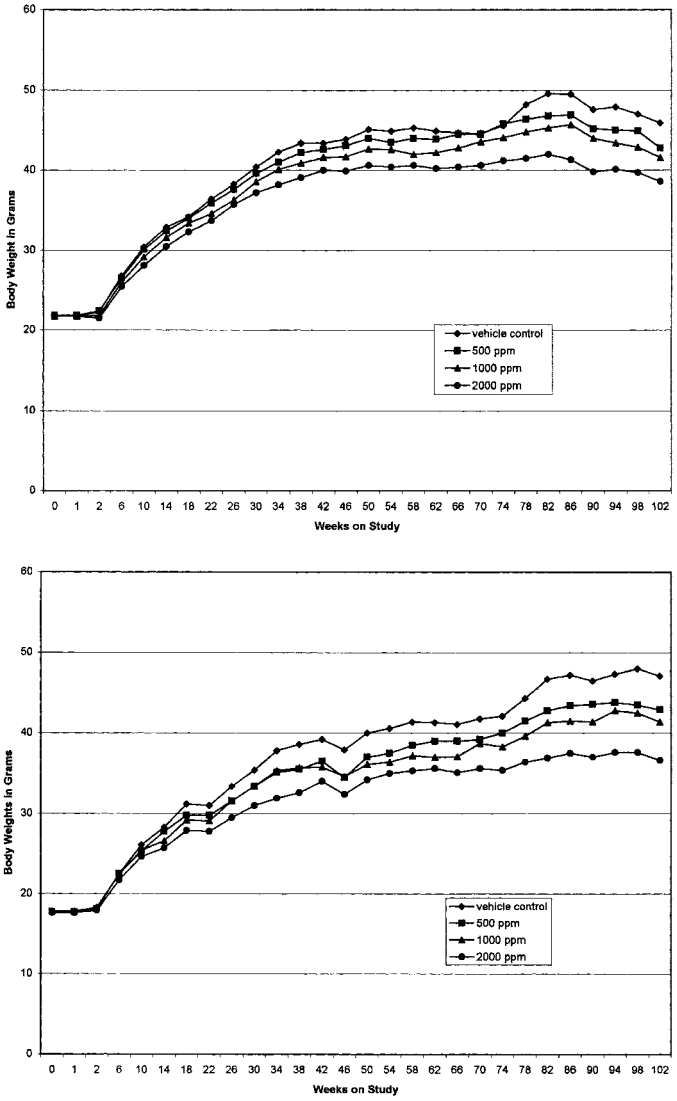

FIG. 2. Growth curves for male (top) and female (bottom) mice exposed to citral in feed for two years.

mucosa. The ulcers were almost always located at the points where hair shafts penetrated the oral mucosa. The inflammation and ulceration are considered to be secondary to embedded hair shafts either present in the actual section or in an adjacent plane of section. These same lesions, with similar severity, were observed in vehicle controls. Thus, the inflammation and ulceration were probably not a direct toxic effect of citral, but may have been exacerbated by citral.

DISCUSSION

Toxicology and carcinogenicity studies of citral were performed because of widespread human exposure from its use as a food and fragrance additive. Because the most significant human exposure to citral occurs through ingestion as a food

TABLE 4
Incidences of Malignant Lymphoma in Female Mice in the Two-Year Feed Study of Citral

			Concentration (ppm)		
	Untreated control	Vehicle control	500	1000	2000
Malignant lymphoma					
Overall rate[a]	4/50 (8%)	3/49 (6%)	5/50 (10%)	9/50 (18%)	12/50 (24%)
Adjusted rate[b]	9.1%	6.5%	10.4%	18.6%	25.7%
Terminal rate[c]	4/40 (10%)	2/41 (5%)	5/45 (11%)	7/43 (16%)	10/40 (25%)
First incidence (days)	733[d]	719	733[d]	469	491
Poly-3 test[e]		$p = 0.004$	$p = 0.376$	$p = 0.070$	$p = 0.011$

Note. Historical incidence for two-year studies with controls given NTP-2000 diet (mean ± SD): 98/659 (14.0 ± 7.1%), range 6–32%; with feed controls given NIH-07 diet: 167/953 (17.5 ± 7.7%), range 6–30%.

[a]Number of animals with neoplasm per number of animals necropsied.

[b]Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality.

[c]Observed incidence at terminal kill.

[d]Terminal sacrifice.

[e]Beneath the vehicle control incidence are the *p* values associated with the trend test. The untreated control group is excluded from the trend test. Beneath the exposed group incidence are the *p* values corresponding to pairwise comparisons between the vehicle controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice.

additive, dosed feed was the preferred route of exposure for the rodent studies. In a stability study of citral in NIH-07 diet, a 41% loss of citral was observed after one day, which was attributed to volatility and reactivity of the aldehydic moiety of citral with components in the feed (Kuhn *et al.,* 1991). However, when citral was given in starch microcapsules mixed with the diet, the stability of citral in the diet increased to 95% after seven days (Kuhn *et al.,* 1991). Microencapsulation represents a novel formulation method that enables the testing of chemicals that are volatile, have an unpleasant taste or odor, or present stability problems. Previous studies have demonstrated that chemical loss from microcapsules is minimal, bioavailability of the chemical is not compromised, and toxic effects are similar to those following gavage administration using corn oil (Dieter *et al.,* 1993; Kuhn *et al.,* 1991; Melnick *et al.,* 1987a,b; Yuan *et al.,* 1993).

TABLE 5
Incidences of Selected Nonneoplastic Lesions of the Oral Mucosa in Mice in the Two-Year Feed Study of Citral

	Vehicle control	Concentration (ppm)		
		500	1000	2000
Male				
n	50	50	50	50
Inflammation, chronic active	12[a] (1.8)[b]	16 (1.9)	21 (1.9)	21* (1.5)
Ulcer	9 (1.8)	8 (1.6)	12 (1.4)	10 (1.6)
Female				
n	49	50	50	50
Inflammation, chronic active	14 (1.4)	32** (1.9)	35** (1.8)	32** (1.5)
Ulcer	6 (1.2)	15* (1.9)	22** (1.6)	15* (1.5)

[a]Number of animals with lesion.

[b]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

*Significantly different ($p \leq 0.05$) from the vehicle control group by the Poly-3 test.

**$p \leq 0.01$.

In this study, only data from vehicle controls were presented. However, statistical analyses were performed to determine differences in the incidences of nonneoplastic or neoplastic lesions between placebo and untreated controls (NTP, 2001). For all nonneoplastic lesions that were significantly different, an informal review of their incidences in other NTP studies was performed to verify that they were within a normal range. In all cases, the lesions occurred at the frequencies expected by chance, suggesting that the differences were due to biological variation and not to ingestion of microcapsules. The only neoplastic lesion that was different between placebo and untreated controls was the incidence of uterine stromal polyps in female rats, which was significantly lower in vehicle controls (10%) when compared to untreated controls (28%) and outside the lower end of the historical control range for the NTP-2000 diet (female: range, 12–31%). While this response is statistically significant, it is not believed to be biologically relevant as the incidences are on the low and high end of the control range and probably reflect normal biological variation.

In the 14-week rat study, nephropathy with renal tubule granular casts was observed in some male rats exposed to 3900 ppm and most male rats exposed to 7800 or 15,600 ppm. The presence of granular casts and exacerbation of spontaneous nephropathy is suggestive of α2u-globulin nephropathy. α2u-Globulin is a protein produced by male rats under the influence of testosterone, therefore production begins with sexual matu-

rity and starts declining later in life (Charbonneau *et al.,* 1987). Some is filtered through the glomerulus with a portion being lost in the urine and a portion reabsorbed via the cytoplasm of the proximal renal tubular epithelium. With chemicals that induce α2u-globulin, the amount of hyaline droplets within the proximal renal tubule epithelium is increased and can be detected microscopically (Charbonneau *et al.,* 1987). There was no apparent increase in the amount of hyaline droplets in this study as determined by H&E and Mallory Heidenhain stains. Additionally, in the two-year study, no compound-related increases in kidney neoplasms were observed in male rats exposed to citral. Therefore, it was considered unlikely that renal lesions were mediated by α2u-globulin.

Citral has been extensively studied for its effect on the induction of benign and atypical hyperplasia in the ventral prostate of male rats (Engelstein *et al.,* 1996; Kessler *et al.,* 1998; Servadio *et al.,* 1986). In the present study, careful examination did not reveal any effect on male accessory glands, including all lobes of the prostate. A comparative study of citral-induced benign and atypical hyperplasia in Wistar, Sprague-Dawley, Fischer 344, and ACI/Ztm rats demonstrated that strain genotype and endocrine background play a role in the development of this disease (Scolnik *et al.,* 1994). The animal model chosen for the current study, the Fischer 344/N rat, was shown to be refractory to citral-induced prostatic hyperplasia (Scolnik *et al.,* 1994).

In the two-year mouse study, the incidences of malignant lymphoma in females occurred with a positive trend. Malignant lymphoma is a common spontaneous systemic neoplasm that most often arises in the spleen and lymph nodes in the B6C3F1 mouse. They may also arise in the thymus, particularly when induced by chemicals. When detected in lymph nodes and thymus, lymphomas were easily diagnosed. However, malignant lymphoma in the spleen was often difficult to distinguish from lymphoid hyperplasia.

Several arguments support an association of malignant lymphoma in female mice with citral administration. In addition to the positive trend in the incidences of malignant lymphoma, the incidence in the 2000 ppm group was significantly greater than that in the vehicle controls and exceeded the incidences of lymphoma in control female mice in all but one study using the NTP-2000 diet. The incidences of malignant lymphoma in 1000 and 2000 ppm females were significantly greater than that in untreated and vehicle control groups combined (Table 4).

Conversely, while the incidence of malignant lymphoma in the 2000 ppm group was significantly increased, it was within the historical ranges for control female mice given the NTP-2000 and NIH-07 diets for two years. In addition, this is a common neoplasm and there was a low incidence in the vehicle controls compared to historical control ranges. Based on these arguments the malignant lymphoma response was considered an equivocal or uncertain finding.

In conclusion, in the 14-week studies, the kidney appeared to be a target organ for citral toxicity in male rats. Other lesions observed in rats and mice were primarily noted in exposure groups that were higher than those selected for the two-year study. In the two-year study, citral appeared to exacerbate kidney mineralization in rats and ulceration of the oral mucosa in mice. Citral was not carcinogenic in F344/N rats or male B6C3F1 mice. However, there was a marginal increase in malignant lymphoma in female mice that may have been related to citral. The daily citral exposures (mg/kg/day) achieved in rats and mice at the lowest dose tested in the two-year study represents approximately 10 times the average daily intake of 5 mg/kg/day in humans (Council of Europe, 1974).

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