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Bioassay for Carcinogenicity of Rotenone in Female Wistar Rats

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Rotenone, a pesticide extracted from the *Derris* root, consistently was reported by a series of investigators to have induced mammary fibroadenomas in female Wistar rats when administered ip or by gavage in a sunflower (SF) oil or SF oil:chloroform vehicle. In contrast, no less than eight bioassays done in other laboratories with rotenone or rotenone-containing powders have given consistently negative carcinogenic results when different strains or species and different modes or vehicles of administration have been used. However, these studies were not designed to address the biological reproducibility of the positive data. Thus, the present study was designed to simulate conditions of the positive studies and to investigate a possible cocarcinogenic interaction between rotenone and chloroform. Each of eight treatment groups was assigned 72 weanling female Wistar rats. Groups were (1) untreated, (2) needle puncture, (3) SF oil:10% chloroform (SF oil:chloroform), (4) 1.0 mg/kg rotenone in SF oil:chloroform, (5) 2.0 mg/kg rotenone in SF oil:chloroform, (6) SF oil, (7) 1.0 mg/kg rotenone in SF oil, and (8) 2.0 mg/kg rotenone in SF oil. Rats were injected ip 5 days a week for 8 weeks (42 injection days) and subsequently held for 16 months. The appearance of palpable tissue masses was recorded; over 50 tissues from each rat were histologically evaluated. There were no statistically significant differences in overall or individual tumor incidences among control and rotenone-treated groups. Specifically, neither incidence nor time-to-palpation of mammary fibroadenoma significantly differed among control and rotenone-treated groups, regardless of the vehicle of administration. Thus, rotenone was not carcinogenic, and rotenone and chloroform did not interact to produce a carcinogenic effect in female Wistar rats in the current study. Thus, previous reports of carcinogenic activity were not reproducible under similar experimental conditions.

Rotenone is the principal active pesticidal constituent of the *Derris* (*Derris elliptica, Derris chinensis*) and Lonchocarpus (*Lonchorcarpus utilis, Lonchorcarpus urucu, Lonchorcarpus nicou-cube*) bark and root (Haley, 1978).

In mammals, rotenone and rotenone-containing powders (Cubé, tuba, and derris) have been extensively examined for chronic toxicity (Haley, 1978). Cubé powder was fed for 104 weeks to Osborne–Mendel rats at five dietary concentrations ranging from 50 to 1000 ppm (Hansen *et al.*, 1965) with no remarkable evidence of toxicity. However, there was a significant decrease in the prevalence of mammary gland tumors in surviving animals as the dose of Cubé powder was increased (control, 14/19; 1000 ppm Cubé, 1/20). Pure rotenone was tested for its chronic effects by feeding to rats of unspecified strain (Lehman, 1952a, b) or by gavage and feeding to C57BL/6 × C3H/AnfF$_1$ and C57BL/6 × AKRF$_1$ mice (Innes *et al.*, 1969), with no discernible evidence of carcinogenicity. On the other hand, when injected ip, rotenone has been reported to induce mammary gland fibroadenomas in female albino or Wistar rats in several subsequent reports from the same laboratory (Gosalvez and Merchan, 1973; Diaz-Gil, 1977; Gosalvez *et al.*, 1977). These positive reports stimulated an extensive research effort in the U.S. to reevaluate the potential carcinogenicity of this pesticide (USEPA, 1979a, b; NCTR, 1979; NTP, 1983). These additional studies included a 14-month oral gavage bioassay in Wistar rats, an 18-month ip injection bioassay in Sprague–Dawley rats, an 18-month rotenone feeding study in Syrian golden hamsters, and 2-year feeding studies in Fischer 344 rats and B6C3F1 mice. No increased incidence of mammary tumors was observed and no discernible evidence of carcinogenicity with respect to any other type of neoplasm was noted in any of these studies (Freudenthal *et al.*, 1981; Abdo *et al.*, 1988). However, in each case, these studies lacked experimental similarity to those studies reported by Gosalvez and colleagues.

Our investigation into the carcinogenic potential of rotenone began after reviewing the dissertation by a student of Gosalvez (Diaz-Gil, 1977), which revealed that rotenone had been dissolved in chloroform and added to sunflower (SF) oil prior to treatment of rats. Since chloroform is a known animal carcinogen (NCI, 1976; IARC, 1979) it was considered appropriate to evaluate the potential for a carcinogenic interaction to take place between chloroform and

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r[illegible]none even though chloroform's toxic effects are asso-c[illegible]d primarily with the liver and kidney, not the mammary gland. Furthermore, since SF oil had been used as the vehicle of administration in studies arising from Gosalvez' laboratory but in none of the other studies, it was felt important to include this oil as the vehicle of administration. Thus, the current study was designed to evaluate the carcinogenic potential of rotenone using an experimental design as similar to that used by the laboratory of Gosalvez as could be determined by careful reading of the reports of Gosalvez and Merchan (1973) and Diaz-Gil (1977). Furthermore, additional groups of rats were added to evaluate any potential cocarcinogenic interactions between rotenone and chloroform.

MATERIAL AND METHODS

Chemicals. Rotenone (Fig. 1), purchased from Penick (Lyndhurst, NJ), was >99% pure (<0.2% rotenolone or dehydrorotenone) when analyzed by high-pressure liquid chromatography (Bowman *et al.*, 1978) and g[illegible]romatography/mass spectroscopy. SF oil (Sunlite Sunflower Oil, H[illegible] Wesson Foods, Inc., Fullerton, CA) was free of pesticide residues (to[illegible] organophosphates, MDL = 50 ppb; total organochlorines, MDL = 50 ppb) and PCBs (MDL = 50 ppb) (by electron capture gas chromatography), estrogenic activity (by RIA and mouse bioassay, MDL = 5 ppb DES equivalents), or heavy metal residues (by atomic absorption, Pb MDL = 1 ppm, Cd MDL = 25 ppb, Hg MDL = 50 ppb). Chloroform (Burdick and Jackson Laboratories, Inc., Muskegon, MI) was nanograde quality, and all other solvents and inorganic salts were reagent grade or better.

Animal husbandry and maintenance. Female Wistar outbred albino rats (Charles River Laboratories, Wilmington, MA) were started on the study when 42–45 days old (average body wt, 91 g). During a 10-day quarantine period prior to starting the experiment, no internal or external parasites or pathogenic bacteria or viruses were identified by routine screening (Tortorich, 1978). Microbiological integrity of animals and animal room conditions was similarly evaluated throughout the experiment by biweekly removal and testing of sentinel animals and samples of bedding, cage waste, feed, water, and room air.

Rats were housed, three per cage, in a conventional animal room supplied with 17 changes/hr of nonrecirculated air with relative humidity and temperature maintained between 40–60% and 70–75°F, respectively. Room lighting was on a 12 hr light/12 hr dark cycle with fluorescent light-ir[illegible]maintained at 100 foot candles. Animals were maintained on hard-[illegible] chip bedding in polycarbonate cages capped with polyester filter bo[illegible]ts. They had free access to autoclaved feed (Purina 5010 pellets) and potable water that had been filtered and pasteurized.

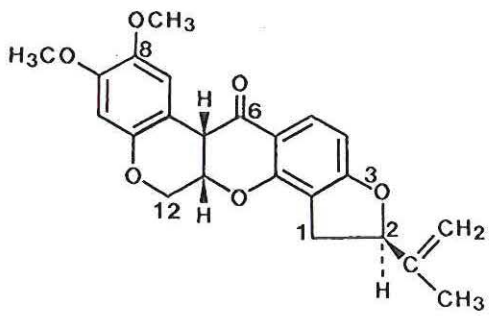

FIG. 1. Rotenone, 1,2,12,12a-tetrahydro-8,9-dimethoxy-2-(1-methylethenyl)-[1]benzopyrano[3,4-*b*]furo[2,3-*h*][1]benzopyran-6(6*H*)-one.

The test animals were weighed and examined for tumors weekly, and all tissue masses or body swellings observed were recorded and monitored until termination. Twice daily cage checks were made for dead or moribund animals, and such animals were sent to pathology for routine necropsy and histologic examination.

Dose preparation. Dose levels of rotenone (1.0 and 2.0 mg/kg) were chosen to bracket the dose level (1.7 mg/kg) used in the study by Gosalvez and Merchan (1973). Preliminary experiments established rotenone to be stable in SF oil or SF oil:chloroform (9:1) for at least 4 weeks at room temperature in the dark, but fresh rotenone doses were prepared weekly. For SF oil:rotenone suspensions, rotenone was added to SF oil in a beaker, stirred by a Teflon-coated magnetic stir-bar for 5 min, and then stirred in an ultrasonic water bath for an additional 15 min. For SF oil:chloroform solutions, rotenone was dissolved in chloroform and added to SF oil to a final ratio of 9:1 SF oil to chloroform. It was then mixed as described above for SF oil suspensions. All operations were carried out at room temperature under incandescent light. All dose formulations were assayed for rotenone (Bowman *et al.*, 1978) and used only if within ±5% of the target concentration. The rotenone dose preparations were stored at room temperature in brown 50-cc serum vials.

Experimental design. Rats were randomly assigned to test group cages and the cages were randomly assigned to racks. Seventy-two animals were assigned to each of eight experimental groups (Table 1). There were four control groups: (1) nontreated, (2) needle puncture, (3) SF oil, and (4) SF oil:chloroform (9:1). Treated groups included animals injected with rotenone dissolved in either SF oil or SF oil:chloroform at dosages of either 1.0 or 2.0 mg per kg per injection. Injections were administered ip in a volume of 0.1 ml each morning, Monday through Friday between the hours of 8 and 10 AM for 42 injection days. After the dosing period the animals were monitored an additional 16 months, and survivors were euthanized with CO_2 and necropsied.

Necropsy and histopathology. All dead, moribund, or terminally euthanized rats were necropsied. Tissue masses, lesions observed at necropsy, and all major organs and tissues were fixed overnight in Bouin's solution, rinsed in saturated lithium carbonate (in 70% ethanol), and stored in 70% ethanol until they were trimmed, processed, and embedded in paraffin. Tissues were sectioned and stained with hematoxylin and eosin and examined by light microscopy.

Statistical analysis. The comparisons of interest for this experiment were as follows: (1) untreated vs puncture control, (2) puncture vs SF oil:chloroform control, (3) puncture vs SF oil control, (4) SF oil:chloroform vs SF oil control, (5) SF oil:chloroform control vs 1.0 mg/kg or (6) 2.0 mg/kg rotenone in SF oil:chloroform, and (7) SF oil control vs 1.0 mg/kg or (8) 2.0 mg/kg rotenone in SF oil.

For each treatment comparison, mean body weights were compared using Student's *t* test. Since no differences were detected it was unnecessary to correct for multiple comparisons. The statistical analysis of mortality data and selected pathology data were performed using the procedure CHRONIC described by Kodell *et al.* (1983). Alternative hypotheses generally dictated one-sided tests. Tests for differences in mortality between treatments of interest were adjusted only for removal due to accidental death and terminal sacrifice. Plots of estimated mortality distributions were produced for selected comparison groups. For the analysis of time-to-onset of mammary tumors (fatal and nonfatal) the time of first positive palpation was used as time-to-tumor, provided that a mammary tumor was identified subsequently, upon microscopic examination. Only the incidence of fibroadenomas provided sufficient data for a time-to-tumor analysis. Pituitary adenoma prevalences (nonfatal tumors) also were compared using CHRONIC.

RESULTS

Body Weight

Body weight means before, during, and after the dosing phase of the experiment are reported in Table 2. Compared

TABLE 1
Treatment and Survival of Rotenone-Treated Female Wistar Rats

Rotenone[a]			No. of rats[b]		
Dosage	Total dose[c]	Vehicle	Dead	Moribund[d]	Killed[e]
None	None	None	3	2	67
None	None	None/puncture[f]	2	7	63
None	None	Sunflower oil:chloroform	2	1	69
1.0 mg/kg	8.5 mg	Sunflower oil:chloroform	2	3	67
2.0 mg/kg	17.6 mg	Sunflower oil:chloroform	4	5	63
None	None	Sunflower oil	2	5	65
1.0 mg/kg	8.7	Sunflower oil	1	2	69
2.0 mg/kg	17.4 mg	Sunflower oil	0	5	67

[a] Injected ip once a day, 5 days/week (M–F) for 42 injections.
[b] Seventy-two rats were originally allocated to each treatment group.
[c] Average total dose from 42 injections.
[d] When tumors became large enough to impair animal movement animals were removed and included in this grouping.
[e] These animals were euthanized 18 months after the start of treatment.
[f] No vehicle administered; needle puncture was given daily, 5 days/week (M–F) 42 times.

.o the appropriate vehicle control, weight gain was apparently depressed in the high-dose rotenone groups, particularly in those receiving the SF oil:chloroform vehicle (17.9%). Statistical comparisons of body weight means were made midway during the course of injections and at the termination of the experiment (Table 2). While no significant ($\alpha = 0.05$) differences in mean body weights among treatment comparison groups were found, two compari-

TABLE 2
Body Weight Comparisons for Selected Weeks of Experiment

Treatment group[a]	Body weight (means ± SEM)[b]				
	Week 0	Week 4	Week 9	Week 18	Week 78
uC	89.9 ± 1.2	198.4 ± 1.9	254.6 ± 2.7	292.2 ± 3.5	423.3 ± 10.0
pC	88.1 ± 1.1	198.1 ± 1.7	254.5 ± 1.9	289.9 ± 2.0	431.4 ± 10.2
ocC	90.4 ± 1.7	196.8 ± 2.1	260.6 ± 2.7	295.4 ± 3.3	409.4 ± 6.5
ocR1	88.3 ± 0.9	193.0 ± 1.7	257.3 ± 2.0	289.9 ± 2.6	403.5 ± 7.5
ocR2	95.6 ± 1.0	191.0 ± 2.2	257.2 ± 2.8	303.8 ± 3.1	436.3 ± 11.8
oC	89.7 ± 1.3	201.1 ± 1.9	257.1 ± 2.4	293.4 ± 3.1	422.6 ± 8.7
oR1	89.3 ± 1.3	202.3 ± 1.9	260.4 ± 2.4	294.1 ± 3.1	408.5 ± 8.0
oR2	93.9 ± 1.1	199.9 ± 1.7	259.1 ± 2.7	295.1 ± 3.5	423.9 ± 8.5

	Week 4		Week 78	
Comparison[c]	Ratio	*p* value	Ratio	*p* value
Control vs control (uC/pC)	1.00	0.898	0.98	0.575
Control vs control (pC/ocC)	1.01	0.661	1.05	0.071
Control vs control (pC/oC)	0.99	0.244	1.02	0.510
Control vs control (ocC/oC)	0.98	0.142	0.97	0.230
Control vs treated (ocC/ocR1)	1.02	0.173	1.01	0.556
Control vs treated (ocC/ocR2)	1.03	0.061	0.94	0.051
Control vs treated (oC/oR1)	0.99	0.662	1.03	0.239
Control vs treated (oC/oR2)	1.01	0.645	1.00	0.917

[a] Treatment groups are uC, untreated control; pC, needle puncture control; ocC, oil:chloroform vehicle control; ocR1, 1.0 mg rotenone/kg in oil:chloroform; ocR2, 2.0 mg rotenone/kg in oil:chloroform; oC, oil vehicle control; oR1, 1.0 mg rotenone/kg in oil; oR2, 2.0 mg rotenone/kg in oil.
[b] Means ± standard error of the mean in grams.
[c] Ratios are the ratios of mean body weights of the treatment groups specified. *p* values are the result of statistical comparisons of the specified group means.

s[illegible]s of interest did approach significance. During the dos[illegible]phase of the experiment (at 4 weeks) rats receiving the high dose of rotenone (in SF oil:chloroform) weighed less than their vehicle controls and the difference approached significance ($p = 0.061$). After the final injection rats in this high-dose group apparently gained weight more rapidly than controls so that at 78 weeks, they weighed more, on the average, than their SF oil:chloroform controls, and this difference approached significance ($p = 0.051$).

Clinical Observations and Survival

The only treatment-related clinical observations noted were recorded during the injection period. These included rough hair and sluggishness, which were noted most frequently in the high-dose rotenone group (dissolved in SF oil:chloroform).

The disposition of animals during the study is reported in Table 1. Ninety-two percent of control and dosed animals [illegible]ived until the terminal sacrifice. Test results for mea[illegible]s[illegible]g differences in mortality, adjusted for euthanasia or accidental removal, indicated increased mortality among needle puncture control animals relative to all other control groups (e.g., $p < 0.008$ for puncture control vs SF oil:chloroform control) (Fig. 2A). Several rats in the puncture control group were removed because of palpable masses, histologically confirmed to be mammary gland fibroadenomas.

Comparison of mortality distributions of vehicle controls (SF oil:chloroform or SF oil) with their respective dose groups showed that the high dose of rotenone, administered in SF oil:chloroform, significantly increased mortality compared to vehicle controls ($p = 0.045$) (Fig. 2B). This difference was due to removal of eight dead or moribund rats from the rotenone-treated group during the dosing phase. These animals had no apparent mammary gland masses. No other rotenone treatment group showed significantly increased mortality relative to its vehicle controls (Figs. 2B and 2C), and only one other animal was found [illegible] or moribund during the dosing phase (in the 2.0 mg r[illegible]ione/kg in SF oil group).

Gross and Microscopic Pathology

No lesions found at necropsy in dead or moribund rats were dose-related. Histopathological evaluations of these animals revealed rats with some or all of the following diagnoses: myocardial degeneration, hepatocellular necrosis, mammary gland inflammation and hyperplasia, pulmonary congestion, edema and hemorrhage, and inflammation with mast cell infiltration of the mesenteric fat and connective tissue.

Microscopic examination of tissues from control and treated animals killed at the end of the study revealed a number of age-related lesions (Kroes, 1981). Most of these

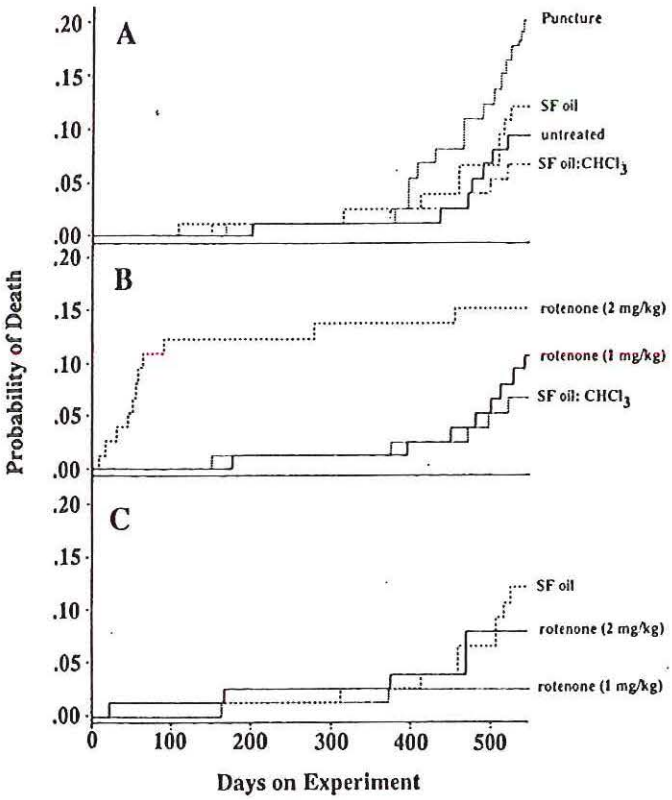

FIG. 2. Plots of estimated mortality distributions for selected control and rotenone treatment groups. (A) No-dose control (—); needle sham control (· · ·); SF oil:chloroform (9:1) vehicle control (-- ·); SF oil vehicle control (---). (B) SF oil:chloroform (9:1) vehicle control (· · ·); 1.0 mg/kg rotenone in SF oil:chloroform (9:1) vehicle (—); 2.0 mg/kg rotenone in SF oil:chloroform (9:1) vehicle (---). (C) SF oil vehicle control (---); 1.0 mg/kg rotenone in SF oil vehicle (· · ·); 2.0 mg/kg rotenone in SF oil vehicle (—).

lesions occurred with low and approximately equal frequency in control and rotenone-treated animals.

The most commonly diagnosed benign neoplasms were pituitary adenomas, mammary gland fibroadenomas, uterine polyps, and adrenal cortical adenomas. Pituitary adenocarcinomas, stromal sarcomas of the reproductive tract, and mammary gland adenocarcinomas were the most common malignant neoplasms.

Mammary Gland Tumors

The types and frequency of mammary neoplasia are reported in Table 3. Only fibroadenomas were found in all treatment groups in sufficient numbers to allow time-to-tumor analyses. Of the 83 rats in which mammary gland fibroadenomas were identified they were first detected as palpable tissue masses in 56 animals. The time of appearance of these palpable fibroadenomas is given in Table 5. There were no statistically significant differences at the 5% level in the time-to-tumor analysis between treatment groups of interest although animals in the needle puncture control

TABLE 3
Mammary Gland Tumors in Rotenone-Treated Female Wistar Rats

Rotenone[a] dosage	Vehicle	No. with mammary gland tumors				
		Fibroadenoma[b]	Adenocarcinoma	Adenoma	Fibroma	Total (%)
None	None	12	2	1	0	15 (21)
None	None/puncture[c]	16	0	0	1	17 (24)
None	Sunflower oil:Chloroform	8	1	0	0	9 (13)
1.0 mg/kg	Sunflower oil:Chloroform	5	0	0	0	5 (7)
2.0 mg/kg	Sunflower oil:Chloroform	8	0	0	0	8 (11)
None	Sunflower oil	11	0	0	0	11 (15)
1.0 mg/kg	Sunflower oil	14	1	0	0	15 (21)
2.0 mg/kg	Sunflower oil	9	0	0	0	9 (13)

[a] Injected ip once a day, 5 days/week (M–F) for 42 injections.
[b] No significant differences were found between control and treatment groups in the incidence of mammary fibroadenomas ($\alpha = 0.05$).
[c] No vehicle administered but needle puncture was given daily, 5 days/week (M–F) 42 times.

group tended to have palpable mammary gland fibroadenomas earlier than other treatment groups (Table 4).

DISCUSSION

Observations made during the dosing phase of the present study suggest that animals receiving the high dose of rotenone, dissolved in SF oil:chloroform, experienced acute toxic effects of the compound. Clinical signs of rough hair and sluggishness, possible weight gain suppression, and several deaths that occurred in this group during the dosing phase all suggest this. Since animals receiving the high dose of rotenone in SF oil (without chloroform) did not exhibit these same endpoints (clinical observations, body weight gain suppression, and mortality) it is concluded that chloroform enhanced the acute toxicity of rotenone. This difference in the toxicity of rotenone may have been due to a difference of solubility in the two vehicles. Microscopic examination of rotenone preparations dissolved in SF oil:chloroform revealed no particles larger than 1 μm but particle sizes ranged from 50 to 200 μm in SF oil preparations. Thus, bioavailability of rotenone may have been greater from SF oil:chloroform than from SF oil. Furthermore, deaths noted in this high-dose group are consistent with the 10 to 20% early death rate among females treated at a dosage of 1.7 mg/kg by Gosalvez and Merchan (1973).

There were no statistically significant differences (α

TABLE 4
Life Table for Time to First Palpation of Mammary Masses Later Confirmed Histologically as Mammary Fibroadenomas[a]

Interval (days)	Treatment group[b]															
	uC		pC		ocC		ocR1		ocR2		oC		oR1		oR2	
	N[c]	n[d]	N	n	N	n	N	n	N	n	N	n	N	n	N	n
0–150	72	0	72	0	72	0	72	0	72	0	72	0	72	0	72	0
151–210	72	0	72	1	72	0	72	0	63	0	72	0	70	0	71	0
211–270	72	0	71	0	72	0	72	0	63	0	72	0	70	0	71	0
271–330	72	0	71	1	72	0	72	1	63	0	71	1	70	0	71	0
331–390	72	0	70	2	71	0	71	0	63	1	70	0	69	1	70	0
391–450	72	2	68	1	71	0	70	0	62	0	70	1	68	1	70	4
451–510	69	3	63	2	70	2	70	1	62	2	68	1	66	1	66	0
511–560	62	3	60	3	66	3	66	3	59	3	66	2	65	6	65	4

[a] Life-table analysis showed no statistically significant ($\alpha = 0.05$) differences in time to palpation of tumor between control and dosed animals.
[b] Treatment groups are uC, untreated control; pC, needle puncture control; ocC, oil:chloroform vehicle control; ocR1, 1.0 mg rotenone/kg in oil:chloroform; ocR2, 2.0 mg rotenone/kg in oil:chloroform; oC, oil vehicle control; oR1, 1.0 mg rotenone/kg in oil; oR2, 2.0 mg rotenone/kg in oil.
[c] N is the number of animals at risk at the beginning of the time interval.
[d] n is the number of animals in the given time interval for which a mammary mass was first detected by palpation. All masses were subsequently confirmed histologically as a mammary fibroadenoma.

TABLE 5
Study Comparison[a]

Strain	Route	Vehicle	Dose	Age or weight at start	Period of exposure	Result	Reference
Albino	ip	SF oil	1.7 mg/kg/d	35 d 100 g	42 d (daily)	+[b]	Gosalvez and Merchan (1973)
Wistar	ip	SF oil	1.7 mg/kg/d	100 g	40–50 d (daily)	+	Gosalvez *et al.* (1977)
Wistar	ip	SF oil	0.1 or 0.2 mg/d	50–65 d	58 d (6 x/wk)	+	Diaz-Gil (1977)
Wistar	ip	SF oil	1.0 or 2.0 mg/kg/d	42–45 d 91 g	58 d (5 x/wk)	–	Greenman *et al.* (current)
S-D	ip	Corn oil	1.7 or 3.0 mg/kg/d	67–156 g	42 d (daily)	–	Freudenthal *et al.* (1981)
Wistar	po	SF oil	0.2 mg/kg/d (45 d) and 0.3 mg/kg/d (15 d)	100 g	60 d (daily)	+	Gosalvez *et al.* (1977)
Wistar	po	Corn oil	1.7 or 3.0 mg/kg/d	75–145 g	42 d (daily)	–	Freudenthal *et al.* (1981)
O–M	Oral	Diet	2, 5, 10, 25, 50, 100 ppm	3 wk	104 wk	+/–[c]	Long (1959)
O–M	Oral	Diet	3, 6, 14, 29, 58 ppm	3 wk	104 wk	–	Hansen *et al.* (1965)
F344	Oral	Diet	38, 75 ppm	5 wk	103 wk	–	Abdo *et al.* (1988)

[a] The following abbreviations are used: SF, sunflower; d, day; wk, week; S–D, Sprague–Dawley rat; O–M, Osborne–Mendel rat; F344, Fischer 344 rat. Only studies carried out in rats are summarized in this table.
[b] Results specified + gave evidence of a positive mammary tumor response.
[c] Results specified +/– gave equivocal evidence of a liver tumor response.

= 0.05) in tumor incidences between control animals and those receiving 1.0 or 2.0 mg rotenone/kg body wt/injection in a vehicle of sunflower oil or sunflower oil:chloroform (9:1), nor was there a significant difference in time-to-onset of palpable mammary fibroadenomas. These data are in contrast to results summarized by Gosalvez (1983) who has reported mammary fibroadenoma incidences as high as 44% in Wistar females treated with rotenone vs 0% in comparable controls. On the other hand, our results are in agreement with more recent studies carried out by the USEPA (Freudenthal *et al.*, 1981) and NTP (Abdo *et al.*, 1988). A comparison of several parameters of the chronic s es thus far carried out with rotenone in rats may be fc d in Table 5. In those studies reporting a rotenone-induced increase in mammary gland fibroadenomas the following parameters were constant: (1) the same primary investigator was associated with each study, (2) female Wistar or inbred albino rats of unspecified strain were used, (3) rotenone was administered in a single daily bolus 6 or 7 days a week for a period of 1.5 to 2 months, (4) animals were relatively young (35 to 65 days old) or weighed about 100 g at the start of treatment, (5) daily doses were likely in the range of 0.8 to 2.5 mg/kg (Gosalvez, 1983), and (6) sunflower oil was used as the vehicle of administration.

Gosalvez (1983) has suggested several factors to explain why other laboratories have been unable to duplicate the mammary tumorigenic response to rotenone reported by his laboratory. These factors include (1) the dosages of rotenone, (2) the period of treatment or observation of animals after termination of treatment, (3) the oil used as a solvent, and (4) the vitamin-enrichment of the diets.

Gosalvez (1983) concluded that the dose–response curve for carcinogenesis of rotenone is bell-shaped and that negative studies had resulted from using doses that were either too high or too low. He suggested that positive results likely would be found only within the daily dosage range of 0.8 to 2.5 mg/kg. Gosalvez' conclusion of a bell-shaped curve seems to be based primarily upon the results of an unpublished report of a feeding study by Long (1959) in Osborne–Mendel rats fed rotenone concentrations of 2, 5, 10, 25, 50, or 100 ppm. In that study four hepatocellular neoplasms (not reported as due to rotenone, even by Gosalvez) were identified among males or females fed 5 or 10 ppm rotenone, but none were found in controls or other dose groups. In addition, the frequency of multiple tumors was very low in controls, highest among animals fed 5 ppm, and intermediate at all other concentrations. In contrast to Gosalvez' hypothesis, Long (1959) suggested that the clumping of hepatocellular tumors in the 5 and 10 ppm groups may have been a false positive result, and she cited poor survival among controls as a possible reason for a lower than normal incidence of general neoplasia in controls. In any event, that study did not reveal an increase in mammary gland tumors. Furthermore, in a later study by Hansen *et al.* (1965), Cubé powder fed to Osborne–Mendel rats at rotenone concentrations of 2.9, 5.8, 14.5, 29, or 58 ppm caused no increase in either mammary gland fibroadenomas or multiple tumors at the lower doses but did depress the inci-

ence of mammary fibroadenomas and neoplasia in general at 5.8 ppm and above. No increase in hepatocellular neoplasms was observed. Thus, except for the Long (1959) report, there is no evidence for a "bell-shaped" dose–response curve.

Gosalvez *et al.* (1977) reported that mammary fibroadenomas usually appeared from 7 to 10 months after the end of treatment with rotenone while controls held for 19 months after treatment with the vehicle did not develop mammary tumors. In a summarization of control data from Wistar rats used in several of his studies he reported an overall incidence of 0/70 (Gosalvez, 1983). In the present study animals were held 16 months after the end of treatment to provide sufficient time for detecting rotenone-induced tumors, yet no effect of rotenone was noted. On the other hand, 11 to 22% of the controls in the current study had mammary fibroadenomas at the end of the 16-month waiting period. Our control incidence seems to be similar to that found in Wistar rats by other laboratories. For example, Sher (1982) has cited incidences ranging from 5 to 41% in studies performed over a 24-month or longer period. Thus, the normal background incidence of mammary fibroadenomas in Wistar rats used in this and other laboratories was markedly higher than in the Gosalvez studies. Differences in control tumor incidence of this magnitude are hard to reconcile, and a unique association with one series of studies makes interpretation of those study results difficult. Although unique genetic or environmental factors could explain the difference, interpretation of the results awaits adequate description of such variables.

In the current study the number of treatments, method of administration, and vehicle for dosing were selected based on information provided by Gosalvez and his co-workers. Unfortunately, some of the details provided may have been incomplete. Work published in the literature gives little information about the methods of dose preparation. Thus, it is not clear that the work published involved chloroform in the dose preparation. That chloroform was used is stated only in the work of Diaz-Gil (1977), and even in that description the proportion of chloroform to sunflower oil was not stated. Our use of 10% chloroform, in part was based on the use of 10% oily solutions of chloroform in rat studies that demonstrated the carcinogenicity of chloroform (IARC, 1979). Since we used sunflower oil, both with and without chloroform, it seems unlikely that the presence of chloroform was responsible for the mammary tumor response in the Gosalvez studies.

Since estrogens are an important cofactor in mammary tumorigenesis (Muhlbock, 1956; Shafie and Hilf, 1979) and numerous oils may naturally possess estrogenic activity (Booth *et al.*, 1960) or pesticide contaminants that are estrogenic (Bitman *et al.*, 1968; Clement and Okey, 1972), it is important to know whether the vehicle contained such compounds. In our study no estrogenic activity or pesticides with estrogenic activity were detected in the sunflower oil. Unfortunately, the Gosalvez studies failed to report any attempt to analyze for estrogens. Thus, one may not discount the possibility that mammary tumorigenesis in their studies resulted from relatively high levels of estrogenic activity in the sunflower oil they used.

In his review of rotenone carcinogenesis, Gosalvez (1983) suggested that positive tumorigenic results with rotenone in studies originating from his laboratory may have been a consequence of using riboflavin-deficient diets. Riboflavin-deficient diets have been associated with an increased incidence of DMBA-induced skin tumors or azo dye-induced liver neoplasia in experimental animals while, in contrast, riboflavin deficiency has been found to decrease spontaneous mammary tumor formation and growth (reviewed in Rivlin, 1973). Thus, both a low spontaneous (control) incidence and a high chemically induced incidence of cancer might be caused by riboflavin deficiency. Gosalvez suggested that such an explanation could account for differences between his and other studies. He reported that diets used in the positive Wistar studies contained riboflavin at a level of 3.6 ppm, while subsequent studies from his laboratory in which the dietary level of riboflavin had been 13 ppm were negative. Although we did not analyze our diet for riboflavin, the manufacture's data sheet for the diet we used indicates a level of approximately 8 ppm. Thus, the suggestion of Gosalvez (1983) cannot be fully discounted on the basis of the present study. However, vitamin B_{12} and folate deficiencies have also been associated with enhanced activity of various carcinogens (Eto and Krumdieck, 1986), and it is premature to suggest that his is the most likely explanation of the disparate results.

In summary, rotenone was not carcinogenic for the mammary gland in female Wistar rats when injected ip, 5 days/week for 8 weeks, at 1.0 or 2.0 mg/kg body wt/injection in vehicles of sunflower oil or sunflower oil:chloroform (9:1). Moreover, chloroform was not cocarcinogenic with rotenone in this bioassay. Furthermore, tumors at other organ/tissue sights were not significantly different from those observed in control animals. Finally, this study does not confirm the observations from Gosalvez' laboratory reporting rotenone to be a potent mammary gland carcinogen in female Wistar rats.

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