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Chronic Toxicity and Carcinogenicity Studies of Gentian Violet in Mice

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Chronic Toxicity and Carcinogenicity Studies of Gentian Violet in Mice. LITTLEFIELD, N. A., BLACKWELL, B-N., HEWITT, C. C., AND GAYLOR, D. W. (1985). *Fundam. Appl. Toxicol.* 5, 902–912. Gentian violet is a dye belonging to a chemical class known as the di- and triaminophenylmethanes. Although it has been used for many years for the control of fungal and intestinal parasites, for various uses in veterinary medicine, and as an additive to the feed of chickens to inhibit propagation of mold and fungus, very few long-term toxicity data are available. A life span dosing study of gentian violet in the diet of 720 males and 720 females of B6C3F$_1$ mice (C57BL/6 × C3H) at dose levels of 0, 100, 300, and 600 ppm was done to determine its toxicity and carcinogenicity. Sacrifices were conducted after 12, 18, and 24 months of continuous dosing. There was no effect on food consumption or body weight gain; however, a dose effect was noted for mortality rates. Mortality (adjusted for sacrifices) in the controls of both sexes was less than 15% at 24 months, but was approximately 64% in the females and 23% in the males given the high dose. Females appeared to be more susceptible than males. A positive dose response for hepatocellular carcinoma was noted in males at 24 months and in females at 18 and 24 months. Statistical tests for dose-related trends with respect to (1) mortality due to liver neoplasms, (2) prevalence of liver neoplasms, and (3) time to onset of liver neoplasms showed positive trends in both males and females. Other dose-related toxicological responses, particularly in the female mice, included erythropoiesis in the spleen, atrophy of the ovaries, adenoma of the Harderian gland, and the presence of type A reticulum cell sarcomas in the urinary bladder, uterus, ovaries, and vagina. The estimation of risk of 10^{-6} over background for malignant liver neoplasms using linear extrapolations showed a lower bound on the virtually safe dose (VSD) to be 2 ppb for the female mice and 1 ppb for the male mice. For benign and malignant liver tumors together, the lower bound on the VSD was essentially the same as for malignant liver neoplasm alone. Under the conditions of the experiment described above, gentian violet appears to be a carcinogen in mice at several different organ sites.

Gentian violet, a dye belonging to a chemical class known as di- and triaminophenylmethanes, is a mixture of crystal violet (crystalline hexamethyl-*p*-rosaniline) and methyl violet (tetramethyl- and pentamethyl-*p*-rosaniline plus hexamethyl-*p*-rosaniline). USP grade gentian violet must be 96% or greater crystal violet, with the remaining percentage consisting of methyl violet. Very few subchronic or chronic definitive toxicity data exists on gentian violet although it has been marketed since 1951.

Gentian violet has been used to control fungal and intestinal parasites in humans, to disinfect umbilical cords in infants, and for various uses in veterinary medicine and is added to the feed of livestock, most commonly chickens feed, to inhibit propagation of mold and fungus.

Fujita (1977) has shown gentian violet to be mutagenic to *Bacillus subtilis, Escherichia coli,* and *Salmonella typhimurium.* Au *et al.* (1979) found the dye to be toxic but not mu-

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tagenic for *Salmonella typhimurium.* Using the Rosenkranz assay (Rosenkranz *et al.,* 1976), which measures the ability of chemicals to inhibit the growth of DNA polymerase A-deficient *E. coli,* Au found gentian violet to be highly active. Also, he determined that the addition of the mammalian liver postmitochondrial supernatant (S-9) fraction resulted in a reduction of inhibition and a loss of gentian violet color, suggesting either its binding to the added protein or metabolism to a noncolored product, possibly the reduced leuco derivative.

Gentian violet is a direct-acting electrophile which reacts with DNA and other nucleophiles. Wolfe (1977) demonstrated that gentian violet inhibits DNA synthesis in an *in vitro* system that was catalyzed by *E. coli* B polymerase 1. Rosenkranz and Carr (1971) demonstrated damage to bacterial DNA *in vitro.* The dye is readily reduced with corresponding decoloration to the leuco form by a variety of inorganic reducing agents according to Kirk–Othmer Encyclopedia of Chemical Technology (1969). This leuco derivative is structurally similar to the classical aromatic amine carcinogens.

Norrby and Mobacken (1972) have detected significant mammalian cell cytotoxicity in an *in vitro* assay. This toxicity could be inhibited by heparin, serum, and other polyanions. Marked inhibition of protein, collagen, and RNA biosynthesis was also detected. In humans, oral ulceration has been noted with infants treated for *Candida* infections. Cytotoxicity (Au *et al.,* 1978) was demonstrated *in vitro* with Chinese hamster ovary cells. Gentian violet caused metaphase chromosome aberrations; but this activity could be virtually eliminated upon addition of the S-9 fraction.

Magenta, a ring-methylated primary amine analog of gentian violet, was one of the first chemicals suspected to be a human bladder carcinogen. Case and Pearson (1954) noted an increased risk of contracting bladder cancer in industrial workers engaged in manufacturing magenta. Benzyl violet 4B, an alkyl sulfonate derivative of gentian violet, primarily induced tumors of the ear duct and mammary gland in female Sprague–Dawley rats (Ikeda *et al.,* 1974).

At least two 90-day feeding studies have been done using gentian violet at levels of up to 500 ppm in rats and 516 ppm in dogs. Other than a slight body weight loss in rats and a liver weight increase in dogs, the results were not conclusive (unpublished data, FDA files). Two chronic studies were conducted in rats; however, no conclusions were drawn since each study suffered from several design and/or conduction deficiencies. Kinosita (1940) detected gastric papillomas and an adenomatous proliferation of hepatic tissue in rats after dosing for over 300 days. The dose level was not stated. Fitzhugh (unpublished data, FDA files, 1949) treated rats for up to 2 years at levels up to 1600 ppm. These inconclusive data revealed dose-related hepatic neoplastic nodules and dysplastic foci that were more severe in females.

The objective of this study was to determine if gentian violet has any oncogenic or other toxicological effects when administered in the diet for the lifetime of the mouse.

MATERIALS AND METHODS

Chemical. Gentian violet was purchased from Hilton Davis, Cincinnati, Ohio, in one 200-pound batch that was packaged in ten 20-pound drums. The chemical was analyzed using high-performance liquid chromatography (HPLC) prior to the start of the study, during the study (11/24/82), and following removal of the last animals in the program. Quantitation in the analytical procedure was performed using a uv-visible spectrophotometer operated at 588 nm. The three analyses indicated there was no change in the bulk material during the term of the study. The purity of the test article on a dry basis was 99% gentian violet and 1% methyl violet.

Dosage preparation. The gentian violet was dissolved in ethyl alcohol and sprayed directly into the feed (Purina 5010M, autoclavable) at dose levels of 0, 100, 300, and 600 ppm. The ethyl alcohol was subsequently removed from the prepared feed during a 30-min blending process by a vacuum. The feed preparation process was conducted in a sanitized chamber. The feed was loaded into small labeled cardboard boxes by dose and packaged in groups of six into stainless-steel carrying cases for transport to the animals in the barrier rooms.

Prior to starting the study, the mixing process was validated by chemical analyses for gentian violet content and also shown to be sufficiently stable when in contact with the feed. During the study, representative feed samples were analyzed and certified to be within a ±10% range of the target dose before being sent to the animal rooms.

Animal care and maintenance. The animals used on this study were B6C3F$_1$ mice (C57BL/6 females × C3H males) and were derived from the specific-pathogen-free (SPF) mice of the NCTR breeding colony. They were approximately 4 to 5 weeks old and weighed between 8 and 15 g at the time of allocation of four animals per cage in a barrier-type animal holding room. Microbiological evaluations were conducted periodically on the mice, and on room air, floors, walls, and cage litter every 2 weeks. Prior to use, the drinking water and the feed were checked for the presence of pathogenic organisms. The temperature of the room was controlled at 72 ± 2°F and the humidity maintained at 50 ± 5%. A slight positive pressure was maintained in the animal room with reference to the corridors. Fourteen to sixteen changes of air per hour provided ventilation in the animal room. The light/dark cycle in the room was set for 12 hr each. Hardwood chips were used as cage bedding. Feed, water, bedding, and cages were changed weekly. Filter tops were used on all cages. Weekly weights of animals, food consumption, clinical signs, and other observations were recorded by a computer-supported program.

Twice-daily room checks were made and animals were removed individually from the experiment as they died or became moribund. All animals in the study received a complete necropsy, histopathological examination, and clinical chemistry analysis at the scheduled sacrifice.

Experimental design. The experimental design is shown in Table 1. Both sexes of mice received a dose of 100, 300, or 600 ppm gentian violet in their feed for 12, 18, or 24 months. A similar group of mice received no gentian violet in their feed and served as controls. There were a total of 720 males and 720 females on the study.

Statistical procedures. The statistical procedure, as outlined by Kodell *et al.* (1983), is a computer program for statistical analysis of carcinogenesis data that was developed into a statistical analysis system (SAS) procedure. This program follows a unified approach for the estimation and testing of the time to onset, prevalence, and mortality distribution functions. The onset and mortality functions represent "net" rather than "crude" probabilities in that they are adjusted for the mortality from causes of death other than from the tumor of interest. The prevalence function represents a probability further adjusted for mortality caused by the tumor. Specifically, the mortality function characterizes the mortality rate due to a tumor, the prevalence function characterizes the incidental (nonfatal) tumor rate, and the time to onset function characterizes the distribution of time to histological appearance of the tumor (disease of interest).

Risk assessment was performed using the method of Farmer *et al.* (1982), which is a linear extrapolation method to determine lower bounds on chemical dose levels associated with specific levels of disease risk. It incorporates the tumor probabilities as derived from the time to onset distribution functions.

A curvilinear multistage model was fit to the experimental dose–response data. The curvilinear model is only used to estimate adjusted tumor rates in the experimental dose range (100–600 ppm) or down to a tumor response of 1% above background. Below these doses the estimates are suspect as they then become highly dependent on the mathematical form of the model. Since there is no means to confirm the model in the low dose range, linear extrapolation is used only below the experimental dose range to provide an upper bound on tumor risk.

The Cochran–Armitage Test (Snedecor and Cochran, 1980) was used to test for positive linear trends and the Fisher's exact test (Randles and Wolfe, 1979) was used to compare each dose group against the controls for analysis of the crude proportions of reticulum cell sarcomas (RCS) in the various organs for the terminally sacrificed animals.

TABLE 1

EXPERIMENTAL DESIGN FOR MICE LIFE SPAN STUDY

Dose (ppm)	Time of sacrifice (months)							
	Total number		12		18		24 (Terminal)	
	Male	Female	Male	Female	Male	Female	Male	Female
Control	288	288	48	48	48	48	192	192
100	144	144	24	24	24	24	96	96
300	144	144	24	24	24	24	96	96
600	144	144	24	24	24	24	96	96
Total	720	720	120	120	120	120	480	480

RESULTS

Body Weights

The average body weights for females and males are shown in Fig. 1. The means are calculated for the allocation day, for weeks 1 through 4 and then for 4-week intervals after the fourth week. The body weights increased gradually throughout the study in all dose groups at essentially the same rate as the control group. There appeared to be no dose effect on body weight gain.

Food Consumption and Dosing

The average food consumption remained essentially the same for each of the dose groups and the controls. When the consumption was calculated on the basis of body weight, the food consumed started out at about 1.3 g food/g body wt/week for females and 1.1 g for males, then gradually decreased during the initial 30 to 40 weeks to about 0.90 and 0.80 g for the females and males, respectively (Fig. 2). The females ingested approximately 500, 250–275, and 100 mg gentian violet/kg body wt/week at each of the respective dose levels while the males had a slightly lower ingestion rate at 450–475, 225–250, and 75–100 mg gentian violet, respectively. The rates were more constant at the lower dose level.

Mortality

Mortality rates in males and females (Figs. 3 and 4) for all causes of death combined (but adjusted for interim sacrifices) was very low up to approximately 450 days, after which time a dose–response trend was noted in both the males (p = 0.01288) and females (p = 0.00005). The dose response was more pronounced in the females. At the end of the 2-year dosing, mortality in the female control group was approximately 13%, and 28, 27, and 64% in the 100-, 300-, and 600-ppm-dose groups, respectively. The males' mortality rates at the 0-, 100-, 300-, and 600-ppm-dose levels were 13, 14, 20, and 23%, respectively. When each dose level was compared with the control, all dose levels in the females were significantly different, i.e., at 100 ppm, p = 0.00088, at 300 ppm, p = 0.00079, and at 600 ppm, p = 0.00005. For males, none of the pairwise comparisons were powerful enough to detect

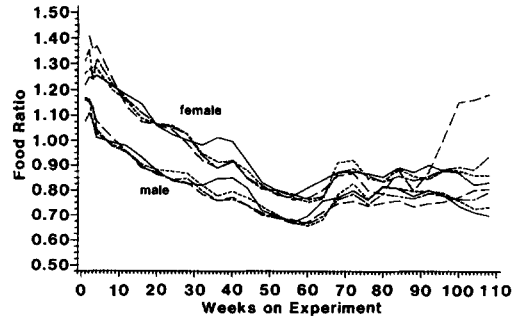

FIG. 2. Average food consumption g food/g average body wt/week in mice.

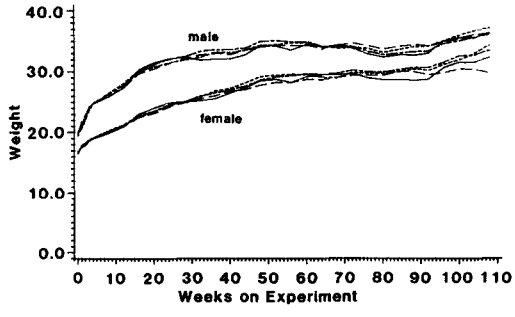

FIG. 1. Average body weight of mice for 24 months.

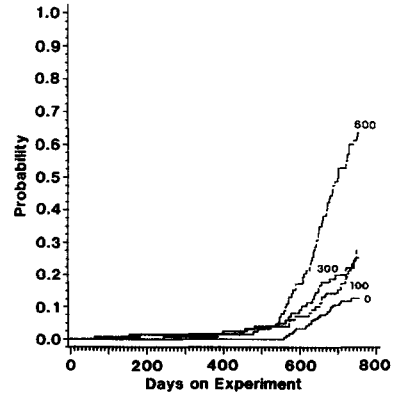

FIG. 3. Mortality of female mice for 24 months.

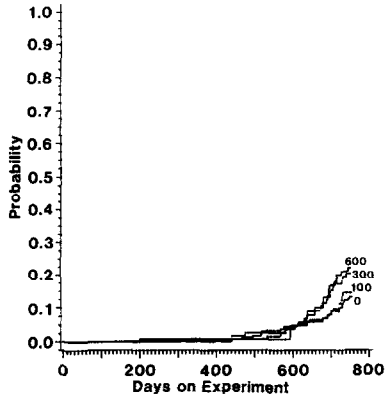

FIG. 4. Mortality of male mice for 24 months.

a significant increase when a Bonferroni (Miller, 1966) correction was applied to the significance level. Since the food consumption and body weights did not deviate between treatment groups or from controls, the dose response in mortality is likely due to the toxic effects of the gentian violet and not related to nutritional factors.

Pathology

The microscopic histopathology examination revealed several lesions showing dose responses (Tables 2 and 3). The greatest response was in liver neoplasms (hepatocellular carcinoma). The females in each instance appeared more susceptible or exhibited a greater response to the gentian violet than the males. Except for a slight dose response at 18 months for the liver neoplasms in females, no other dose responses were noted in the 12- or 18-month sacrifice groups. Essentially, all dose-related lesions were noted in the 24-month

TABLE 2

MICROSCOPIC HISTOPATHOLOGY SUMMARY OF FEMALE MICE[a]

	18-Month sacrifice[b] (Dose, ppm)				24-Month sacrifice[b] (Dose, ppm)			
Site, (lesion)	0	100	300	600	0	100	300	600
Liver, neoplasm,	3/47	0/22	3/24	8/24	8/185	8/93	36/93	20/95
benign	6[c]	0	13	33	4	9	39	21
Liver, neoplasm,	1/47	0/22	1/24	3/24	7/185	5/93	30/93	73/95
malignant	2	0	4	13	4	5	32	77
Uterus, RCS Type A	0/47	0/22	1/24	1/24	0/188	2/95	6/90	12/93
	0	0	4	4	0	2	7	13
Bladder, RCS Type A	0/47	1/22	1/24	0/23	0/188	2/92	3/89	5/91
	0	0	4	0	0	2	3	6
Spleen erythropoiesis	2/47	1/21	1/24	0/23	13/190	15/96	18/92	42/95
	4	5	4	0	7	16	20	44
Ovaries, Atrophy	0/45	0/21	0/22	1/21	11/178	13/90	25/89	37/89
	0	0	0	5	6	15	28	42
Harderian gland,	2/46	2/21	3/23	1/23	8/186	11/93	18/89	15/94
adenoma	4	10	13	4	4	12	20	16
Ovaries, RCS Type A	0/45	0/21	0/22	0/21	0/178	1/90	3/89	5/89
	0	0	0	0	0	1	3	6
Vagina, RCS Type A	0/46	0/22	1/23	0/22	1/182	1/90	4/88	8/87
	0	0	4	0	0.5	1	5	9

[a] All incidences in the 12-month sacrifice group were 0% except a 4% incidence in Harderian gland adenoma at 0 and 300 ppm, vagina RCS (Type A) at 100 ppm, and vagina RCS (total) at 100 ppm.

[b] Includes dead or moribund animals that were removed from the study prior to the scheduled sacrifice dates.

[c] No. positive for lesion/total No., and percentage.

TABLE 3

MICROSCOPIC HISTOPATHOLOGY SUMMARY OF MALE MICE[a]

Site, lesion	18 Month sacrifice[b] (Dose – ppm)				24 Month sacrifice[b] (Dose – ppm)			
	0	100	300	600	0	100	300	600
Liver, neoplasm benign	3/48	0/24	2/24	2/22	17/183	14/92	20/93	37/93
	6[c]	0	8	9	10	15	22	38
Liver, neoplasm	5/48	1/24	2/24	2/22	27/183	15/92	17/93	33/93
malignant	0	4	8	9	15	17	18	35
Harderian gland, adenoma	2/47	2/24	2/23	0/21	7/187	7/92	10/94	9/89
	4	8	9	0	4	7	11	10

[a] All incidences in the 12-month sacrifice group were 0% except an 8% incidence in liver neoplasm (benign) at 100 ppm.

[b] Includes dead or moribund animals that were removed from the study prior to the scheduled sacrifice dates.

[c] No. positive for lesion/total No., and percentage.

sacrifice groups. Figure 5 shows that malignant liver neoplasms occurred with an incidence of 4, 5, 32, and 77% in the female controls, 100-, 300-, and 600-ppm-dose groups, respectively, by 24 months. A background incidence of 15% was noted in the control males by 24 months compared to an incidence of 17, 18, and 35% in the 100-, 300-, and 600-ppm-dose groups, respectively. The incidence in the females by 18 months was 2, 0, 4, and 13% for the controls, 100-, 300-, and 600-ppm-dose groups, respectively. The incidence of liver neoplasms in males by 18 months did not rise above the background levels of 10% observed in the controls.

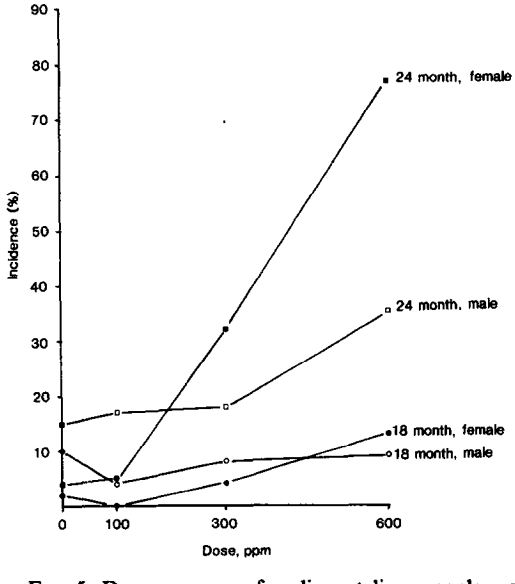

FIG. 5. Dose response of malignant liver neoplasms (unadjusted) for males and females at 18 and 24 months.

Extensive statistical analyses, as outlined under Methods, were conducted on the liver tumor data with respect to time to response. All corresponding p values, as shown in Table 4, demonstrate the levels of significance for positive trends across dose groups for (1) mortality due to the lesion, (2) prevalence, and (3) time to onset. In reporting statistical significance, the Bonferroni correction is applied to the nominal 0.05 significance level to adjust the false positive error rate for multiple comparisons to the controls, in this case requiring a p value of less than $0.05(3) = 0.0167$ for statistical significance with a false positive rate < 0.05.

For the females, the same trend was noted both for malignant liver neoplasms and for the combination of benign and malignant liver neoplasms. Significant positive trends were noted in mortality, prevalence, and onset in the overall comparison (i.e., all doses) as well as in the pairwise test for 300 ppm vs control

TABLE 4

LEVELS OF SIGNIFICANCE (p VALUES) FOR POSITIVE TRENDS AMONG DOSE GROUPS FOR (1) MORTALITY DUE TO A SPECIFIC DISEASE, (2) PREVALENCE (NONFATAL), AND (3) TIME TO ONSET[a]

	Females			Males		
	Mortality	Prevalence	Onset	Mortality	Prevalence	Onset
Mortality: overall	0.00005			0.01288		
Control vs 100 ppm	0.00088			0.48759		
Control vs 300 ppm	0.00079			0.10099		
Control vs 600 ppm	0.00005			0.03062		
Liver: malignant neoplasm overall	0.00005	0.00005	0.00005	0.01354	0.00045	0.00005
Control vs 100	0.10195	0.35780	0.42967	0.25258	0.25808	0.50502
Control vs 300	0.00818	0.00005	0.00005	0.24563	0.43369	0.30665
Control vs 600	0.00005	0.00005	0.00005	0.01993	0.00157	0.00009
Liver: malignant and benign tumor	0.00005	0.00005	0.00005	0.01354	0.00005	0.00005
Control vs 100	0.10195	0.47510	0.27258	0.25258	0.37448	0.23748
Control vs 300	0.00818	0.00005	0.00005	0.24563	0.01667	0.00956
Control vs 600	0.00005	0.00005	0.00005	0.01993	0.00005	0.00005
Spleen: erythropoiesis		0.00005			0.04342	
Control vs 100		0.01738			0.00743	
Control vs 300		0.00147			0.05695	
Control vs 600		0.00005			0.02248	
Ovaries: atrophy		0.00005				
Control vs 100		0.01490				
Control vs 300		0.00005				
Control vs 600		0.00005				
Harderian gland: adenoma		0.00110			0.06159	
Control vs 100		0.03754			0.16234	
Control vs 300		0.00005			0.03070	
Control vs 600		0.00323			0.10644	

[a] Trend tests were performed across all dose groups and controls.

and 600 ppm vs control. In the comparison of the 100-ppm-dose group to control, none of the three functions showed a statistically significant difference.

The males demonstrated a lower susceptibility than females to liver carcinogenicity from gentian violet. While the overall comparisons for both malignant tumors alone and malignant plus benign tumors showed a significant positive trend for mortality, prevalence, and onset in both sexes, the pairwise comparisons of doses to controls showed less positive trends in the males. For malignant liver neoplasms, positive increases from control were noted only in prevalence and onset at 600 ppm. Mortality at this dose level showed a borderline p value of 0.02. The results for malignant plus benign lesions were essentially the same as malignant only except positive increases from control were noted for prevalence and onset in the 300 ppm also.

Other dose-related lesions were noted by 24 months, almost exclusively in the females (Fig. 6 and Table 2). Dose responses were noted for erythropoiesis in the spleen with 7, 16, 20, and 44% responding for the 0-, 100-, 300-, and 600-ppm-dose levels, respectively, and for atrophy of the ovaries with 6, 15, 28, and 42% respon-

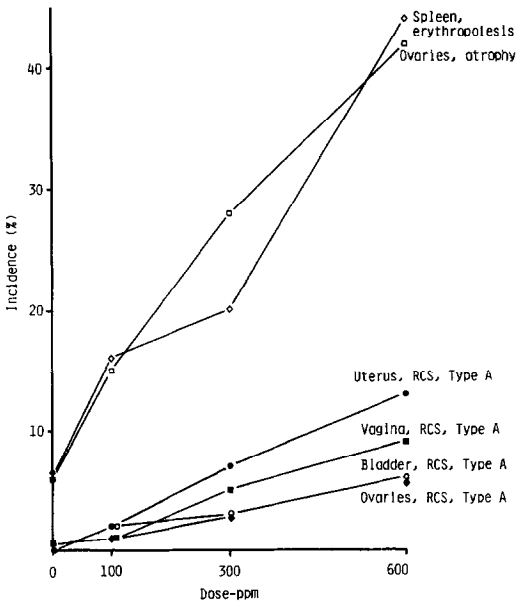

FIG. 6. Pathology summary (unadjusted) of other lesions at the 24-month sacrifice.

ses for the same respective dose groups. Lesser, but significant dose responses were also noted for the occurrence of reticulum cell sarcomas (Type A) in the uterus, vagina, bladder, and ovaries. These lesions were absent from the controls, except for 1/182 for RCS in the vagina. The following incidences were noted for the respective dose levels of 0, 100, 300, and 600 ppm: RCS of the uterus (Type A), 0, 2, 7, and 13%; RCS of the vagina (Type A), 0.5, 1, 5, and 9%; RCS of the bladder (Type A), 0, 2, 3, and 6%; RCS of the ovaries (Type A) 0, 1, 3, and 6%. Adenoma of the Harderian gland was noted in 4, 12, 20, and 16% of the females and in 4, 7, 11, and 10% of the males (Table 3) in the 0-, 100-, 300-, and 600-ppm doses, respectively, by 24 months.

The levels of significance for trend and for comparison of dosed groups to controls are listed in Table 4. The trend for prevalence of erythropoiesis of the spleen in females is highly significant overall and also at each of the comparisons of dose vs control, although the level at the 100-ppm dose is borderline. This same test in males was not significant by any of the tests. The prevalence of atrophy of the ovaries was significant overall and at each dose level. Adenoma of the Harderian gland showed a significant positive trend overall and at 300 and 600 ppm in the females but not in the males.

The statistical analysis of the incidences (unadjusted tumor rates) of Type A RCS in the terminally sacrificed animals in the bladder, uterus, ovaries, and vagina demonstrated a positive linear trend (Table 5). When the incidence of Type A RCS in these same organs was compared between specific dosed groups vs controls, significant increases were noted for all organs at 600 ppm, and for the uterus at 300 ppm. There were no significant differences noted at 100 ppm for any of the organs.

Tumor Morphology

Lymphoreticular tissue and the liver were target tissues. The lymphoreticular lesions are classified according to Dunn's classification (Dunn, 1954).

Benign hepatocellular neoplasms (adenomas) were usually small, and they compressed

TABLE 5

LEVELS OF SIGNIFICANCE (p VALUES) FOR RETICULUM CELL SARCOMA (RCS) IN SPECIFIC ORGANS AT THE 24-MONTH SACRIFICE

Organ	Dose			Trend[b]
	100 ppm[a]	300 ppm[a]	600 ppm[a]	
Bladder, Type A	0.1072	0.0324	0.0034[c]	0.0036
Uterus, Type A	0.1119	0.0010[c]	0.0001[c]	0.0001
Ovaries, Type A	0.3358	0.0362	0.0038[c]	0.002
Vagina, Type A	0.5531	0.0402	0.0006[c]	0.0001

[a] Fisher's exact test, one-sided test of comparison to control.

[b] Cochran–Armitage test for linear trend.

[c] Significant with Bonferroni correction, $p \leq 0.05$.

adjacent parenchyma focally. The adenomas were composed of well-differentiated cells in which the cytoplasm was either basophilic, eosinophilic, clear or vacuolated. Little pleomorphism of nuclei was present. The cells were of uniform size and formed either regular cords of not more than two cell layers thick or solid masses.

Most of the hepatocellular carcinomas of this study were trabecular in pattern while a few were solid. The trabecular pattern consists of cords more than two cell layers thick. The cells were well to poorly differentiated hepatocytes arranged in trabecular, acinar, or papillary form. Hemorrhage and necrosis occurred in some of the cases. In this study all 24 liver tumors that metastasized to the lung were trabecular tumors. There were 28 cases of metastases to the lung in animals having a liver tumor only or liver tumor with other tumors elsewhere in the animals, and only 4 out of these 28 cases were from tumors other than hepatocellular carcinoma. The 4 nonhepatocellular metastases in the lung were from an undifferentiated sarcoma of muscle and subcutis of the thigh, from a mammary gland tumor, from a Harderian gland tumor, and from a granulosa cell tumor of the right ovary.

In the remaining 24 animals, the metastatic lesion was from the liver. They were all from the 24-month sacrifice group (Table 6). The other tumor cellular pattern also found in this study was a solid pattern which was composed of either small immature neoplastic hepatocytes or extremely large anaplastic hepatocytes. The cytoplasm was either acidophilic or vacuolated and the nuclei were large with prominent nucleoli. Mitotic figures were not numerous.

The RCS (Type A) of the female genital organs was similar to those described by Dunn (1954). The tumor was composed of sheets of elongated spindled cells with basophilic ovoid nuclei and scanty acidophilic cytoplasm, involving the wall of the vagina, cervix, and uterus.

TABLE 6

METASTATIC LESIONS

	Males	Females
Controls	3	0
100 ppm	0	1
300 ppm	1	3
600 ppm	3	13

Clinical Chemistry

The statistical analysis (Dunnett, 1964) of the positive findings in the clinical chemistry analysis is summarized in Table 7. Due to the low volume of serum available from mice and also to the presence of a blue/purple color in the serum, apparently from the gentian violet, the numbers of samples analyzed were variable. The blue color in the serum interfered with the procedures that involved a spectrophotometric assay. There were no observed effects at 12 months. At 18 months there was a small increase in direct bilirubin; however, this was probably caused by interference of the blue pigment in the serum.

At 24 months, positive effects were noted for AST–GOT, ALT–GPT, serum cholesterol, $\alpha 1$ serum protein, $\alpha 2$ serum protein, and triglycerides. All of these effects related to abnormalities of the liver. In addition, most of the statistically positive trends were in the females at 24 months and at 600 ppm. This correlates well with the histopathological examination data.

Estimation of Risk

Risk assessment extrapolation was performed according to procedures described by Farmer *et al.* (1982) for each sex with malignant liver neoplasms and benign plus malignant neoplasms as the endpoints. The last day on study, i.e., 753 days, was chosen as the time point to do the risk extrapolation. A multistage dose–response model was fit in the experimental range to the estimated time to onset

TABLE 7

DUNNETT'S *t* (COMPARISON TO CONTROL) APPLIED IN CONNECTION WITH THE UNWEIGHTED MEANS ANALYSIS (SIGNIFICANT TWO-TAILED *p* VALUES AT 24-MONTH SACRIFICE)

	Females			Males		
	100	300	600	100	300	600
AST–GOT	NS[a]	.05 P	.01 P	NS	NS	.01 P
ALT–GPT	NS	.01 P	.01 P	NS	NS	.01 P
ALP	QNS	QNS	QNS	NS	NS	NS
Triglycerides	NS	NS	.01 N	NS	NS	NS
Cholesterol	NS	NS	.01 P	NS	.05 N	NS
αP2-%	NS	.01 P	.01 P	NS	NS	NS
α1-CONC	NS	NS	.01 P	NS	NS	NS
α2-CONC	NS	NS	.01 P	NS	NS	NS

[a] QNS, quantity (of serum) not sufficient; NS, not significant at $p = 0.05$; N, change in negative direction; P, change in positive direction.

probabilities and an upper 95% confidence limit on increased risk over background was calculated. Then linear extrapolation of the upper confidence limit was used to obtain a lower bound on the virtually safe dose (VSD) corresponding to an increased risk of 10^{-6}. The extrapolation was done from either the lowest dose (100 ppm) or the dose which was estimated to produce an excess risk of 1%, whichever was larger.

For malignant liver neoplasms, the lower bound on the VSD for the female mice was 2 ppb and for the male mice, 1 ppb. For benign and malignant liver tumors together, the lower bound on the VSD for the females was 2 ppb and for the males, 1 ppb.

DISCUSSION

From the obvious differences in this study between the males and females, gentian violet appeared to have a sex-related effect. There were dose-related lesions in the liver, Harderian gland, and lymphoreticulum in females, with only liver and Harderian gland lesions in the males. Incidences of liver neoplasms were much greater in the treated females, despite a larger background in the control males.

Preliminary results of metabolism studies performed on chickens at the National Center for Toxicological Research indicate that gentian violet is deposited largely in the liver and in subcutaneous fat. Since one of the principal uses of gentian violet is as a feed additive for inhibition of mold and fungus growth in chicken feed, this must be a consideration in the calculation of risk to the consumer. Chicken livers are considered as an edible portion of the chicken and the fat is used as a base in commercial preparation of soups. Therefore, it is possible that gentian violet is consumed by humans.

The pathology data indicated the occurrence of reticulum cell sarcoma, (Type A) in several organs. These were dose-related in the vagina, uterus, ovaries, and bladder. This reaction was characterized by Dunn (1954) when she pointed out that the incidence of these lesions in the C57BL/6 mouse increased and the life span decreased in the presence of a chemical carcinogen. She reported that the spleen was also involved, as occurred in this study.

An important factor in considering the relative risk of this compound is the time to tumor development. Since there were interval sacrifices at 12 and 18 months, and the study

was terminated at 24 months, the time to tumor is fairly well defined. Almost all of the pathology results occurred only by the 24-month sacrifice. Under the conditions of this study, 100 ppm gentian violet for 12 months could be considered the "no-observed effect" dose and time for female mice. For male mice, a no-observed effect level would be 300 ppm for 18 months. It would appear that the choice of dose levels in the experimental design of this study provided sufficient information for estimation of risk as well as identification of a possible no-observed effect level. In addition, food consumption did not appear to be influenced by the presence of the test agent, thereby providing an accurate comparison between doses.

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