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13-WEEK SUBCHRONIC TOXICITY STUDIES OF DIRECT BLUE 6, DIRECT BLACK 38, AND DIRECT BROWN 95 DYES

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U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
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13-WEEK SUBCHRONIC TOXICITY STUDIES

DIRECT BLUE 6, DIRECT BLACK 38, AND DIRECT BROWN 95 DYES

Carcinogenesis Testing Program
Division of Cancer Cause and Prevention
National Cancer Institute
National Institutes of Health
Bethesda, Maryland 20014

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FOREWORD: This report presents the results of 13-week subchronic toxicity studies of direct blue 6, direct black 38, and direct brown 95 dyes conducted for the Carcinogenesis Testing Program, Division of Cancer Cause and Prevention, National Cancer Institute (NCI), National Institutes of Health, Bethesda, Maryland. These subchronic studies were conducted as part of the bioassay protocol to establish concentrations for use in 2-year bioassays of the test chemicals in rats and mice. In view of the results obtained in the 13-week studies, the 2-year bioassays will not be performed.

The Carcinogenesis Testing Program is designed to determine whether selected chemicals have the capacity to produce cancer in animals. Negative results, in which the test animals do not have a greater incidence of cancer than control animals, do not necessarily mean that the test chemical is not a carcinogen, inasmuch as the experiments are conducted under a limited set of circumstances. Positive results demonstrate that the test chemical is carcinogenic for animals under the conditions of the test and indicate that exposure to the chemical is a potential risk to man. The actual determination of the risk to man from animal carcinogens requires a wider analysis.

CONTRIBUTORS: The 13-week subchronic toxicity studies of direct blue 6, direct black 38, and direct brown 95 dyes were conducted by Battelle Memorial Institute, Columbus Laboratories, Columbus, Ohio, under a subcontract to Tracor Jitco, Inc., prime contractor for the NCI Carcinogenesis Testing Program.

The principal investigator for the studies of the direct dyes was Dr. A. C. Peters[1]. Drs. Peters and J. F. Robens[2] were responsible for the selection of the doses administered. Drs. Peters and G. S. Dill[1] prepared the reports of the methods and results from the laboratory. Mr. M. Hughes[1] was responsible for the care of the animals. Histopathologic examinations were performed by Dr. Dill, rat hepatic lesions were reviewed by Dr. J. M. Ward[3]; the diagnoses included in this report represent their interpretation.

Animal pathology tables were compiled at EG&G Mason Research Institute[4]. The statistical analyses were performed by Dr. J. R. Joiner[2], using methods selected for the bioassay program by Dr. J. J. Gart[5]. Chemicals used in the studies were analyzed at Midwest Research Institute under the direction of Dr. E. Murrill[6]; stability tests were performed by Mr. J. R. Wagner[6] and Dr. T. C. Carpenter[6], and tests for benzidine in the dyes were performed by Ms. J. Huerner[6]. Test diets were analyzed for dye content at Battelle, Columbus Laboratories, by Mr. D. Emmerling[1]. Tests for benzidine in the urine were performed at Battelle, Columbus Laboratories, by Dr. A. P. Leber[1] and tests for methemoglobin in the blood were performed by Ms. S. D. Guthrie[1]. The results of the analyses were reviewed by Dr. S. S. Olin[2].

This report was prepared at Tracor Jitco[2] under the direction of NCI. Those responsible for the report at Tracor Jitco were Dr. L. A. Campbell, Director of the Bioassay Program; Dr. S. S. Olin, Deputy Director for Science; Dr. J. F. Robens, toxicologist; Dr. R. L. Schueler, pathologist; Dr. G. L. Miller and Mr. W. D. Reichardt, bioscience writers; and Dr. E. W. Gunberg, technical editor, assisted by Ms. Y. E. Presley and Ms. P. J. Graboske.

The statistical analysis was reviewed by members of the Mathematical Statistics and Applied Mathematics Section of NCI[5]: Dr. John J. Gart, Mr. Jun-mo Nam, Dr. Hugh M. Pettigrew, and Dr. Robert E. Tarone.

The following other scientists at NCI were responsible for evaluating the studies, interpreting the results, and reporting the findings: Dr. Kenneth C. Chu, Dr. Cipriano Cueto, Jr., Dr. J.

Fielding Douglas, Dr. Dawn G. Goodman, Dr. Richard A. Griesemer, Dr. Harry A. Milman, Dr. Thomas W. Orme, Dr. Jerrold M. Ward, and Dr. Carrie E. Whitmire.

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[1]Battelle Memorial Institute, Columbus Laboratories, 505 King Avenue, Columbus, Ohio.

[2]Tracor Jitco, Inc., 1776 East Jefferson Street, Rockville, Maryland.

[3]Carcinogenesis Testing Program, Division of Cancer Cause and Prevention, National Cancer Institute, National Institutes of Health, Bethesda, Maryland.

[4]EG&G Mason Research Institute, 1530 East Jefferson Street, Rockville, Maryland.

[5]Mathematical Statistics and Applied Mathematics Section, Biometry Branch, Field Studies and Statistics, Division of Cancer Cause and Prevention, National Cancer Institute, National Institutes of Health, Bethesda, Maryland.

[6]Midwest Research Institute, 425 Volker Boulevard, Kansas City, Missouri.

SUMMARY

Thirteen-week subchronic toxicity studies of direct blue 6, direct black 38, and direct brown 95 dyes were conducted by administering the test chemicals in feed to Fischer 344 rats and B6C3F1 mice.

Groups of 10 rats and 10 mice of each sex were administered one of the three dyes at one of five concentrations for 13 weeks and then necropsied, beginning the second day after the end of the dosing period. The concentrations used for the rats were 190, 375, 750, 1,500, and 3,000 ppm. The concentrations used for the mice were 750, 1,500, 3,000, 6,000, and 12,500 ppm, except for the female mice administered direct brown 95 dye, which were given concentrations of 375, 750, 1,500, 3,000, and 6,000 ppm. Matched controls consisted of groups of 10 untreated rats and 10 untreated mice of each sex.

Mean body weights of the male and female rats administered the two or three highest doses of any one of the test dyes were lower than mean body weights of the corresponding controls, and the depressions in mean body weight were dose related. Mean body weights of the male and female mice administered the highest dose of any one of the test dyes were slightly lower than mean body weights of the corresponding controls; mean body weights of mice administered lower doses were generally unaffected.

All male and female rats administered 3,000 ppm of any one of the dyes or 1,500 ppm of direct brown 95 dye died before the end of the studies. One male administered 1,500 ppm direct blue 6 dye, six males administered 1,500 ppm direct black 38 dye, and two males administered 750 ppm direct brown 95 dye also died by the end of the studies. No deaths occurred in any other dosed group or in any control group of rats. All male and female mice administered the test dyes survived to the end of the studies, except for one male whose death was attributed to bacterial infection.

Benzidine and monoacetyl benzidine were detected in the urine of male and female rats and mice administered the test dyes, but neither compound was detected in the urine of control rats and mice. Determinations of methemoglobin in control and dosed rats showed no differences.

In rats, neoplastic lesions occurred only in dosed groups and consisted of hepatocellular carcinomas and neoplastic nodules of the liver. The incidences of hepatocellular carcinomas in female rats administered 3,000 ppm direct blue 6 dye (4/9) and male rats administered 1,500 ppm direct black 38 dye (4/9) were significant ($P = 0.033$) when related to the incidences of the tumors in the corresponding controls (0/10); hepatocellular carcinomas were also observed in two male rats administered 1,500 ppm direct blue 6 dye and in one female rat administered 1,500 ppm direct brown 95 dye. No control rats from any of the three studies developed hepatocellular carcinomas.

When incidences of neoplastic nodules were combined with those of hepatocellular carcinomas, the significance increased to $P < 0.001$ for male rats administered 1,500 ppm direct blue 6 dye, $P = 0.001$ for females administered 3,000 ppm direct blue 6 dye, $P < 0.001$ for males administered 1,500 ppm direct black 38 dye, and $P = 0.007$ for females administered 1,500 ppm direct brown 95 dye. No controls developed neoplastic nodules. Female rats administered direct black 38 dye developed no hepatocellular carcinomas, but had an incidence of neoplastic nodules of 5/10, with a significance of $P = 0.016$. Male rats administered direct brown 95 dye developed neither hepatocellular carcinomas nor neoplastic nodules, but as indicated below, had significant incidences of preneoplastic lesions. The failure of groups of rats administered 3,000 ppm dye to develop tumors when other groups administered 1,500 ppm did develop tumors may be due to earlier deaths at the higher dose.

Preneoplastic hepatic lesions (basophilic foci) occurred only in dosed rats and did not occur in the controls. The incidences of the basophilic foci were significant ($P \leq 0.033$) in male (4/9) and female (7/9) rats administered 3,000 ppm direct blue 6 dye and in male rats (7/8) administered 1,500 ppm direct brown 95 dye. Basophilic foci also occurred, at lower incidences, in

males (1/10) administered 1,500 ppm direct blue 6 dye, in males (3/9) administered 1,500 ppm direct black 38 dye, in females (1/8) administered 3,000 ppm direct black 38 dye, in males administered 750 ppm (3/10) or 3,000 ppm (2/9) direct brown 95 dye, and in females administered 1,500 ppm (3/8) or 3,000 ppm (3/8) direct brown 95 dye. When incidences of foci of cellular alteration, a possible preneoplastic lesion, were added to those of basophilic foci, significance occurred in additional dosed groups.

In mice, no neoplastic lesions occurred in the liver or other tissues of groups administered the different dyes. However, three mice administered 12,500 ppm direct black 38 dye and one mouse administered 12,500 ppm direct brown 95 dye had foci of cellular alteration, in which the cells were basophilic when compared with surrounding normal cells.

It is concluded that under the conditions of these 13-week subchronic toxicity studies, direct blue 6 and direct black 38 dyes were carcinogenic in male and female Fischer 344 rats and direct brown 95 was carcinogenic in female rats; all three dyes induced hepatocellular carcinomas and neoplastic nodules in the liver. The test dyes were not carcinogenic for B6C3F1 mice in the 13-week subchronic toxicity studies.

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I. INTRODUCTION

Direct blue 6 (CAS 2602-46-2; NCI C54579), direct black 38 (CAS 1937-37-7; NCI C54557), and direct brown 95 (CAS 16071-86-6; NCI C54568) are azo dyes used on textiles such as cotton, silk, wool, nylon, and acetate. All three dyes also have commercial use on leather. In addition to use as textile dyes, direct blue 6 and direct black 38 are used in aqueous printing inks and as biological stains, and one or another of these dyes has been used in plastics (direct black 38, direct brown 95), paper (direct blue 6, direct brown 95), wood stains (direct black 38), and wood flour (direct black 38) (Society of Dyers and Colourists, 1971).

Two of these dyes, direct blue 6 and direct black 38, have been used in hair dyes (Comptroller General of the U.S., 1977).

The United States International Trade Commission (1977a) reports that 70,753 pounds of direct black 38 and 8,205 pounds of direct brown 95 were imported for use in the United States in 1976. In the same year, U.S. manufacturers produced 3,759,000 pounds of direct black 38, and 595,000 pounds of direct brown 95. Similar data are not available for direct blue 6, although its production is believed to be greater than 5,000 pounds annually (USITC, 1977a and 1977b).

These three dyes were selected for study in the Carcinogenesis

Testing Program because they are derived from benzidine, which is known to be carcinogenic in animals and man (IARC, 1972), because large quantities were used industrially, and because of the potential for long-term human exposure both through industrial use and through contact with products containing the dyes.

II. MATERIALS AND METHODS

A. Chemicals

The chemicals used were technical-grade factory-strength (unformulated) dyes, manufactured by GAF Corporation (New York, N.Y.). Direct blue 6 (Phenamin Blue BB-FS) was obtained in one batch (Lot No. 43762), direct black 38 (Phenamin Black E-FS) in one batch (Lot No. 43761), and direct brown 95 (Fastusal Brown LBRS-FS) in one batch (Lot No. 43763). The molecular structures of these test dyes are given in Appendix E and show the occurrence of the benzidine moiety in each structure. The identity and purity of each chemical were determined by analyses at Midwest Research Institute. According to the manufacturer, the purities by dyestuff assay of direct blue 6, direct black 38, and direct brown 95 were 66%, 86%, and 79%, respectively; according to analyses performed at Midwest Research Institute, the corresponding purities by titration of azo groups with titanous chloride were 59.9 $\pm$ 1.9%, 87.1 $\pm$ 3.4%, and 72.2 $\pm$ 7.0%. Elemental analyses (for all elements except oxygen) were reasonably consistent with the molecular formulas of direct blue 6 ($C_{32}H_{20}O_{14}N_6Na_4S_4$), direct black 38 ($C_{34}H_{25}N_9O_7S_2Na_2$), and direct brown 95 ($C_{31}H_{20}N_6O_9SNa_2 \cdot Cu$), after correction for the percent dye determined by titanous chloride titration, the water content, and sodium chloride (estimated from analyses for Na and

Cl); direct blue 6 and direct brown 95 were somewhat high in C, H, and Na. Water analyses (Karl Fischer) were 9.18 $\pm$ 0.51%, 7.13 $\pm$ 0.54%, and 4.99 $\pm$ 0.22%, respectively, and NaCl concentrations were estimated at 20.8%, 7.9%, and 14.9%. The infrared spectra of direct black 38 and direct brown 95 were consistent with those in the literature (Sadtler, 1960); the infrared spectrum for direct blue 6 was not consistent with that in the literature and could not be taken as assurance of identity. Thin-layer chromatographic analyses using two different solvent systems showed 8-15 minor or trace impurities. No attempt was made to identify or quantitate these impurities. High-pressure liquid chromatography showed several small impurity peaks in each of the dyes, but no benzidine (detection limit, 0.004%). The methodology used would have detected total amounts of benzidine — that is, both benzidine salts and free benzidine.

Mazola® corn oil (Best Foods, Division of CPC International, Inc., Englewood Cliffs, N. J.) was added to the dyes as a dust suppressant. The concentration of corn oil in the dye was 1.3%. Bulk dyes containing the corn oil were stored at 5°C.

B. Dietary Preparation

A 1-week supply of each diet was formulated 1 or 2 days before use by mixing Purina® Laboratory Chow® animal meal (Ralston

Purina Co., St. Louis, Mo.) and dye containing 1.3% corn oil. Weighed amounts of animal meal were combined with weighed amounts of dye containing the corn oil and mixed in a Patterson-Kelly twin shell blender for 15 minutes to assure homogeneity. Formulated diets were stored at 23°C until used. The control diets contained corn oil in amounts equal to that in the highest dose groups for each species; i.e., 39 ppm for rats and 163 ppm for mice. Corn oil was present in the diets containing the dyes at 3 to 39 ppm for rats and 5 to 163 ppm for mice.

Stability of diets formulated with 10% of the bulk dyes containing 1.3% corn oil was determined by analyses performed after storage for 2 weeks at -20°, 5°, 25°, or 45°C. Spectrophotometric analysis of extracts of the diets showed that each of the dyes was stable in feed for 2 weeks at all temperatures tested. Analyses for benzidine were not performed.

As a quality control test on the accuracy of diet preparations, the concentration of dye in one sample at each dose level was determined for each dye during the studies and verified to be within $\pm$ 10% of the required concentration.

C. Animals

Fischer 344 rats and B6C3F1 mice of each sex were obtained from Frederick Cancer Research Center, Frederick, Maryland, through

contracts with the Division of Cancer Treatment, National Cancer Institute. On arrival at the laboratory, the rats were 4 weeks of age and the mice were 4-5 weeks of age. All animals were quarantined (rats for 12 days, mice for 13 days) prior to the start of the studies. During the quarantine periods, all animals of each species and sex were examined, and several were necropsied to detect observable disease. For the study of each chemical, rats and mice of each sex were randomized into dosed or control groups from the quarantine pool by tables of random numbers, and marked to assure individual identification.

D. Animal Maintenance

All animals were housed in temperature- and humidity-controlled rooms. The temperature range was 21-23°C, and the relative humidity was maintained at 40-60%. The air in each room was filtered with high-efficiency particulate air (HEPA) filters and changed 20-25 times per hour. Fluorescent light provided 12 hours of illumination per day. Tap water was available ad libitum, and diets were replenished as necessary, usually at 2-day intervals. Fresh control and test diets were provided every week.

Both rats and mice were housed five per cage in solid polycarbonate cages (Lab Products, Inc., Garfield, N.J.) suspended from

stainless steel racks. Rack shelves were covered with spun-bonded polyester filters (Dupont 2024). Absorb-Dri® hardwood chip bedding (Lab Products, Inc., Garfield, N.J.) was used for all cages and was changed two times per week. All rat and mouse cages were changed two times per week and were mechanically washed at temperatures not less than 82°C using Exceed® detergent (Economics Labs, Inc., Osborn Building, St. Paul, Minn.). All feed hoppers were changed once per week. Automatic watering systems provided water for both the rats and the mice.

All rats were housed in one room, and the mice were housed in another room. No animals administered any other test compounds were housed in these rooms. Neither cage positions within the racks nor rack positions within the rooms were rotated.

E. Two-Week Toxicity Tests

Two-week toxicity tests were conducted with Fischer 344 rats and B6C3F1 mice to estimate the toxicity of each of the test dyes; on the basis of these tests various concentrations were selected for use in 13-week studies. In the 2-week tests, the dyes were administered in the feed at concentrations of 3,000, 6,000, 12,500, 25,000, and 50,000 ppm. Five males and five females of each species were administered each dose, and five males and five females of each species were given basal diets. After the

administration of the dyes for 2 weeks, all animals were killed and necropsied.

Rats administered the dyes had severe dose-related decreases in food consumption and dose-related depressions in mean body weight. The effects appeared even at the lowest concentrations administered. In rats administered direct blue 6 dye, one male given 50,000 ppm died on day 6; in rats administered direct black 38 dye, all males given 50,000 ppm except one and all females given 50,000 ppm except one died by day 9; and in rats administered direct brown 95 dye, one male given 50,000 ppm died on day 12 and all females given 12,500 ppm died by day 11. All other animals survived to the end of the tests. Gross observations of rats administered the different dyes included thymic atrophy, splenic enlargement, and darkening of the spleen and kidneys. In rats administered the direct black 38, pigmentation of the liver also was noted. The pigmentation of the spleen and kidneys was dose related; the thymic atrophy was attributed to the low consumption of food. Methemoglobin was measured in four animals from tests using each of the dyes and was found to be elevated.

Mice administered the dyes had depressions in mean body weights in all groups except the males administered direct blue 6 dye. The effects were generally dose related and extended in most

cases to all but the lowest doses administered. In mice administered direct blue 6 or direct brown 95 dyes, no deaths occurred; in mice administered direct black 38 dye, three males given 25,000 ppm died by day 10 and two females given 50,000 ppm died by day 12. All other animals survived to the end of the tests. Hunched appearance and lethargic body movement were noted in mice at the higher concentrations. Gross observations of mice administered the different dyes consisted primarily of pigmentation of spleen and kidneys in mice at the higher doses of the dyes, related directly to the dye. The brown-colored viscera and blood of mice administered direct black 38 dye was attributed to methemoglobin, although tests for concentration of methemoglobin in the blood were not performed for this species.

Concentrations for the 13-week subchronic toxicity studies were selected mainly on the basis of the effects of the dyes on mean body weight. Because of generally excessive weight losses in the male and female rats at 6,000 ppm or higher, the concentrations set for the rats were 190, 375, 750, 1,500, and 3,000 ppm; similarly, because of generally excessive weight losses in the male and female mice at 25,000 ppm or higher, the concentrations set for the mice were 750, 1,500, 3,000, 6,000, and 12,500 ppm, except for the females administered the direct brown 95 dye, for which the highest concentration was set at 6,000 ppm.

F. Thirteen-Week Subchronic Toxicity Studies

The test groups, doses administered, and times on study of the 13-week subchronic toxicity studies are shown in tables 1 and 2. These studies were conducted as a part of the bioassay protocol to establish concentrations for use in the 2-year bioassays of the test chemicals in both rats and mice.

G. Clinical and Pathologic Examinations

Inspections for mortality and morbidity were carried out twice daily. Clinical observations were recorded daily. Body weights of individual animals were determined weekly. Tests for benzidine in the urine were performed at weeks 4 and 12 of the studies for the rats and at weeks 3 and 11 for the mice. Tests for methemoglobin of the rats were performed at the end of the studies.

Moribund animals and those animals that survived to the end of the studies were killed using CO_2 anesthesia and necropsied. Necropsies were also performed on all animals found dead, except one that was cannibalized. The tissues were preserved in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The following tissues were examined microscopically in all control rats and in rats administered 1,500 or 3,000 ppm of each dye and 750 ppm of direct brown 95

Table 1. Thirteen-Week Subchronic Toxicity Studies of Direct Blue 6, Direct Black 38,and Direct Brown 95 Dyes Administered in Feed to Rats

Sex and Test Group[a]	Initial No. of Animals[b]	Time on Study	
		Dosed (days)	Observed (days)[c]
Male			
Matched-Control[d]	10		93
190 ppm	10	91	2
375 ppm	10	91	2
750 ppm	10	91	2
1,500 ppm	10	91	2
3,000 ppm	10	91	2
Female			
Matched-Control[d]	10		93
190 ppm	10	91	2
375 ppm	10	91	2
750 ppm	10	91	2
1,500 ppm	10	91	2
3,000 ppm	10	91	2

[a]Each dye was mixed with animal meal to give the concentrations indicated. Corn oil was also present in the various dosed diets, with concentrations ranging from 3 to 39 ppm.

[b]Male and female rats were 6 weeks of age when placed on study.

[c]Surviving animals were necropsied beginning the second day after the end of the dosing period.

[d]Matched-control rats were fed animal meal containing 39 ppm corn oil.

Table 2. Thirteen-Week Subchronic Toxicity Studies of Direct Blue 6, Direct Black 38, and Direct Brown 95 Dyes Administered in Feed to Mice

Sex and Test Group[a]	Initial No. of Animals[b]	Time on Study	
		Dosed (days)	Observed (days)[c]
Male			
Matched-Control[d]	10		93
750 ppm	10	91	2
1,500 ppm	10	91	2
3,000 ppm	10	91	2
6,000 ppm	10	91	2
12,500 ppm	10	91	2
Female			
Matched-Control[d]	10		93
750 ppm	10	91	2
1,500 ppm	10	91	2
3,000 ppm	10	91	2
6,000 ppm	10	91	2
12,500 ppm	10	91	2

[a]Each dye was mixed with animal meal to give the concentration indicated. The concentrations given the female mice administered diet containing direct brown 95 dye were 375 to 6,000 ppm instead of the concentrations indicated. Corn oil was also present in the various dosed diets, with concentrations ranging from 5 to 163 ppm.

[b]Male and female mice were 6-7 weeks of age when placed on study.

Table 2. Thirteen-Week Subchronic Toxicity Studies of Direct Blue 6, Direct Black 38, and Direct Brown Dyes Administered in Feed to Mice

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[c]Surviving animals were necropsied beginning the second day after the end of the dosing period.

[d]Matched-control mice were fed animal meal containing 163 ppm corn oil.

dye: skin, lung, bone marrow, spleen, mandibular lymph node, mesenteric lymph node, thymus, heart, salivary gland, liver, pancreas, stomach, small intestine, colon, kidney, bladder, adrenals, thyroids, testes, and epididymis. Microscopic examination was also performed on the above tissues (plus bile duct) of control mice, males and females administered 12,500 ppm of each dye, females administered 6,000 ppm of direct brown 95 dye, and a male mouse that was administered 750 ppm of direct brown 95 dye and that died early. In addition, certain tissues were examined in rats and mice administered lower doses, as indicated in Appendix A, tables A1-A6 and Appendix B, tables B1-B7.

A few tissues from some animals were not examined. Thus, the number of animals from which particular organs or tissues were examined microscopically varies, and does not necessarily represent the number of animals that were placed on study in each group.

H. Data Recording and Statistical Analyses

Pertinent data on this experiment have been recorded in an automatic data processing system, the Carcinogenesis Bioassay Data System (Linhart et al., 1974). The data elements include descriptive information on the chemicals, animals, experimental design,

clinical observations, survival, body weight, and individual pathologic results, as recommended by the International Union Against Cancer (Berenblum, 1969). Data tables were generated for verification of data transcription and for statistical review.

Data on the incidences of neoplastic and nonneoplastic lesions were analyzed using the statistical techniques described in this section. Those analyses of the experimental results that bear on the possibility of carcinogenicity are discussed in the statistical narrative sections.

The incidence of neoplastic or nonneoplastic lesions has been given as the ratio of the number of animals bearing such lesions at a specific anatomic site (numerator) to the number of animals in which that site is examined (denominator). In most instances, the denominators included only those animals for which that site was examined histologically. However, when macroscopic examination was required to detect lesions prior to histologic sampling (e.g., skin or mammary tumors), or when lesions could have appeared at multiple sites (e.g., lymphomas), the denominators consist of the numbers of animals necropsied.

The purpose of the statistical analyses of tumor incidence is to determine whether animals receiving the test chemical developed a significantly higher proportion of tumors than did the control

animals. As a part of these analyses, the one-tailed Fisher exact test (Cox, 1970) was used to compare the tumor incidence of a control group with that of a group of dosed animals at each dose level. When results for a number of dosed groups (k) are compared simultaneously with those for a control group, a correction to ensure an overall significance level of 0.05 may be made. The Bonferroni inequality (Miller, 1966) requires that the P value for any comparison be less than or equal to 0.05/k. In cases where this correction was used, it is discussed in the narrative section.. It is not, however, presented in the tables, where the Fisher exact P values are shown.

The approximate 95 percent confidence interval for the relative risk of each dosed group compared to its control was calculated from the exact interval on the odds ratio (Gart, 1971). The relative risk is defined as p_t/p_c where p_t is the true binomial probability of the incidence of a specific type of tumor in a dosed group of animals and p_c is the true probability of the spontaneous incidence of the same type of tumor in a control group. The hypothesis of equality between the true proportion of a specific tumor in a dosed group and the proportion in a control group corresponds to a relative risk of unity. Values in excess of unity represent the condition of a larger proportion in the dosed group than in the control.

The lower and upper limits of the confidence interval of the relative risk have been included in the tables of statistical analyses. The interpretation of the limits is that in approximately 95% of a large number of identical experiments, the true ratio of the risk in a dosed group of animals to that in a control group would be within the interval calculated from the experiment. When the lower limit of the confidence interval is greater than one, it can be inferred that a statistically significant result ($P < 0.025$ one-tailed test when the control incidence is not zero, $P < 0.050$ when the control incidence is zero) has occurred. When the lower limit is less than unity, but the upper limit is greater than unity, the lower limit indicates the absence of a significant result while the upper limit indicates that there is a theoretical possibility of the induction of tumors by the test chemical, which could not be detected under the conditions of this test.

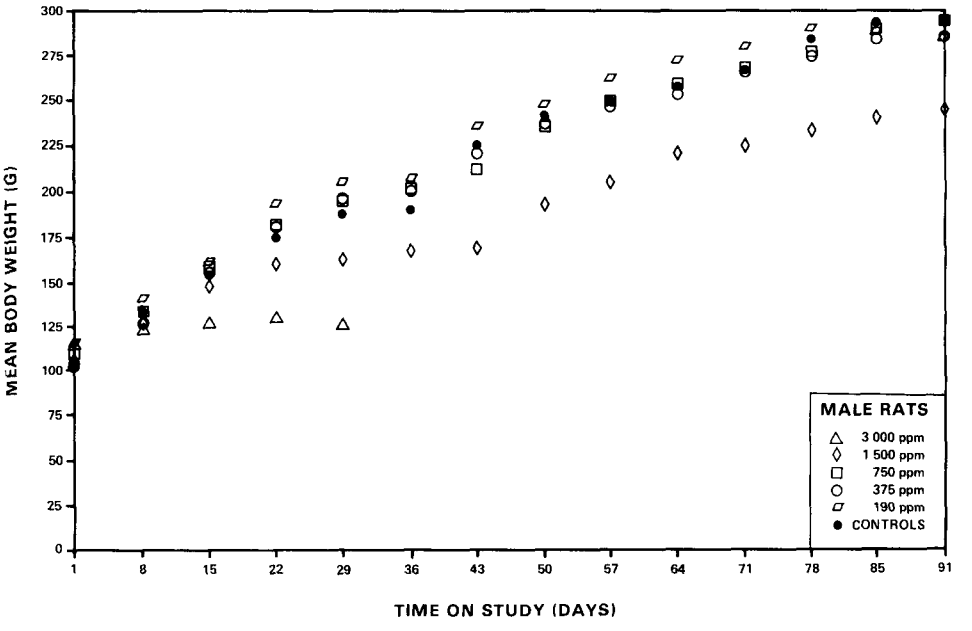
III. RESULTS - RATS

A. Body Weights and Clinical Signs (Rats)

Mean body weights of the male and female rats dosed with the two or three highest doses of any one of the test dyes were lower than mean body weights of the corresponding controls, and the depressions in mean body weight were dose related (figures 1, 2, and 3). No other clinical signs related to administration of the dyes were reported.

B. Benzidine and Methemoglobin Studies (Rats)

Urine collected over a 24-hour period during weeks 4 and 12 of the subchronic toxicity studies from male and female rats receiving each of three respective test dyes contained benzidine and monoacetyl benzidine, while specimens of urine taken from corresponding controls contained neither compound. The benzidine and monoacetyl benzidine were identified by thin-layer chromatography and mass spectroscopy. Quantities excreted in the urine were determined by combined extraction and spectrometric procedures. In most tests, the amounts excreted were dose related for each of the dyes administered. No consistent differences in results were found between males and females. Details of the methods and results are given in Appendix D.



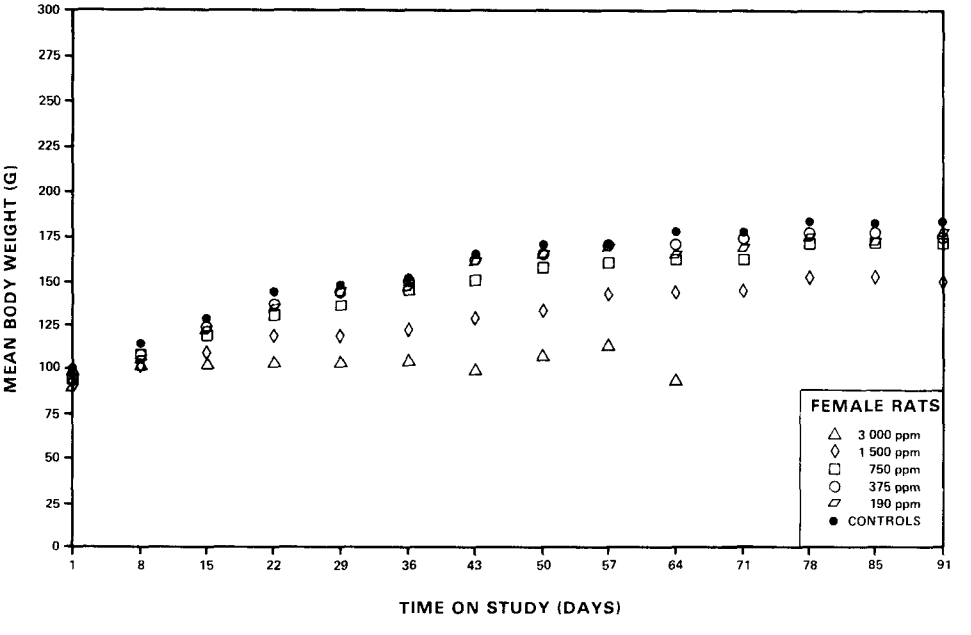


Figure 1. Growth Curves for Rats Administered Direct Blue 6 in the Diet

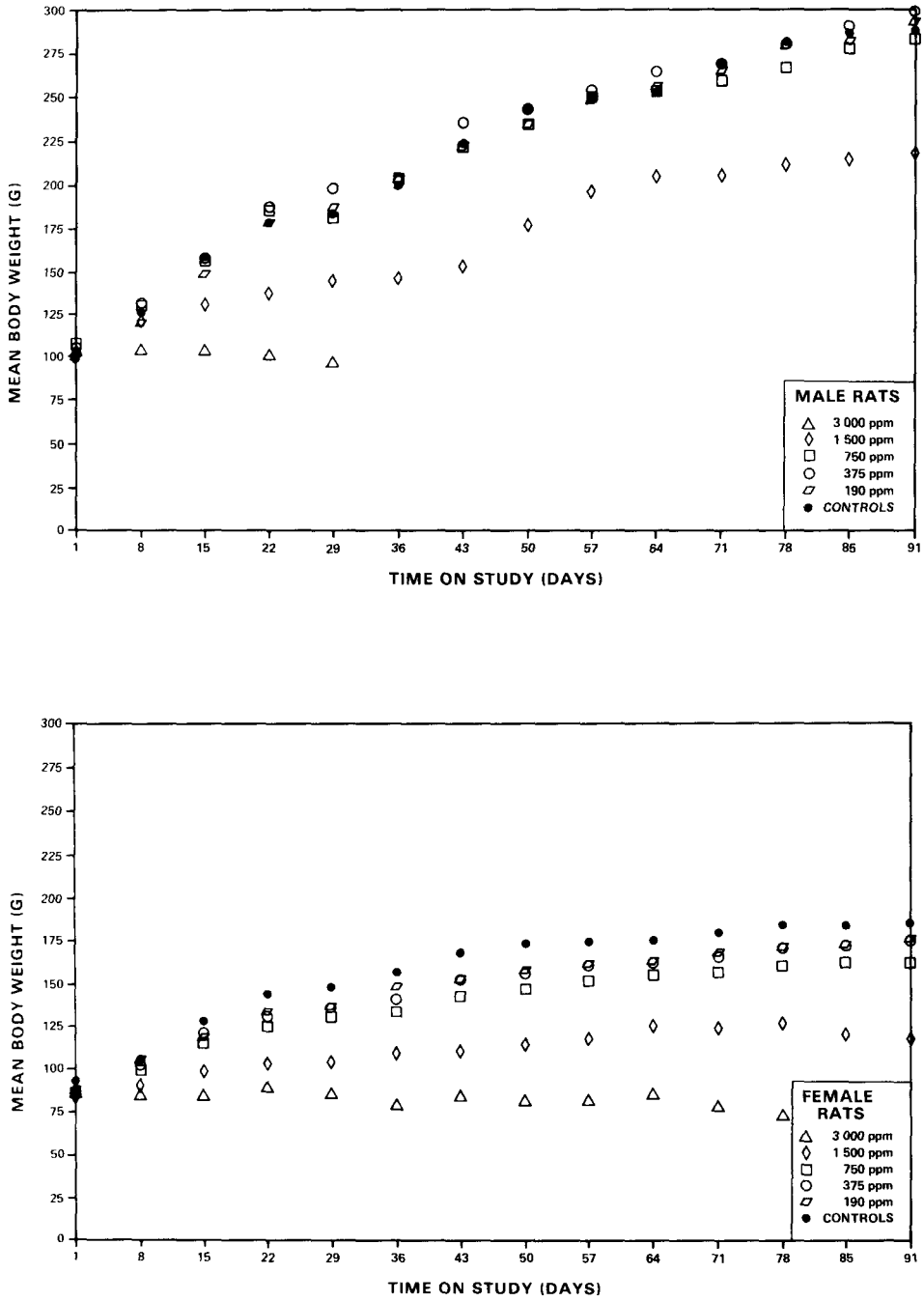
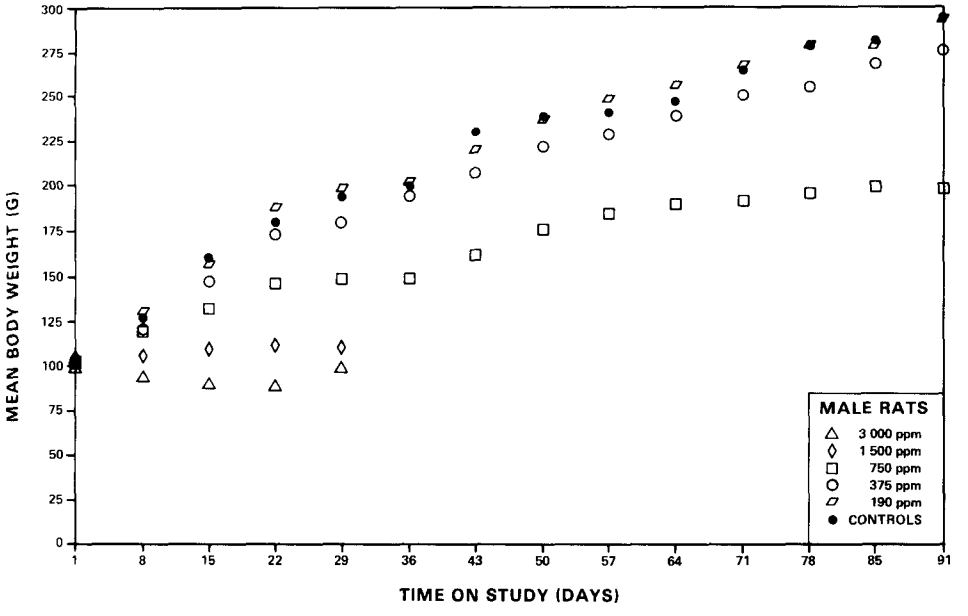


Figure 2. Growth Curves for Rats Administered Direct Black 38 in the Diet



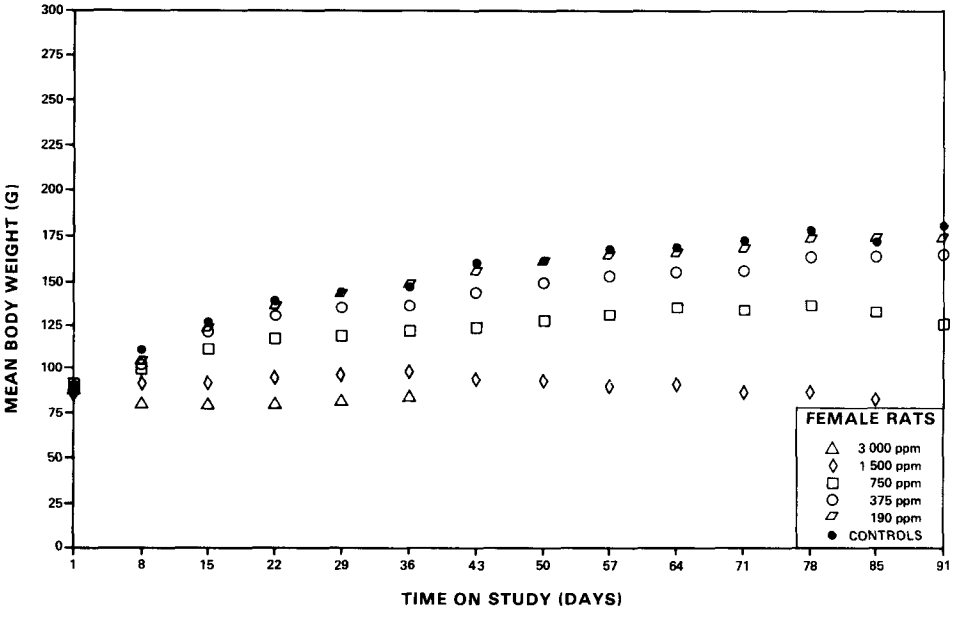
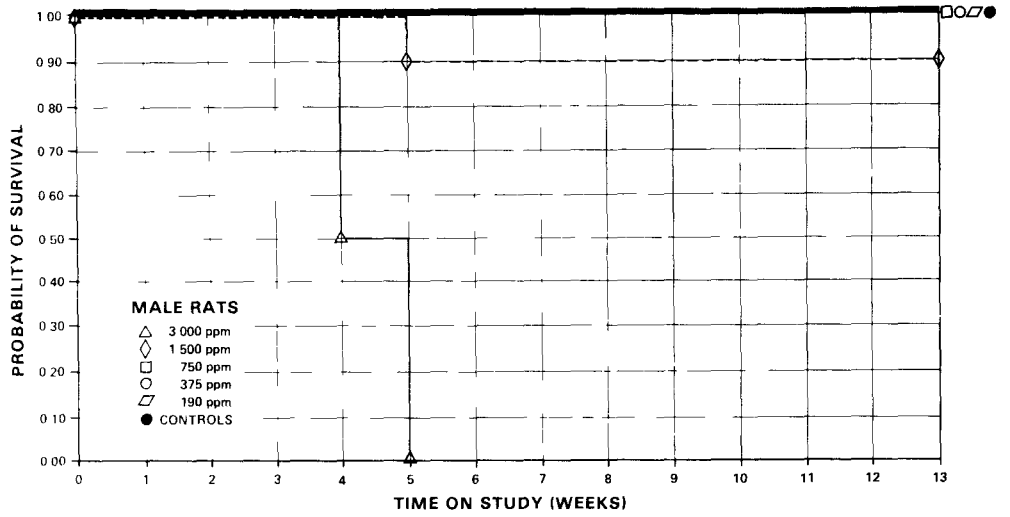


Figure 3. Growth Curves for Rats Administered Direct Brown 95 in the Diet

Concentrations of methemoglobin (Evelyn and Malloy, 1938) were measured because methemoglobin was elevated in selected rats at the higher dose concentration in the 2-week toxicity test. However, the dye concentrations administered in the 13-week studies were much lower, and determinations of methemoglobin in rats administered various doses of each of the dyes were not different from those of control rats.

C. Survival (Rats)

Curves of survival of control rats and of rats dosed with each of the test dyes are shown in figures 4, 5, and 6. All male and female rats administered 3,000 ppm of any one of the dyes and all male and female rats administered 1,500 ppm direct brown 95 dye died before the termination of the studies. One male administered 1,500 ppm direct blue 6 dye, six males administered 1,500 ppm direct black 38 dye, and two males administered 750 ppm direct brown 95 dye also died before the termination of the studies. No deaths occurred in any other dosed group or in any control group. The mortality was dose related, and, based on times and incidences of deaths, direct brown 95 dye was most toxic, followed in order by direct black 38 dye, then direct blue 6 dye.



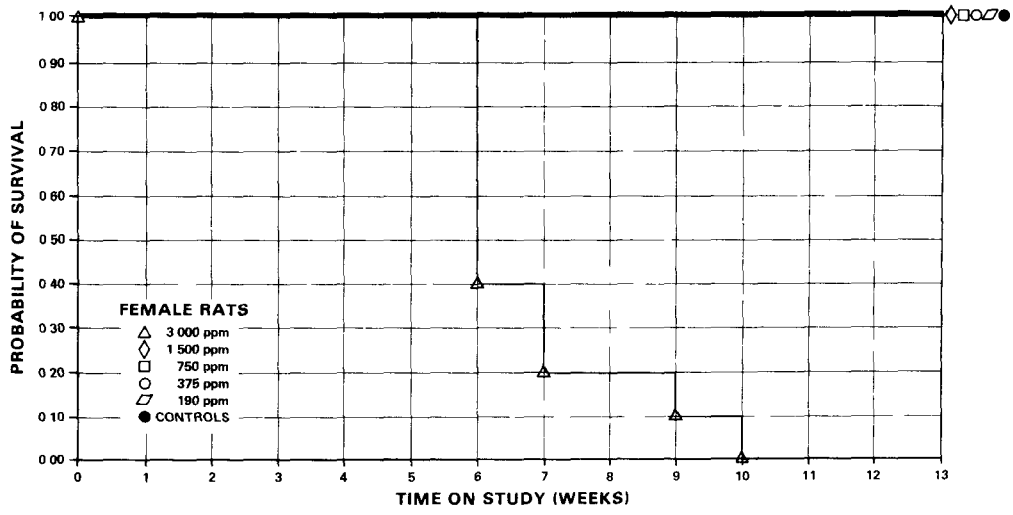
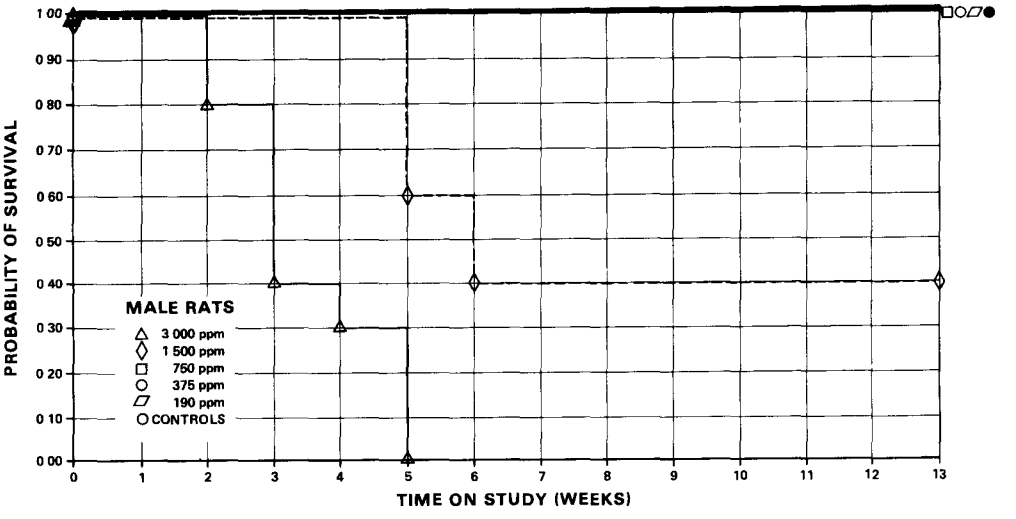


Figure 4. Survival Curves for Rats Administered Direct Blue 6 in the Diet



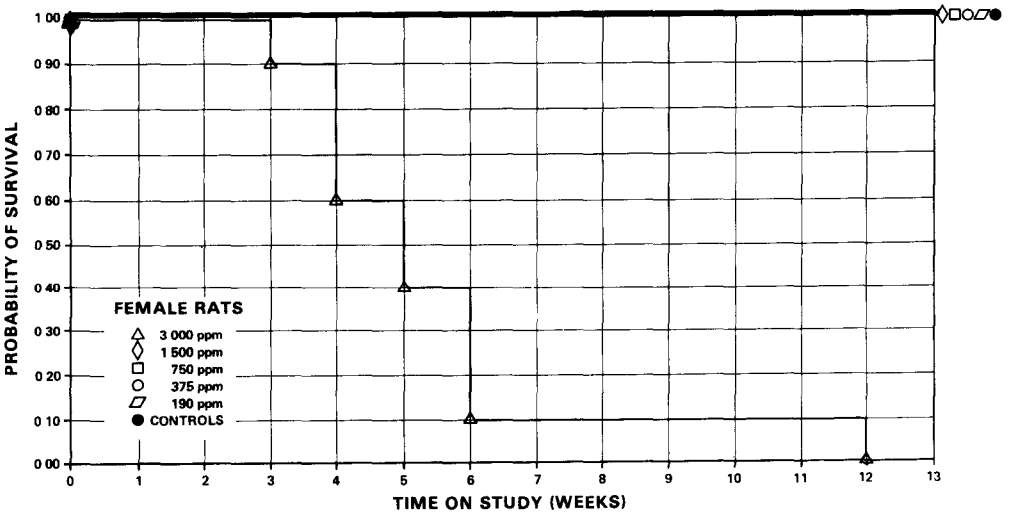
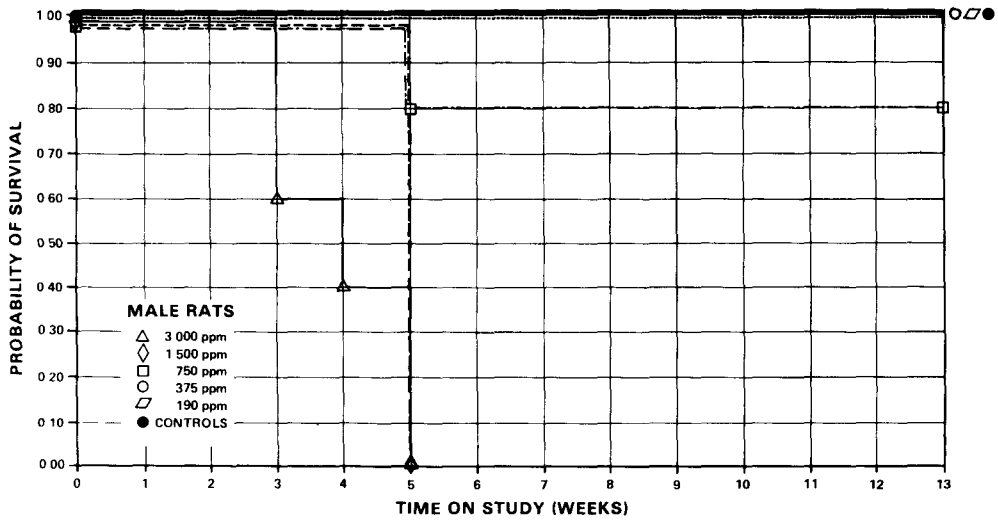


Figure 5. Survival Curves for Rats Administered Direct Black 38 in the Diet



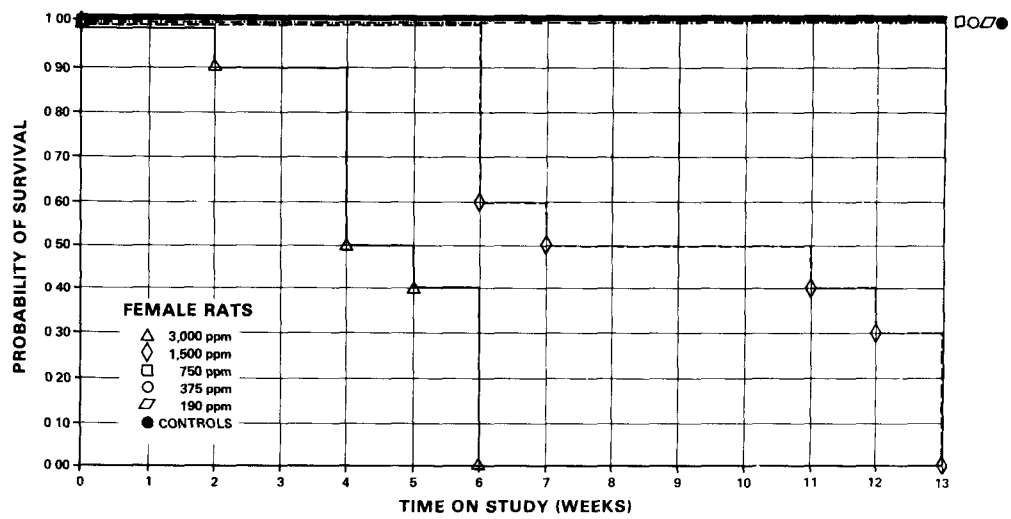


Figure 6. Survival Curves for Rats Administered Direct Brown 95 in the Diet

D. Pathology (Rats)

Gross Lesions. Gross lesions that were related to administration of any one of the dyes varied, depending on the dose and the length of time the animals survived. Livers of the rats given the highest doses and dying first (generally the males) were pale, yellow, or tan; these animals had ascites, hydrothorax, and edema of subcutaneous tissues and intestinal submucosa. Rats surviving longer but not to the end of the studies had, in addition, roughened surfaces on their livers. Rats given the highest doses at which survival was complete had livers with more severely roughened surfaces, due to random, multiple, pale, 2- to 3-mm spherical nodules that were scattered throughout the hepatic parenchyma and that elevated the capsule.

Histopathologic Lesions. Histopathologic lesions observed in control and dosed rats are summarized in Appendix A. Tables A1, A3, and A5 list those lesions that were observed only in rats administered dye in the diet; tables A2, A4, and A6 list other lesions. The histopathologic lesions varied, depending largely on the time of death. The terms "basophilic foci," "foci of cellular alteration," "neoplastic nodule," "hepatocellular carcinoma," and "cholangiofibrosis," applying to lesions of the liver, are used as defined by Squire and Levitt (1975). The term "basophilic foci" is used separately from the term "foci of

cellular alteration," because of the possible greater significance of the basophilic lesion. The term "nodular regeneration" refers to lobules containing hepatocytes that appear (1) to be normal in arrangement, size, shape, and tinctorial quality, but often lacking in central veins or portal areas or both, (2) to be pushing against adjacent areas, and (3) to be larger than normal lobules. They are delineated by focal biliary hyperplasia and fibrosis.

The first animals to die during administration of each of the dyes had varying degrees of biliary hyperplasia, lymphoid depletion of the spleen and the thymus, and myeloid depletion of the bone marrow. Animals that survived longer had more numerous proliferative changes of the liver, including biliary hyperplasia, cholangiofibrosis, nodular regeneration, foci of cellular alteration, neoplastic nodules, and hepatocellular carcinomas. Some of the rats had histopathologic evidence of bacterial septicemia just prior to death.

The most severely affected livers were usually of one of two types: (1) a liver with severe oval cell (biliary, cholangiolar) hyperplasia, multiple foci of cellular alteration, and nodules or (2) a liver with cirrhosis and nodules. In the first type (oval cell), hyperplasia started as a mild increase in periportal oval cells and progressed to large numbers of these cells,

proliferating along the sinusoids in such a fashion as to almost obscure the hepatocytes throughout the lobule. The initial proliferative hepatocellular lesions seen were multiple foci consisting of 10 to 20 or more cells that were larger and much more basophilic than normal hepatocytes. These cells also had larger, more vesicular nuclei, and some mitotic figures were seen. The basophilic foci appeared to progress to neoplastic nodules with basophilic hepatocytes, compressing adjacent parenchyma. The larger nodules with foci of prominent trabecular formation and acini were diagnosed as hepatocellular carcinomas. At least one carcinoma invaded the wall of a vein. None metastasized. The other foci of cellular alteration of hepatocytes included cells with clear cytoplasm, with cytoplasm containing eosinophilic droplets, or with cytoplasm having an eosinophilic, ground-glass appearance.

The second type of severely affected liver occurred in those rats that survived to the end of the studies after receiving the highest dose. These rats had cirrhosis (nodular regeneration and biliary hyperplasia, focal) characterized by multifocal, roughly spherical, nodular aggregations of hepatocytes; the hepatocytes generally appeared normal, although the lobules sometimes lacked central veins. Oval cells and connective tissue separated the regenerative nodules from each other. These livers contained

neoplastic nodules composed of large hepatocytes with eosinophilic cytoplasm. Hepatocellular carcinomas were diagnosed when neoplastic nodules contained foci of basophilic hepatocytes forming prominent trabeculae. Bizarre mitotic figures were occasionally noted. Cholangiofibrosis was commonly seen in livers of rats with neoplastic nodules and hepatocellular carcinomas. The severity of the proliferative changes of the liver decreased as the doses decreased; rats administered 190 or 375 ppm direct blue 6 dye and rats administered 190 ppm direct black 38 dye had essentially normal livers.

Lesions of the spleen, thymus, and bone marrow were characterized by a marked decrease in the number of mature lymphocytes in the white pulp of the spleen and in the cortex of the thymus and of the myeloid elements in the bone marrow.

Those rats receiving 1,500 ppm direct black 38 dye or 750 ppm direct brown 95 dye and surviving to the end of the studies had subacute glomerulonephropathy characterized by an eosinophilic amorphous material in Bowman's space and in the lumen of adjacent tubules. Some affected glomeruli had parietal epithelial cells in Bowman's capsule. There was some thickening of glomerular basement membranes. These kidney lesions were not observed in the rats administered direct blue 6 dye.

All females administered 375 ppm direct brown 95 dye had a degenerative change in pancreatic acinar epithelial cells. Individual cells had separated from the basement membrane and were rounded, with pyknotic nuclei.

Other lesions were considered incidental and not related to administration of the test dyes.

Based on the histopathologic examination, it was concluded that proliferative and neoplastic lesions were induced in the livers of Fischer 344 rats by each of the three test dyes administered for 13 weeks.

E. Statistical Analyses of Results (Rats)

Tables C1-C6 in Appendix C contain the statistical analyses of liver tumors which, along with other morphology concerning changes in liver cells, were observed in the studies of the three chemicals. Since each group for each chemical contained a maximum of 10 animals, the power of the Fisher exact test to determine significance of results is low; for example, with 0/10 incidence of a lesion in the controls, a significant result of $P \leq 0.05$ is not seen until the incidence of the lesion in a dosed group is over 4/10 (40%), at which incidence $P = 0.043$. The higher dosed groups developed neoplastic or nonneoplastic lesions that did not appear in the controls or in lower dose groups. In

some instances, mostly those involving nonneoplastic morphology, Fisher exact test results have P values lower than the 0.01 level required for an overall 0.05 significance level, taking into account the criterion for multiple comparisons of five dosed groups with a single control.

In male rats, liver tumors were observed in the 1,500 ppm- (8/10, 80%; $P < 0.001$) and the 3,000 ppm- (1/9, 11%; P is not significant) dose groups fed direct blue 6 as well as in the 1,500 ppm- (9/9, 100%; $P < 0.001$) dose group fed direct black 38 (see figures 7, 8, and 9). No incidence of these tumors appeared in any of the three control groups or in any dosed group fed direct brown 95. Foci of cellular alteration or basophilic foci were observed in significant incidences ($P < 0.01$) in the 750 ppm- and 1,500 ppm-dose groups fed direct blue 6, in the 375 ppm- and 750 ppm-dose groups fed direct black 38, and in the 375 ppm- and 1,500 ppm-dose groups fed direct brown 95, when compared with corresponding control groups. Incidences of these foci were also observed in the 3,000 ppm-dose group fed direct blue 6, the 1,500 ppm-dose group fed direct black 38, and in the 750 ppm- and 3,000 ppm-dose groups fed direct brown 95. These observed incidences are in contrast to the absence of such incidences in any of the control and 190 ppm-dose groups. In some of the higher dosed groups, occurrences of either neoplastic nodules or cell changes

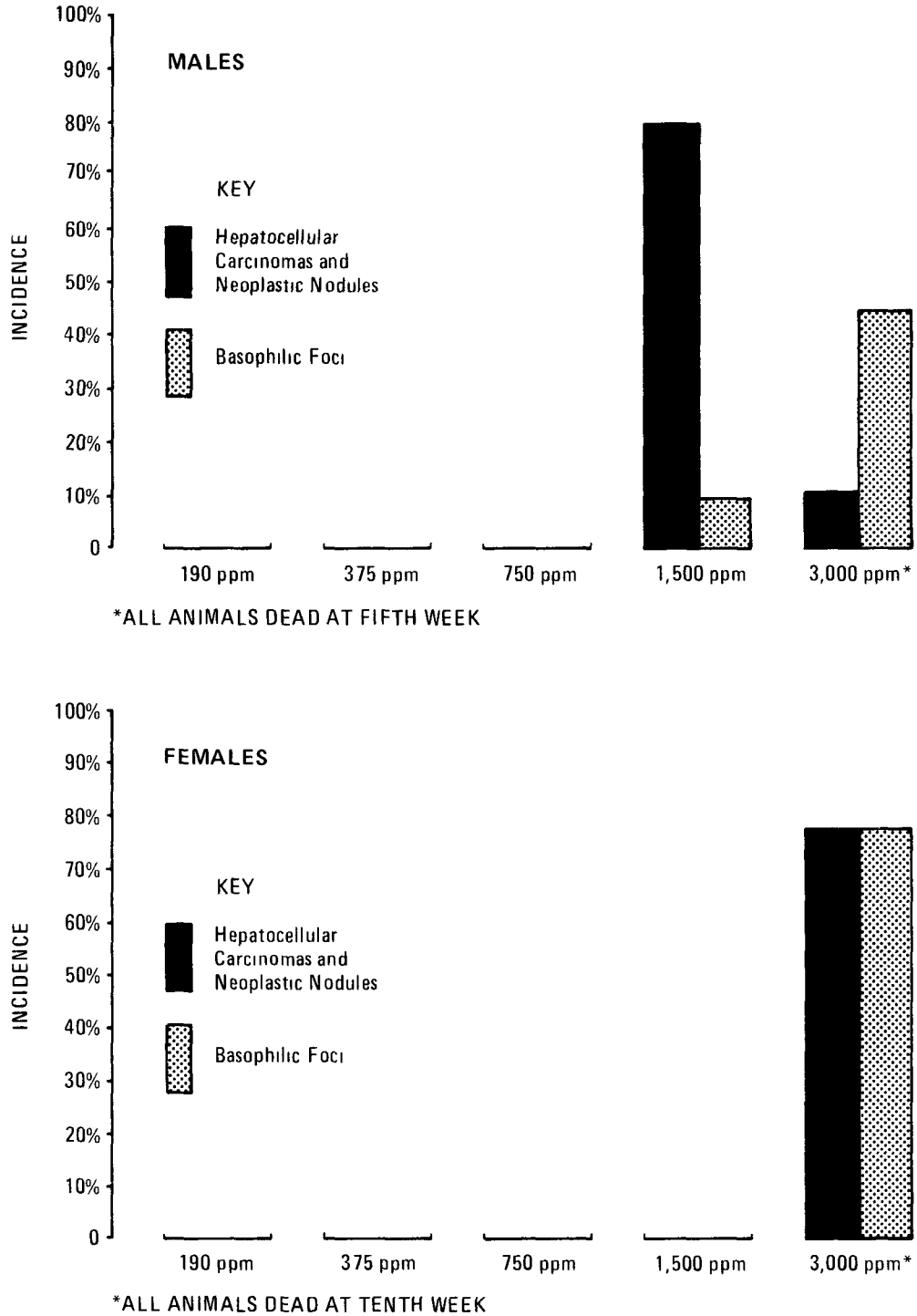


Figure 7. Hepatic Lesions Observed in Rats Administered Direct Blue 6 in the Diet

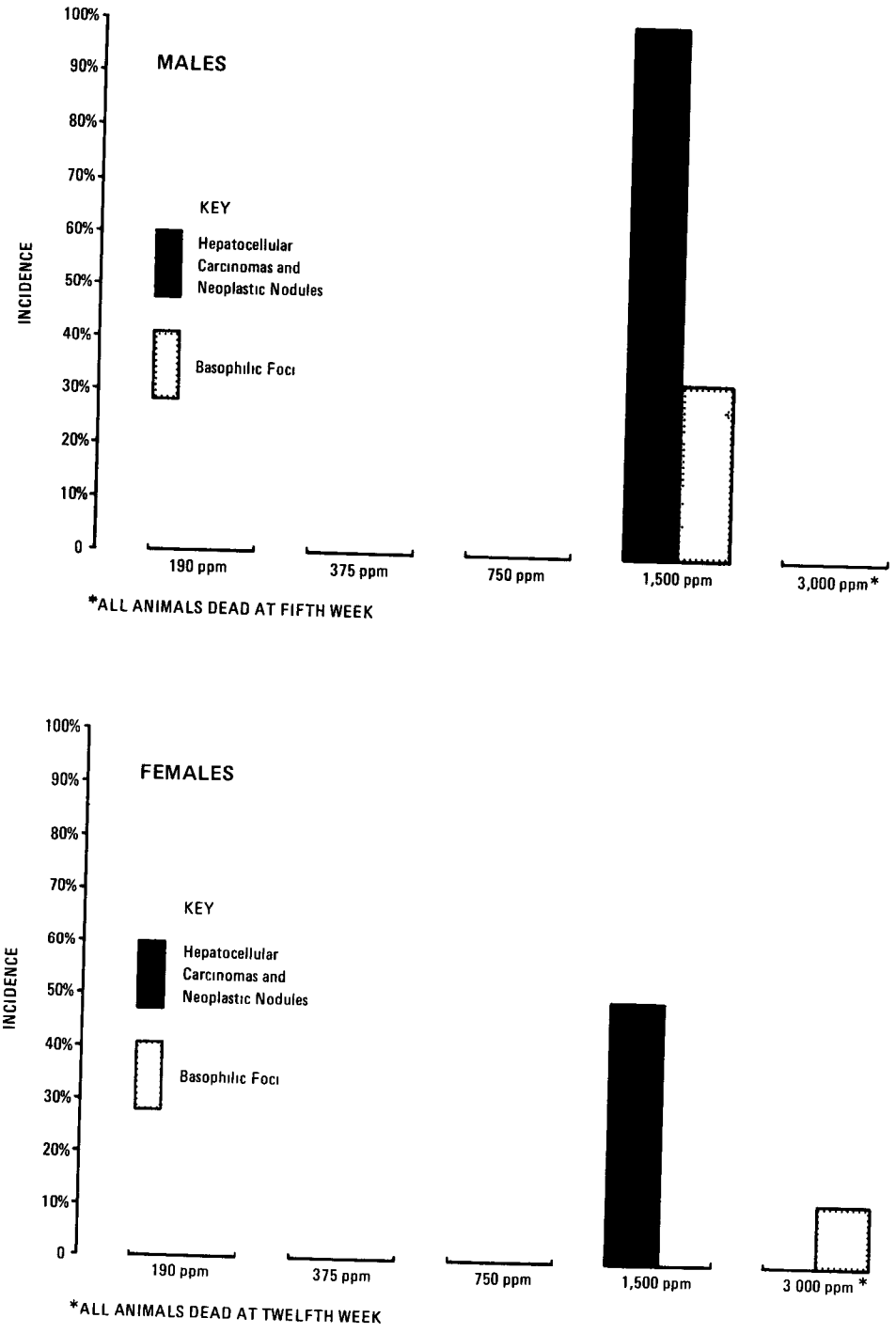


Figure 8. Hepatic Lesions Observed in Rats Administered Direct Black 38 in the Diet

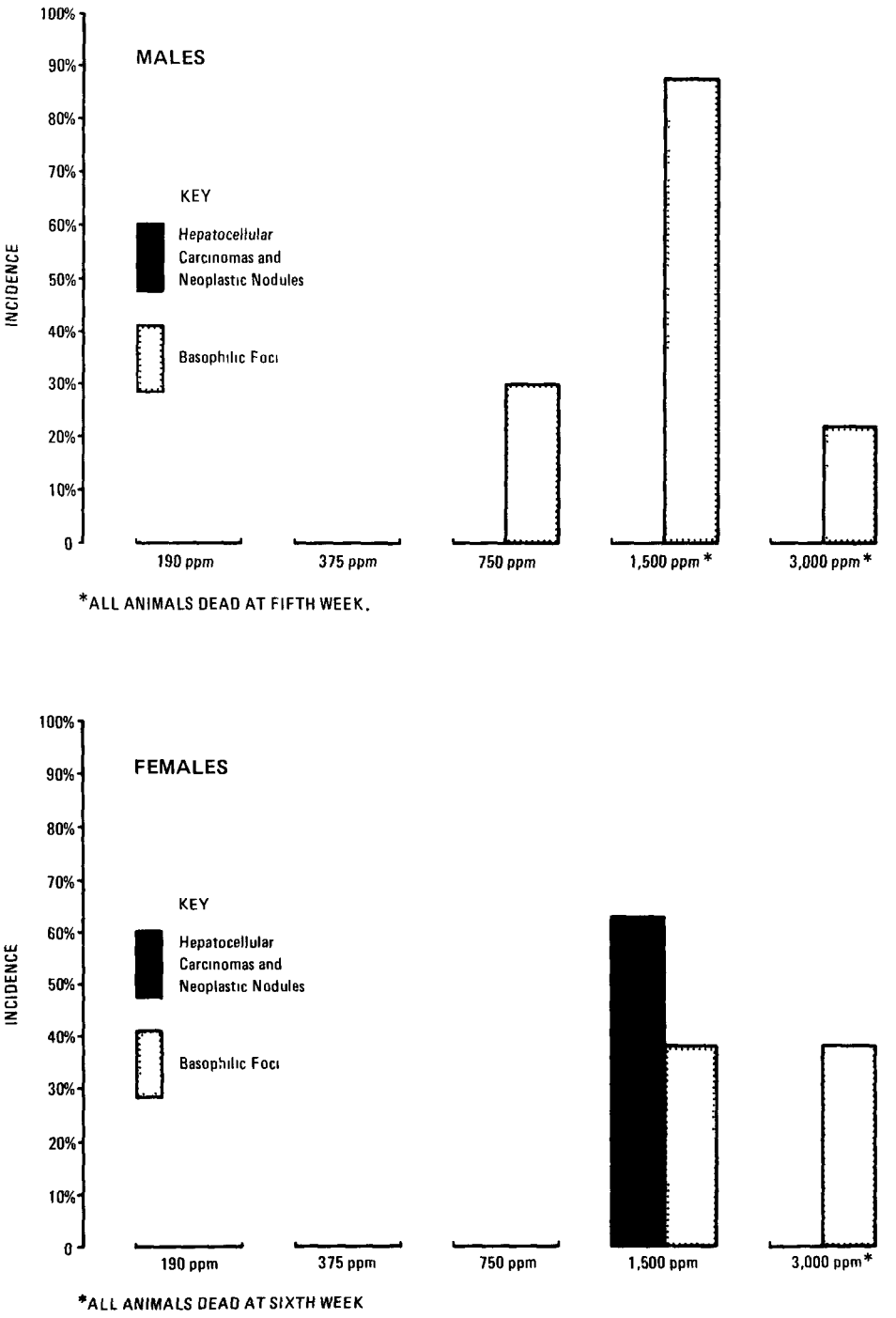


Figure 9. Hepatic Lesions Observed in Rats Administered Direct Brown 95 in the Diet

were observed as early as week 4 on study. The data on control male Fisher 344 rats, compiled to date from 2-year studies performed by all laboratories in the Carcinogenesis Testing Program, indicate an incidence of neoplastic nodules or hepatocellular adenomas or carcinomas of 32/1,806 (1.8%). There were no such tumors in the 220 male rats that died before week 78.

In female rats, liver tumors were observed in the 3,000 ppm-dose group (7/9, 77%; P = 0.001) fed direct blue 6, in the 1,500 ppm-dose group (5/10, 50%; P = 0.016) fed direct black 38, and in the 1,500 ppm-dose group (5/8, 63%; P = 0.007) fed direct brown 95, but in none of the controls or the three lower dosed groups of each study in the females. Foci of cellular alteration or basophilic foci occurred in significant incidences ($P < 0.01$) in the 750 ppm-, 1,500 ppm-, and 3,000 ppm-dose groups fed direct blue 6, in the 750 ppm- and 1,500 ppm-dose groups fed direct black 38, and in the 1,500 ppm-dose group fed direct brown 95. Some incidences of these foci were observed in the 375 ppm- and 3,000 ppm-dose groups fed direct black 38 and in the 375 ppm-, 750 ppm-, and 3,000 ppm-dose groups fed direct brown 95. Historical records from 2-year studies indicate that in control animals the incidence of neoplastic nodules or hepatocellular adenomas or carcinomas was 55/1,765 (3.1%). There were two such

tumors in the 182 female rats that died prior to week 78 on study.

In summary, the occurrence of lesions of the liver at statistically significant levels in dosed rats when compared with controls as well as the comparison of incidences of the lesions in these present 13-week subchronic toxicity studies with those in historical records indicate that the observed hepatocellular carcinomas, neoplastic nodules, and related proliferative lesions are associated with the administration of the test dyes.

IV. RESULTS - MICE

A. Body Weights and Clinical Signs (Mice)

Mean body weights of the male and female mice administered the highest dose of any one of the test dyes were slightly lower than mean body weights of the corresponding controls; mean body weights of mice administered lower doses were generally unaffected (figures 10, 11, and 12). No other clinical signs related to administration of the dyes were reported.

B. Benzidine Studies (Mice)

Urine collected over a 24-hour period during weeks 3 and 11 of the subchronic toxicity studies from male and female mice dosed with any one of the test dyes was found to contain benzidine and monoacetyl benzidine, while specimens of urine taken from corresponding control groups contained neither compound. The benzidine and monoacetyl benzidine were identified by thin-layer chromatography and mass spectroscopy. Quantities excreted in the urine were determined by combined extraction and spectrometric procedures. In most tests, the amounts excreted were dose related for each of the dyes administered. No consistent differences in results were found between males and females. Details of the methods and results are given in Appendix D.

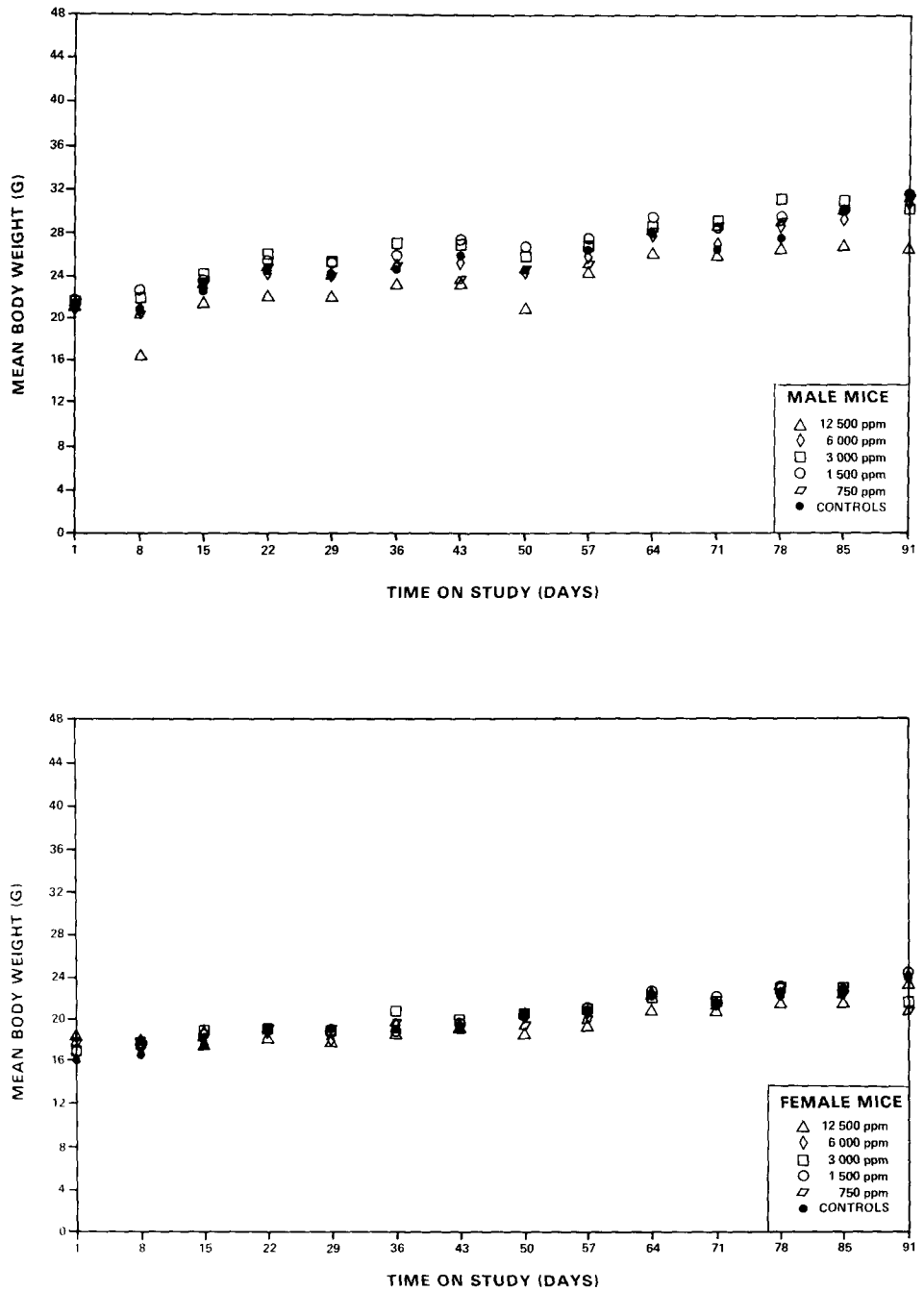


Figure 10. Growth Curves for Mice Administered Direct Blue 6 in the Diet

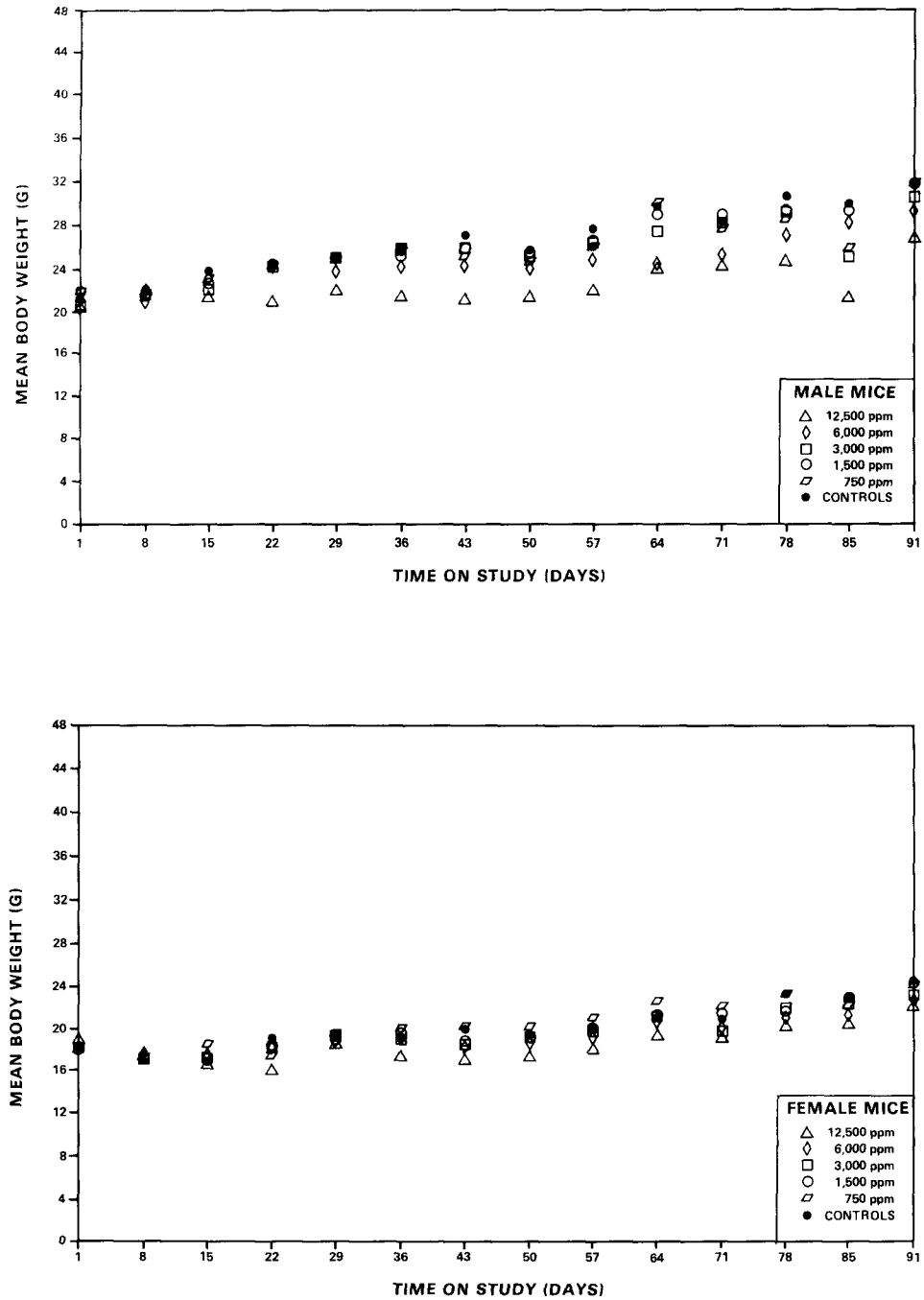


Figure 11. Growth Curves for Mice Administered Direct Black 38 in the Diet

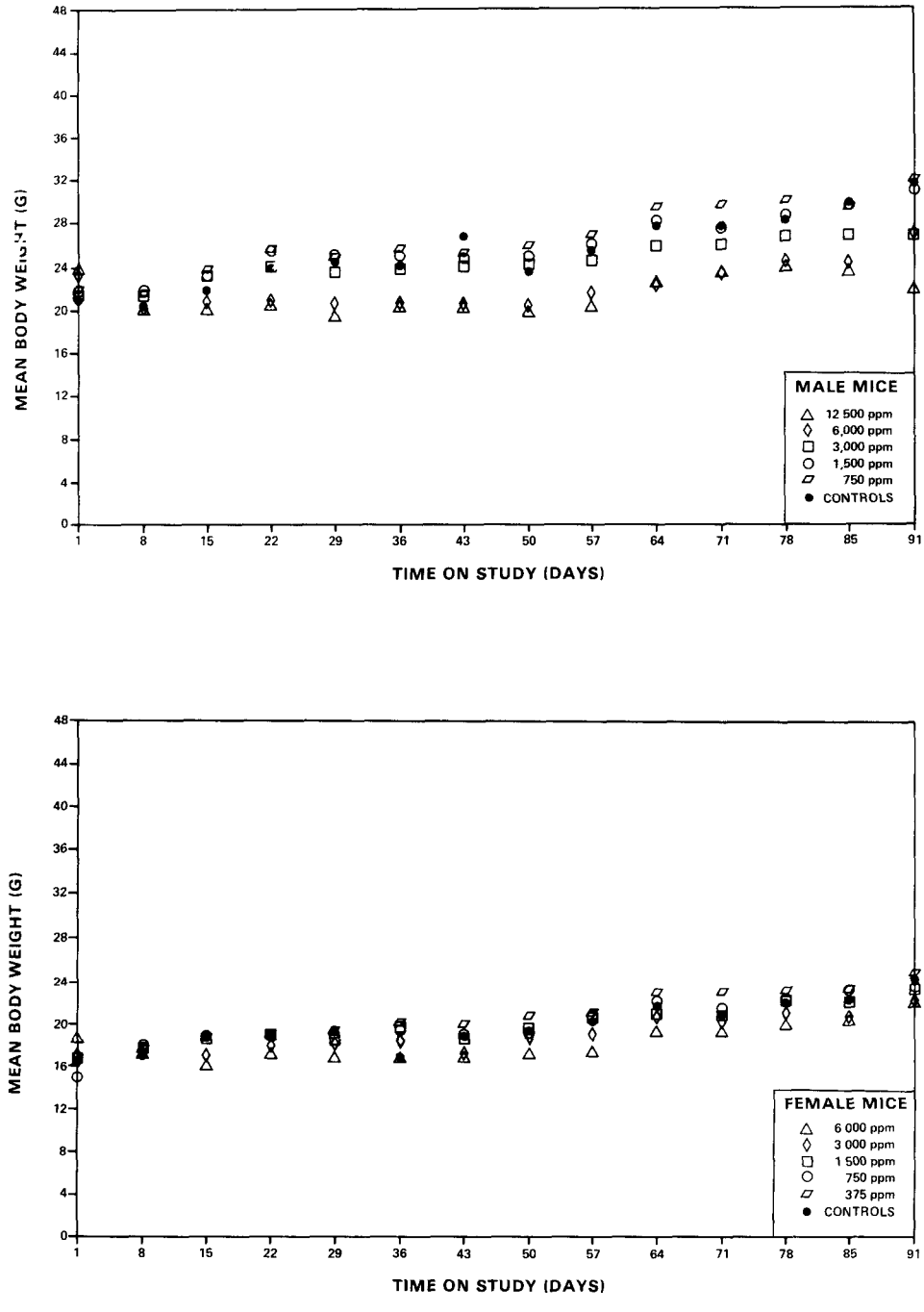


Figure 12. Growth Curves for Mice Administered Direct Brown 95 in the Diet

C. Survival (Mice)

All male and female mice administered any one of the doses of the dyes survived to the end of the subchronic toxicity studies, except for one male administered 750 ppm direct brown 95 dye. The death of this animal was attributed, however, to bacterial infection, and was not related to administration of the dye.

D. Pathology (Mice)

Gross Lesions. Gross lesions observed in mice administered direct blue 6 at doses of 6,000 or 12,500 ppm consisted of bluish-black and slightly enlarged spleens. Those observed in mice administered direct black 38 dye at 12,500 ppm consisted of slightly darkened livers and of enlarged and darkened spleens; those observed in the mice administered 6,000 ppm consisted of slightly enlarged and darkened spleens. Similar lesions were not reported in mice administered direct brown 95 dye.

Histopathologic Lesions. Histopathologic lesions observed in control and dosed mice are summarized in Appendix B. Tables B1, B3, and B5 list those lesions that were observed only in mice administered dye in the diet; tables B2, B4, B6, and B7 list other lesions. The findings observed in animals administered any one of the test dyes consisted mainly of hemosiderosis of the kidney and pigmentation of the liver at the highest doses and of

hemosiderosis of the spleen at low as well as high doses. The splenic hemosiderosis was characterized by an increase in the golden brown, iron-positive pigment that is normally seen in macrophages in the red pulp. The kidney hemosiderosis consisted of a finely granular, iron-positive pigment in epithelial cells of the proximal convoluted tubules; the pigment was difficult to detect without use of the Prussian-blue reaction. The hepatic pigment was yellow to yellowish-green and occasionally iron-positive; it occurred in occasional macrophages lining the sinusoids. Pigment of the thyroid, observed only in mice administered the two highest doses of direct black 38 dye, was finely granular, yellow, iron-negative, and located largely in the follicular cells.

Biliary hyperplasia was observed in the mice administered the highest doses of direct black 38 and direct brown 95 dyes, but not in mice administered direct blue 6 dye. The hyperplasia consisted of a modest increase in the number of biliary cells adjacent to most portal areas. Other hepatic lesions were observed in mice administered direct black 38 dye or direct brown 95 dye, but not in mice administered direct blue 6 dye. Hepatocellular degeneration, observed in 9/10-10/10 male and female mice administered 3,000, 6,000, or 12,500 ppm direct black 38 dye and in 10/10 male mice administered 12,500 ppm direct brown 95

dye, was characterized by pleomorphic nuclei, cytoplasmic vacuolization, eosinophilic droplet formation, and hydropic change, and, in the mice administered the direct black 38 dye, also an increased mitotic index. Three mice administered the highest dose of direct black 38 dye and one mouse administered the highest dose of direct brown 95 dye had foci of cellular alteration in their livers; in these instances, the hepatocytes were distinctly basophilic when compared with surrounding normal cells.

Based on the histopathologic examination, hemosiderosis of the spleen and kidney and pigmentation of the liver were the principal lesions occurring in B6C3F1 mice administered any one of the test dyes. The hepatocellular degeneration found in large numbers of mice given high doses of direct black 38 and direct brown 95 dyes also was related to administration of dye. No hepatic lesions occurred in mice administered direct blue 6 dye.

E. Statistical Analyses of Results (Mice)

No neoplasms occurred in the mice administered any one of the test dyes.

V. DISCUSSION

These subchronic toxicity studies of direct blue 6 dye, direct black 38 dye, and direct brown 95 dye were conducted as a part of the bioassay protocol for testing for possible carcinogenicity. Thirteen-week studies are conducted to establish respective doses of test chemicals to use in 2-year studies with both rats and mice.

In these feeding studies of the three dyes, mean body weights of the male and female rats administered the two or three highest concentrations of the test dyes were markedly lower than mean body weights of the corresponding controls throughout the studies, and the depressions in mean body weight were dose related. Mean body weights of the male and female mice administered the highest dose of any one of the test dyes were slightly lower than mean body weights of the corresponding controls; mean body weights of mice administered lower doses were generally unaffected.

All male and female rats administered 3,000 ppm of any one of the dyes or 1,500 ppm direct brown 95 dye died before the end of the studies. One male rat administered 1,500 ppm direct blue 6 dye, six males administered 1,500 ppm direct black 38 dye, and two males administered 750 ppm direct brown 95 dye also died by the

end of the subchronic toxicity studies. No deaths occurred in any other dosed group or in any control group of rats. Mortality in the rats was dose related, and based on times and incidences of deaths, direct brown 95 dye was most toxic, followed in order by direct black 38 dye, then direct blue 6 dye. All male and female mice administered the test dyes survived to the end of the studies, except for one male whose death was attributed to bacterial infection.

In rats, neoplastic lesions occurred only in dosed groups and consisted of hepatocellular carcinomas and neoplastic nodules of the liver. The time to onset of the tumors was remarkably short. The incidences of the hepatocellular carcinomas in female rats administered 3,000 ppm direct blue 6 dye (4/9) and male rats administered 1,500 ppm direct black 38 dye (4/9) were significant ($P = 0.033$) when related to the incidences of the tumors in the corresponding controls (0/10); hepatocellular carcinomas were also observed in two male rats administered 1,500 ppm direct blue 6 dye and in one female rat administered 1,500 ppm direct brown 95 dye. No control rats from any of the three studies developed hepatocellular carcinomas.

When incidences of neoplastic nodules were combined with those of hepatocellular carcinomas, the significance increased to $P < 0.001$ for male rats administered 1,500 ppm direct blue 6 dye, $P =$

0.001 for females administered 3,000 ppm direct blue 6 dye, $P < 0.001$ for males administered 1,500 ppm direct black 38 dye, and $P = 0.007$ for females administered 1,500 ppm direct brown 95 dye. No controls developed neoplastic nodules. Female rats administered direct black 38 dye developed no hepatocellular carcinomas, but had an incidence of neoplastic nodules of 5/10, with a significance of $P = 0.016$. Male rats administered direct brown 95 dye developed neither hepatocellular carcinomas nor neoplastic nodules, but as indicated below, had significant incidences of preneoplastic lesions. The failure of groups of rats administered 3,000 ppm dye to develop tumors when other groups administered 1,500 ppm did develop tumors may be due to earlier deaths at the higher dose.

Preneoplastic hepatic lesions (basophilic foci as described by Squire and Levitt, 1975) occurred only in dosed rats and did not occur in controls. The incidences of the basophilic foci were significant ($P \leq 0.033$) in male (4/9) and female (7/9) rats administered 3,000 ppm direct blue 6 dye and in male rats (7/8) administered 1,500 ppm direct brown 95 dye. Basophilic foci also occurred, at lower incidences, in males (1/10) administered 1,500 ppm direct blue 6 dye, in males (3/9) administered 1,500 ppm direct black 38 dye, in females (1/8) administered 3,000 ppm direct black 38 dye, in males administered 750 ppm (3/10) or

3,000 ppm (2/9) direct brown 95 dye, and in females administered 1,500 ppm (3/8) or 3,000 ppm (3/8) direct brown 95 dye. When incidences of foci of cellular alteration, a possible preneoplastic lesion, were added to those of basophilic foci, significance occurred in additional dosed groups.

In mice, no neoplastic lesions occurred in the liver or other tissues of groups administered the different dyes. The principal nonneoplastic lesions found in mice consisted of hemosiderosis of the kidney and pigmentation of the liver at doses of 6,000 or 12,500 ppm and of hemosiderosis of the spleen at low as well as high doses. Other nonneoplastic lesions in the dosed mice involved the liver. Both biliary hyperplasia and hepatocellular degeneration occurred in mice given high doses of direct black 38 dye or direct brown 95 dye. In addition, three mice administered 12,500 ppm direct black 38 dye and one mouse administered 12,500 ppm direct brown 95 dye had foci of cellular alteration, in which the cells were basophilic when compared with surrounding normal cells. No mice administered direct blue 6 dye had these lesions of the liver.

In previous work, Rinde and Troll (1975) reported that when azo dyes direct blue 6, direct black 38, direct brown 95, or an additional azo dye (direct red 28) were administered by gavage to rhesus monkeys, benzidine appeared in the urine in yields that

approximated those of animals administered equivalent amounts of free benzidine. In Wistar rats, the metabolic breakdown of benzidine-derived azo dyes to free benzidine has been demonstrated in incubation mixtures of such dyes with intestine (Miyakawa et al., 1973). In the present studies, benzidine and monoacetyl benzidine were detected in the urine of male and female rats and mice administered the test dyes, but neither compound was detected in the urine of control rats and mice.

The biliary (oval cell) lesions observed in Fischer 344 rats in the present studies have been previously reported to be induced in SHR, Wistar, Sprague-Dawley, and Buffalo rats by several chemicals that cause hepatocellular carcinoma (Ito et al., 1973) and by benzidine itself in Sherman rats (Spitz et al., 1950). The foci of cellular alteration, nodules, and carcinomas are identical to those caused by benzidine in Sherman rats (Spitz et al., 1950). In addition, the administration of benzidine in the diet of Wistar rats and hamsters has been reported to induce cholangiomas and hepatocytic tumors (Boyland et al., 1954; Saffiotti et al., 1967). Direct blue 6 dye and direct black 38 dye were reported not to induce tumors in female mice when the dyes were administered by implantation in the bladder in wax pellets (Niitsu, 1973); however, foci of alteration as well as hepatocellular carcinomas have been described in both male and

female mice given benzidine (Frith and Dooley, 1976). The failure of the dyes tested in the present studies to induce tumors in mice may have been due, however, to the short period of administration and/or observation. Papillomas and carcinomas of the bladder were found to develop in 3/7 dogs administered benzidine orally by capsule (Bonser et al., 1956; Spitz et al., 1950), although no control dogs were tested at the same time. Humans exposed to benzidine during its manufacture or industrial use have a significantly high incidence of cancer of the bladder (Case et al., 1954; Goldwater et al., 1965; Hueper, 1969; Mancuso and El-Attar, 1967; Scott, 1952; Uebelin and Pletscher, 1954).

The presence of benzidine in the urine of rats and mice and of liver lesions in rats and mice identical to those caused by benzidine alone suggests that the benzidine released from the metabolism of the dyes may be responsible for the liver lesions. The failure of male rats receiving direct brown 95 dye to develop hepatocellular carcinomas or neoplastic nodules may be due to the toxicity of the chemical, which resulted in deaths of all animals in the highest two dose groups by week 5 of the study. Two-year studies of these three dyes in mice were not conducted, since benzidine was detected in the urine of mice in these studies, and since there is evidence from prior studies that benzidine can produce hepatocellular carcinomas in mice (Frith and Dooley, 1976).

It is concluded that under the conditions of these 13-week subchronic toxicity studies, direct blue 6 and direct black 38 dyes were carcinogenic in male and female Fischer 344 rats and direct brown 95 was carcinogenic in female rats; all three dyes induced hepatocellular carcinomas and neoplastic nodules in the liver. The test dyes were not carcinogenic for B6C3F1 mice in the 13-week subchronic toxicity studies.

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APPENDIX A

LESIONS IN RATS ADMINISTERED DIRECT DYES IN THE DIET

Table A1. Lesions Observed Only in Rats Administered Direct Blue 6 in the Diet

Tissue/Lesion	190 ppm		375 ppm		750 ppm		1,500 ppm		3,000 ppm	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Liver										
Hepatocellular Carcinomas	0/10	0/10	0/10	0/10	0/10	0/10	2/10	0/10	0/9	4/9
Neoplastic nodules	0/10	0/10	0/10	0/10	0/10	0/10	6/10	0/10	1/9	3/9
Foci of cellular alteration	0/10	0/10	0/10	0/10	10/10	9/10	7/10	10/10	0/9	0/9
Basophilic foci	0/10	0/10	0/10	0/10	0/10	0/10	1/10	0/10	4/9	7/9
Biliary hyperplasia	0/10	0/10	0/10	0/10	4/10	0/10	10/10	8/10	9/10	9/9
Nodular regeneration	0/10	0/10	0/10	0/10	0/10	0/10	9/10	10/10	0/9	0/9
Hepatocellular degeneration	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	2/9	0/9
Cholangiofibrosis	0/10	0/10	0/10	0/10	0/10	0/10	8/10	0/10	0/9	0/9
Portal fibrosis	0/10	0/10	0/10	0/10	0/10	0/10	5/10	3/10	0/9	2/9

Table A1. Lesions Observed Only in Rats Administered Direct Blue 6 in the Diet

(continued)

Tissue/Lesion	190 ppm		375 ppm		750 ppm		1,500 ppm		3,000 ppm	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Spleen										
Lymphoid depletion	a	a	a	a	a	a	1/9	0/10	9/9	5/7
Lymphoid necrosis	a	a	a	a	a	a	0/9	0/10	1/9	0/8
Bacterial septicemia	a	a	a	a	a	a	0/9	0/10	1/9	0/8
Thymus										
Lymphoid depletion	a	a	a	a	a	a	1/8	0/4	6/7	2/2
Bone Marrow										
Myeloid depletion	a	a	a	a	a	a	1/10	0/10	10/10	8/9
Kidney										
Subactute glomerulo-nephritis	a	a	a	a	a	a	1/10	0/10	0/10	0/9
Bacterial septicemia	a	a	a	a	a	a	0/10	0/10	1/10	0/9

Table A1. Lesions Observed Only in Rats Administered Direct Blue 6 in the Diet

(continued)

Tissue/Lesion	190 ppm		375 ppm		750 ppm		1,500 ppm		3,000 ppm	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Pancreas										
Edema	a	a	a	a	a	a	0/8	0/9	0/9	1/4
Bacterial septicemia	a	a	a	a	a	a	0/8	0/9	0/9	1/4
Acinar cell necrosis	a	a	a	a	a	a	0/8	0/9	1/8	1/4
Acinar cell atrophy	a	a	a	a	a	a	1/10	0/9	0/8	1/4
Bladder										
Edema	a	a	a	a	a	a	0/8	0/10	1/9	0/9
Epithelial necrosis	a	a	a	a	a	a	0/8	0/10	0/9	1/7
Heart										
Bacterial septicemia	a	a	a	a	a	a	0/10	0/10	1/10	0/9

Table A1. Lesions Observed Only in Rats Administered Direct Blue 6 in the Diet

(continued)

Tissue/Lesion	190 ppm		375 ppm		750 ppm		1,500 ppm		3,000 ppm	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Lung										
Bacterial septicemia	a	a	a	a	a	a	0/10	0/10	1/10	2/9
Pulmonary edema	a	a	a	a	a	a	0/10	0/10	1/10	0/9
Salivary Gland										
Acinar cell necrosis	a	a	a	a	a	a	1/9	0/10	7/10	9/9
Acinar cell atrophy	a	a	a	a	a	a	0/9	0/10	6/10	0/9
Skin										
Subcutaneous edema	a	a	a	a	a	a	1/10	0/10	7/10	0/9
Lymph Nodes										
Lymphoid depletion	a	a	a	a	a	a	0/10	0/10	1/9	8/9
Large Intestinal Lymphoid Follicle										
Lymphoid necrosis	a	a	a	a	a	a	0/7	0/8	1/10	0/8

Table A1. Lesions Observed Only in Rats Administered Direct Blue 6 in the Diet

(continued)

Tissue/Lesion	190 ppm		375 ppm		750 ppm		1,500 ppm		3,000 ppm	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Testes										
Interstitial hemorrhage	a		a		a		1/10		6/9	
Large Intestine										
Edema	a	a	a	a	a	a	0/7	0/8	3/9	1/6
Colon										
Acute enteritis	a	a	a	a	a	a	0/7	0/8	1/10	0/8

[a]Not examined.

Table A2. Other Lesions in Rats Administered Direct Blue 6 in the Diet

Tissue	Lesion	Control Groups Male	Control Groups Female	Combined Dose Groups[a] Male	Combined Dose Groups[a] Female
Lung	Nodular lymphoid hyperplasia	9/10	10/10	9/20	9/19
	Lymphocytic perivasculitis	4/10	1/10	3/20	1/19
	Acute hemorrhage	0/10	0/10	3/20	2/19
	Acute congestion	0/10	0/10	1/20	0/19
Liver	Subacute hepatitis	1/10	1/10	0/49	2/49
	Focal granuloma	0/10	0/10	0/49	1/49
	Extramedullary hematopoiesis	0/10	0/10	3/49	2/49
Kidney	Tubular sclerosis, focal	1/10	0/10	0/20	0/18
	Tubular regeneration	8/10	0/10	4/20	0/18
	Interstitial nephritis	1/10	0/10	0/20	0/18
Heart	Subacute myocarditis	5/10	1/10	4/20	1/19
	Subendocardial hemorrhage	0/10	0/10	3/20	0/19
Adrenals	Acute congestion	0/10	0/10	2/10	0/18
	Acute hemorrhage	0/10	0/10	6/20	3/18
Tracheobronchial Lymph Nodes	Acute hemorrhage	0/10	0/10	3/20	0/19
Submandibular Lymph Node	Acute hemorrhage	0/10	0/10	1/20	1/19

Table A2. Other Lesions in Rats Administered Direct Blue 6 in the Diet

(continued)

Tissue	Lesion	Control Groups Male	Control Groups Female	Combined Dose Groups[a] Male	Combined Dose Groups[a] Female
Mesenteric Lymph Node	Acute hemorrhage	0/10	1/10	2/19	0/19
Mesenteric Fat	Necrosis	0/10	0/10	5/19	1/19
Salivary Gland	Lymphocytic sialoadenitis	0/10	0/10	1/20	0/19
Seminal Vesicle	Spermatic granuloma	1/10		0/20	
Testicle	Degeneration and mineralization	0/10		1/20	
Stomach	Acute hemorrhage	0/10	0/10	4/19	1/18
Colon	Nematodiasis	1/10	0/10	1/19	1/18

[a]For lesions of the liver, the combined group consisted of all dosed groups. In lesions of all other tissues, the combined dose groups consisted of the 1,500 ppm dose and 3,00 ppm dose groups.

Table A3. Lesions Observed Only in Rats Administered Direct Black 38 in the Diet

Tissue/Lesion	190 ppm		375 ppm		750 ppm		1,500 ppm		3,000 ppm	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Liver										
Hepatocellular carcinomas	0/10	0/10	0/10	0/9	0/10	0/10	4/9	0/10	0/9	0/8
Neoplastic nodules	0/10	0/10	0/10	0/9	0/10	0/10	5/9	5/10	0/9	0/8
Foci of cellular alteration	0/10	0/10	7/10	5/9	9/10	10/10	2/9	10/10	0/9	0/8
Basophilic foci	0/10	0/10	0/10	0/9	0/10	0/10	3/9	0/10	0/9	1/8
Biliary hyperplasia	1/10	0/10	0/10	0/9	9/10	6/10	9/9	9/10	9/9	7/8
Nodular regeneration	0/10	0/10	0/10	0/9	2/10	0/10	4/9	10/10	0/9	1/8
Cholangiofibrosis	0/10	0/10	0/10	0/9	1/10	0/10	3/9	0/10	0/9	0/8
Portal fibrosis	0/10	0/10	0/10	0/9	1/10	0/10	4/9	9/10	0/9	1/8
Bacterial septicemia	0/10	0/10	0/10	0/9	0/10	0/10	0/9	0/8	4/9	4/8
Fatty metamorphosis	0/10	0/10	0/10	0/9	0/10	0/10	0/9	0/8	0/9	1/8
Multifocal necrosis	0/10	0/10	0/10	0/9	0/10	0/10	0/9	0/8	1/9	4/8

Table A3. Lesions Observed Only in Rats Administered Direct Black 38 in the Diet

(continued)

Tissue/Lesion	190 ppm		375 ppm		750 ppm		1,500 ppm		3,000 ppm	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Spleen										
Lymphoid depletion	a	a	a	a	0/10	0/10	7/9	10/10	9/9	6/7
Bacterial septicemia	a	a	a	a	0/10	0/10	0/9	0/10	5/9	3/7
Acute congestion	a	a	a	a	0/10	0/10	0/9	0/10	1/9	1/7
Hemosiderosis	a	a	a	a	0/10	0/10	0/9	0/10	2/9	3/7
Thymus										
Lymphoid depletion	a	a	a	a	a	a	2/4	0/2	5/5	5/5
Lymphoid necrosis	a	a	a	a	a	a	1/4	0/2	0/5	0/5
Bone Marrow										
Myeloid depletion	a	a	a	a	0/10	0/10	3/9	7/10	9/9	8/8
Kidney										
Subactute glomerulo-nephritis	a	a	a	a	2/10	a	4/9	10/10	0/9	0/8

Table A3. Lesions Observed Only in Rats Administered Direct Black 38 in the Diet

(continued)

Tissue/Lesion	190 ppm		375 ppm		750 ppm		1,500 ppm		3,000 ppm	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Pancreas										
Necrosis of ancinar epithelium	a	a	a	a	1/10	0/9	0/9	0/8	1/8	0/8
Atrophy of acinar epithelium	a	a	a	a	0/10	0/9	4/9	2/8	0/9	0/8
Edema	a	a	a	a	0/10	0/9	2/9	0/8	1/8	0/8
Lung										
Acute pneumonia	a	a	a	a	a	a	0/8	0/10	1/9	0/8
Leucocytosis	a	a	a	a	a	a	1/9	0/10	1/9	0/8
Bacterial septicemia	a	a	a	a	a	a	0/8	0/10	0/9	1/8
Skin										
Subcutaneous edema	a	a	a	a	a	a	1/9	0/10	0/9	0/8

Table A3. Lesions Observed Only in Rats Administered Direct Black 38 in the Diet

(continued)

Tissue/Lesion	190 ppm		375 ppm		750 ppm		1,500 ppm		3,000 ppm	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Adrenals										
Congestion	a	a	a	a	a	a	0/9	0/10	5/9	5/8
Hemorrhage	a	a	a	a	a	a	2/9	0/10	6/9	0/8
Acute cortical necrosis	a	a	a	a	a	a	0/9	0/10	0/9	2/8
Salivary Gland										
Atrophy of acinar epithelium	a	a	a	a	0/10	0/10	1/7	2/9	7/9	5/7
Acinar epithelial necrosis	a	a	a	a	0/10	0/10	3/7	0/9	0/9	0/7
Lymph Nodes										
Lymphoid depletion	a	a	a	a	a	a	0/8	0/9	0/9	1/7
Acute hemorrhage	a	a	a	a	a	a	1/8	0/9	0/9	1/7
Mandibular lymph nodes	a	a	a	a	a	a	0/8	0/9	4/9	1/7

Table A3. Lesions Observed Only in Rats Administered Direct Black 38 in the Diet

(continued)

Tissue/Lesion	190 ppm		375 ppm		750 ppm		1,500 ppm		3,000 ppm	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Testes										
Seminiferous tubular degeneration	a		a		a		1/9		3/9	
Acute interstitial hemorrhage	a	a	a	a	a	a	1/9	a	1/9	a
Small Intestine										
Edema	a	a	a	a	a	a	1/9	0/10	0/9	0/8
Colon										
Submuscosal edema	a	a	a	a	a	a	0/9	0/10	1/6	0/8
Mesentery										
Fat necrosis	a	a	a	a	a	a	1/9	0/10	0/9	0/8

[a]Not examined.

Table A4. Other Lesions in Rats Administered Direct Black 38 in the Diet

Tissue	Lesion	Control Groups Male	Control Groups Female	Combined Dose Groups[a] Male	Combined Dose Groups[a] Female
Lung	Nodular lymphoid hyperplasia	8/10	9/10	0/17	0/18
	Lymphocytic perivasculitis	0/10	5/10	0/17	1/18
	Acute hemorrhage	0/10	1/10	1/17	0/18
	Proliferative interstitial pneumonia	0/10	0/10	0/27	1/18
Kidney	Focal subacute glomerulitis	1/10	0/10	0/28	0/28
	Tubular regeneration	8/10	0/10	4/28	2/28
	Focal interstitial nephritis	0/10	0/10	1/28	0/28
	Focal tubular degeneration	0/10	0/10	1/28	0/28
Liver	Subacute hepatitis	0/10	0/10	2/48	0/47
Heart	Subacute myocarditis	2/10	0/10	1/27	1/28
	Acute hemorrhage	0/10	0/10	0/27	2/28
Thyroid	Acute interstitial hemorrhage	0/10	0/10	0/17	1/18
	Thyroglossal duct cyst	0/10	0/10	1/17	0/18
Bladder	Acute submucosal hemorrhage	0/10	0/10	1/18	0/18
Stomach	Acute hemorrhage	0/10	0/10	2/18	0/18
Colon	Nematodiasis	1/10	0/10	0/18	0/18
Testes	Atrophy	1/10		1/18	

Table A4. Other Lesions in Rats Administered Direct Black 38 in the Diet

(continued)

Tissue	Lesion	Control Groups Male	Control Groups Female	Combined Dose Groups[a] Male	Combined Dose Groups[a] Female
Epididymis	Spermatic granuloma	0/10		1/18	
	Spermatic cyst	0/10		1/18	

[a]In lesions of the liver, the combined dose groups consisted of all dosed groups. For tissues of the kidney and heart, the combined dose groups consisted of the 750 ppm, 1,500 ppm, and 3,000 ppm dose groups. For lesions of the remaining tissues given in the table, the combined dose groups consisted of the 1,500 ppm and 3,000 ppm dose groups.

Table A5. Lesions Observed Only in Rats Administered Direct Brown 95 in the Diet

Tissue/Lesion	190 ppm		375 ppm		750 ppm		1,500 ppm		3,000 ppm	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Liver										
Hepatocellular carcinomas	0/10	0/10	0/10	0/10	0/10	0/10	0/9	1/8	0/9	0/8
Neoplastic nodules	0/10	0/10	0/10	0/10	0/10	0/10	0/8	4/8	0/9	0/8
Foci of cellular alteration	0/10	0/10	10/10	3/10	5/10	4/10	0/8	3/8	0/9	0/8
Basophilic foci	0/10	0/10	0/10	0/10	3/10	0/10	7/8	3/8	2/9	3/8
Biliary hyperplasia	0/10	0/10	2/10	0/10	5/10	0/10	8/9	8/8	9/9	8/8
Nodular regeneration	0/10	0/10	0/10	0/10	6/10	6/10	0/9	5/8	0/9	0/8
Portal fibrosis	0/10	0/10	0/10	0/10	8/10	4/10	0/9	5/8	0/9	0/8
Hepatic necrosis	0/10	0/10	0/10	0/10	0/10	0/10	0/9	0/8	3/9	0/8
Bacterial septicemia	0/10	0/10	0/10	0/10	0/10	0/10	0/9	0/8	0/9	3/10
Spleen										
Lymphoid depletion	0/10	0/10	0/10	0/10	5/10	5/10	6/8	7/8	5/8	5/8
Lymphoid necrosis	0/10	0/10	0/10	0/10	0/10	0/10	1/8	0/8	1/8	0/8
Bacterial septicemia	0/10	0/10	0/10	0/10	0/10	0/10	0/8	0/8	0/8	3/8

Table A5. Lesions Observed Only in Rats Administered Direct Brown 95 in the Diet

(continued)

Tissue/Lesion	190 ppm		375 ppm		750 ppm		1,500 ppm		3,000 ppm	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Thymus										
Lymphoid depletion	a	a	a	a	2/8	0/10	4/4	2/2	7/7	6/6
Bone Marrow										
Myeloid depletion	0/10	0/10	0/10	0/10	1/10	6/10	10/10	8/8	9/8	9/10
Kidney										
Subacute glomerulo-nephritis	0/10	0/10	0/10	0/10	8/10	10/10	9/10	2/8	0/9	0/10
Bacterial septicemia	0/10	0/10	0/10	0/10	0/10	0/10	6/10	0/8	1/9	0/10
Pancreas										
Degeneration of individual acinar epithelial cells	0/10	0/10	0/10	9/10	0/8	0/10	0/6	0/6	0/7	0/5
Acinar cell atrophy	0/10	0/10	0/10	0/10	4/8	3/10	0/6	3/6	0/7	0/5
Edema	0/10	0/10	0/10	0/10	1/8	0/10	0/6	2/6	0/7	0/5

Table A5. Lesions Observed Only in Rats Administered Direct Brown 95 in the Diet

(continued)

Tissue/Lesion	190 ppm		375 ppm		750 ppm		1,500 ppm		3,000 ppm	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Salivary Gland										
Acinar cell atrophy	a	a	a	a	0/9	0/10	2/10	3/7	5/5	9/10
Necrosis of acinar epithelium	a	a	a	a	1/9	0/10	5/10	3/7	1/5	1/10
Heart										
Bacterial septicemia	a	a	a	a	1/10	0/10	5/9	0/7	1/8	0/10
Testes										
Interstitial hemorrhage	a		a		1/10		2/9		3/9	
Degeneration of germinal epithelium	a		a		1/10		0/9		2/9	
Large Intestine										
Submucosal edema	a	a	a	a	1/10	0/9	a	1/5	1/7	1/7
Epithelial necrosis	a	a	a	a	0/10	0/9	2/7	0/5	0/9	0/7
Submucosal hemorrhage	a	a	a	a	0/10	0/9	2/7	0/5	0/9	0/7

Table A5. Lesions Observed Only in Rats Administered Direct Brown 95 in the Diet

(continued)

Tissue/Lesion	190 ppm		375 ppm		750 ppm		1,500 ppm		3,000 ppm	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Skin										
Subcutaneous edema	a	a	a	a	1/10	0/10	0/10	1/8	0/9	1/10
Lung										
Bacterial septicemia	a	a	a	a	0/10	0/10	5/8	0/7	1/8	0/9
Mandibular Lymph Nodes										
Bacterial septicemia	a	a	a	a	0/9	0/10	1/10	0/5	0/5	1/9
Lymphoid necrosis	a	a	a	a	1/9	0/10	0/10	0/5	0/5	2/9
Bronchial Lymph Nodes										
Bacterial septicemia	a	a	a	a	0/9	0/10	0/10	0/5	1/5	0/9
Mesenteric Lymph Nodes										
Lymphoid necrosis	a	a	a	a	0/9	0/10	0/10	0/5	0/5	1/9

Table A5. Lesions Observed Only in Rats Administered Direct Brown 95 in the Diet

(continued)

Tissue/Lesion	190 ppm		375 ppm		750 ppm		1,500 ppm		3,000 ppm	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Stomach										
Submucosal edema	a	a	a	a	0/10	0/9	0/8	2/7	0/9	0/9
Bladder										
Submucosal edema	a	a	a	a	0/6	0/6	0/9	0/7	1/9	0/8

[a]Not examined.

Table A6. Other Lesions in Rats Administered Direct Brown 95 in the Diet

Tissue	Lesion	Control Groups Male	Control Groups Female	Combined Dose Groups[a] Male	Combined Dose Groups[a] Female
Lung	Lymphoid hyperplasia, nodular	10/10	7/10	5/29	5/27
	Subacute focal pneumonia	2/10	0/10	0/29	0/27
	Acute focal hemorrhage	1/10	0/10	5/29	2/27
	Lymphocytic perivasculitis	4/10	0/10	0/29	0/27
	Congestion	0/10	0/10	0/29	3/27
Heart	Subacute to chronic myocarditis	1/10	0/10	3/29	0/27
	Subendocardial hemorrhage	0/10	0/10	5/29	2/27
	Myocardial degeneration and mineralization	0/10	0/10	1/29	1/27
Liver	Extramedullary hematopoiesis	0/10	0/10	1/49	3/46
	Subacute hepatitis	0/10	0/10	0/49	1/46
	Hemorrhage	0/10	0/10	1/49	0/46
Kidney	Tubular regeneration	8/10	1/10	13/49	9/48
	Focal tubular sclerosis	0/10	1/10	0/49	0/48
	Focal glomerulitis	0/10	0/10	0/49	2/48
	Tubular degeneration	0/10	0/10	0/49	1/48
Spleen	Hemosiderosis	1/10	0/10	0/59	1/57
	Extramedullary hematopoiesis	0/10	0/10	0/59	1/57
	Congestion	0/10	0/10	2/59	1/57

Table A6. Other Lesions in Rats Administered Direct Brown 95 in the Diet

(continued)

Tissue	Lesion	Control Groups Male	Control Groups Female	Combined Dose Groups[a] Male	Combined Dose Groups[a] Female
Brain	Neuronal necrosis and mineralization	0/10	1/10	0/29	0/28
	Hemorrhage	0/10	0/10	1/29	0/28
Thymus	Focal acute hemorrhage	0/10	1/10	0/29	0/24
Adrenals	Acute hemorrhage	0/10	0/10	7/29	5/28
	Acute cortical congestion	0/10	0/10	3/29	7/28
Bladder	Epithelial necrosis	0/10	0/10	0/29	2/28
	Hemorrhage	0/10	0/10	0/29	3/28
Mesentery	Fat necrosis	0/10	0/10	1/29	2/27
	Acute peritonitis	0/10	0/10	0/29	1/27
Mesenteric Lymph Node	Acute hemorrhage	0/10	0/10	1/29	0/27
	Lymphodenitis	0/10	0/10	0/29	1/27
Bronchial Lymph Node	Acute hemorrhage	0/10	0/10	5/29	1/28
Mandibular Lymph Node	Acute hemorrhage	0/10	0/10	6/29	3/28
Stomach	Epithelial necrosis	0/10	0/10	0/26	1/29
	Acute focal hemorrhage	0/10	0/10	4/26	1/29
Small Intestine	Congestion	0/10	0/10	1/29	0/26

Table A6. Other Lesions in Rats Administered Direct Brown 95 in the Diet

(continued)

Tissue	Lesion	Control Groups Male	Control Groups Female	Combined Dose Groups[a] Male	Combined Dose Groups[a] Female
Colon	Nematodiasis	1/10	1/10	0/29	0/26
Epididymis	Interstitial hemorrhage	0/10		1/29	

[a]For lesions of the liver, kidney, or spleen, the combined dose groups consisted of all dosed groups. For lesions of the other tissues given in the table, the combined dose groups consisted of 750 ppm, 1,300 ppm, and 3,000 ppm dose groups.

APPENDIX B

LESIONS IN MICE ADMINISTERED DIRECT DYES IN THE DIET

Table B1. Lesions Observed Only in Mice Administered Direct Blue 6 in the Diet

Tissue/Lesion	750 ppm		1,500 ppm		3,000 ppm		6,000 ppm		12,500 ppm	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Liver										
Pigment deposition	a	a	a	a	a	a	0/10	0/9	9/10	7/10
Spleen										
Hemosiderosis	a	a	3/10	1/10	10/10	10/10	10/10	10/10	10/10	10/10
Kidney										
Hemosiderosis	a	a	a	a	a	a	a	a	6/10	10/10

[a]Not examined.

Table B2. Other Lesions in Mice Administered Direct Blue 6 in the Diet

Tissue	Lesion	Control Groups Male	Control Groups Female	Combined Dose Groups[a] Male	Combined Dose Groups[a] Female
Liver	Focal dystrophic mineralization	1/10	0/10	0/20	0/19
	Subacute hepatitis	0/10	2/10	3/20	5/19
	Hepatic necrosis	0/10	0/10	1/20	0/19
	Extramedullary hematopoiesis	0/10	0/10	0/20	5/19
	Lymphocytic hepatitis	0/10	0/10	1/20	0/19
	Acute hepatitis	0/10	0/10	1/20	0/19
Pancreas	Focal pancreatic necrosis	0/10	0/10	1/10	0/10
Prostate	Interstitial prostatitis	0/10		1/10	
Stomach	Acute focal gastritis	0/10	0/10	0/10	1/10

[a]For lesions of the liver, the combined dose groups consisted of the 6,000 ppm and the 12,500 ppm dose groups. For lesions of the other tissues given in the table, only the 12,500 ppm dose groups were examined.

Table B3. Lesions Observed Only in Mice Administered Direct Black 38 in the Diet

Tissue/Lesion	750 ppm		1,500 ppm		3,000 ppm		6,000 ppm		12,500 ppm	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Liver										
Diffuse hepatocellular degeneration	a	a	0/10	0/10	9/10	10/10	10/10	9/10	10/10	10/10
Foci of cellular alteration	a	a	0/10	0/10	0/10	0/10	1/10	0/10	2/10	1/10
Biliary hyperplasia	a	a	0/10	0/10	0/10	0/10	0/10	0/10	9/10	10/10
Pigment deposition	a	a	0/10	0/10	1/10	1/10	10/10	10/10	10/10	10/10
Spleen										
Hemosiderosis	1/10	1/10	4/10	9/10	10/10	10/10	10/10	10/10	10/10	10/10
Kidney										
Hemosiderosis	a	a	a	a	0/10	0/10	10/10	10/10	10/10	10/10
Thyroid										
Pigment deposition	a	a	a	a	0/10	0/8	8/10	10/10	8/9	9/10

[a]Not examined.

Table B4. Other Lesions in Mice Administered Direct Black 38 in the Diet

Tissue	Lesion	Control Groups Male	Control Groups Female	Combined Dose Groups[a] Male	Combined Dose Groups[a] Female
Liver	Subacute hepatitis	1/10	2/10	5/40	12/40
	Focal hepatocellular necrosis	0/10	0/10	0/40	1/40
	Acute hepatitis	0/10	0/10	1/40	4/40
	Focal granuloma	0/10	0/10	0/40	1/40
Stomach	Acute gastritia	0/10	0/10	3/10	0/10
Cerebellum	Focal lipodystrophy	0/10	0/10	0/10	1/10
Lung	Lymphoid hyperplasia	1/10	0/10	0/10	0/10
Kidney	Tubular regeneration	1/10	0/10	1/30	0/30
	Amyloidosis, focal	0/10	0/10	0/30	1/30
	Lymphocytic interstitial nephritis	0/10	0/10	2/30	0/30

[a]For lesions of the liver, the combined dose groups consisted of the 1,500 ppm, 3,000 ppm, 6,000 ppm, and 12,500 ppm dose groups. For lesions of the kidney, the combined dose groups consisted of the 3,000 ppm, 6,000 ppm, and 12,500 ppm dose groups. For lesions of the remaining tissues given in the table, only the 12,500 ppm dose groups were examined.

Table B5. Lesions Observed Only in Male Mice Administered Direct Brown 95 in the Diet

Tissue/Lesion	750 ppm	1,500 ppm	3,000 ppm	6,000 ppm	12,500 ppm
Liver					
Pigment deposition	0/1	a	1/10	10/10	10/10
Biliary hyperplasia	0/1	a	0/10	0/10	3/10
Hepatocellular degeneration	0/1	a	0/10	0/10	10/10
Foci of cellular alteration	0/1	a	0/10	0/10	1/10
Spleen					
Hemosiderosis	7/10	10/10	10/10	10/10	10/10
Kidney					
Hemosiderosis	0/1	a	0/10	10/10	10/10

[a]Not examined.

Table B6. Lesions Observed Only in Female Mice Administered Direct Brown 95 in the Diet

Tissue/Lesion	375 ppm	750 ppm	1,500 ppm	3,000 ppm	6,000 ppm
Liver					
Pigment deposition	a	a	0/10	10/10	7/10
Biliary hyperplasia	a	a	0/10	0/10	0/10
Hepatocellular degeneration	a	a	0/10	0/10	0/10
Foci of cellular alteration	a	a	0/10	0/10	0/10
Spleen					
Hemosiderosis	5/9	10/10	10/10	10/10	10/10
Kidney					
Hemosiderosis	a	a	0/10	0/10	9/10

[a]Not examined.

Table B7. Other Lesions in Mice Administered Direct Brown 95 in the Diet

Tissue	Lesion	Control Groups Male	Control Groups Female	Combined Dose Groups[a] Male	Combined Dose Groups[a] Female
Liver	Subacute hepatitis	0/10	1/10	2/31	13/30
	Acute hepatitis	0/10	0/10	2/31	1/30
	Extramedullary hematopoiesis	0/10	0/10	1/31	1/30
	Hepatocellular necrosis	0/10	0/10	1/31	1/30
	Hepatocellular cytoplasmic vacuolization	0/10	0/10	1/31	1/30
	Focal granuloma	0/10	0/10	1/31	1/30
Heart	Myocardial degeneration	0/10	0/10	1/11	0/10
Thymus	Lymphoid depletion	0/10	0/10	1/11	0/10
Salivary Gland	Multiple abscesses	0/10	0/10	1/11	0/10
	Lymphocytic sialoadenitis	0/10	0/10	0/11	1/10
Bone Marrow	Granulocytic hyperplasia	0/10	0/10	1/11	0/10
Submandibular Lymph Node	Acute hemorrhage	0/10	0/10	0/11	1/10
Kidney	Lymphocytic interstitial nephritis	0/10	0/10	1/31	0/30
Parathyroid	Cyst	0/10	0/10	0/11	0/10

Table B7. Other Lesions in Mice Administered Direct Brown 95 in the Diet

(continued)

Tissue	Lesion	Control Groups Male	Control Groups Female	Combined Dose Groups[a] Male	Combined Dose Groups[a] Female
Testes	Degeneration of germinal epithelium	0/10		1/11	
Pancreas	Lymphocytic pancreatitis	0/10	0/10	2/11	0/10

[a]For lesions of the liver or kidney, the combined dose groups consisted of the 3,000 ppm, 6,000 ppm, and 12,500 ppm dose groups of the males and the 1,500 ppm, 3,000 ppm, and 6,000 ppm dose groups of the females. For lesions of other tissues given in the table, only the 12,500 ppm dose group of males and the 6,000 ppm dose group of females were exmained.

APPENDIX C

ANALYSES OF THE INCIDENCES OF TUMORS OR FOCI ALTERATIONS IN THE LIVER OF RATS FED DIRECT DYES IN THE DIET

Table C1. Analyses of the Incidence of Tumors or Foci Alterations in the Liver of Male Rats Fed Direct Blue 6 in the Diet[a]

Compound	Matched Control	190 ppm Dose	375 ppm Dose	750 ppm Dose	1,500 ppm Dose	3,000 ppm Dose
Liver; Hepatocellular Carcinoma[b]	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	2/10 (20)	0/9 (0)
P Values[c]		--	--	--	N.S.	--
Relative Risk[d]					Infinite	
Lower Limit					0.330	
Upper Limit					Infinite	
Weeks to First Observed Tumor	--	--	--	--	4	--
Liver: Hepatocellular Carcinoma or Neoplastic Nodule[b]	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	8/10 (80)	1/9 (11)
P Values[c]		--	--	--	P < 0.001	N.S.
Relative Risk[d]					Infinite	Infinite
Lower Limit					2.747	0.064
Upper Limit					Infinite	Infinite
Weeks to First Observed Tumor		--	--	--	4	4

Table C1. Analyses of the Incidence of Tumors or Foci Alterations in the Liver of Male Rats Fed Direct Blue 6 in the Diet[a]

(continued)

Compound	Matched Control	190 ppm Dose	375 ppm Dose	750 ppm Dose	1,500 ppm Dose	3,000 ppm Dose
Liver: Basophilic Foci[b]	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (10)	1/10 (10)[e]	4/9 (44)
P Values[c]		--	--	--	N.S.	P = 0.033
Realtive Risk[d]					Infinite	Infinite
Lower Limit					0.058	1.183
Upper Limit					Infinite	Infinite
Weeks to First Observed Lesion	--	--	--	--	4	4
Liver: Foci of Cellular Alteration or Basophilic Foci[b]	0/10 (0)	0/10 (0)	0/10 (0)	10/10 (100)	8/10 (80)[f]	4/9 (44)
P Values[c]		--	--	P < 0.001	P < 0.001	P = 0.033
Relative Risk[d]				Infinite	Infinite	Infinite
Lower Limit				3.968	2.747	1.183
Upper Limit				Infinite	Infinite	Infinite
Weeks to First Observed Lesion				13	4	4

[a]Dosed groups received 190, 375, 750, 1,500, or 3,000 ppm.

[b]Number of lesion-bearing animals/number of animals examined at site (percent).

Table C1. Analyses of the Incidence of Tumors or Foci Alterations in the Liver of Male Rats Fed Direct Blue 6 in the Diet[a]

(continued)

[c]Beneath the incidence of lesions in a dosed group is the probability level for the Fisher exact test for the comparison of that dosed group with the matched-control group when $P < 0.05$; otherwise, not significant (N.S.) is indicated.

[d]The 95% confidence interval of the relative risk between each dosed group and the control group.

[e]This animal was also reported to have a liver tumor.

[f]Six of these animals were also reported to have liver tumors.

Table C2. Analyses of the Incidence of Tumors or Foci Alterations in the Liver of Female Rats Fed Direct Blue 6 in the Diet[a]

Compound	Matched Control	190 ppm Dose	375 ppm Dose	750 ppm Dose	1,500 ppm Dose	3,000 ppm Dose
Liver: Hepatocellular Carcinoma[b]	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	4/9 (44)
P Values[c]		--	--	--	--	P = 0.033
Relative Risk[d]						Infinite
Lower Limit						1.183
Upper Limit						Infinite
Weeks to First Observed Tumor	--	--	--	--	--	5
Liver: Hepatocellular Carcinoma or Neoplastic Nodule[b]	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	7/9 (77)
P Values[c]		--	--	--	--	P = 0.001
Relative Risk[d]						Infinite
Lower Limit						2.590
Upper Limit						Infinite
Weeks to First Observed Tumor	--	--	--	--	--	5

Table C2. Analyses of the Incidence of Tumors or Foci Alterations in the Liver of Female Rats Fed Direct Blue 6 in the Diet[a]

(continued)

Compound	Matched Control	190 ppm Dose	375 ppm Dose	750 ppm Dose	1,500 ppm Dose	3,000 ppm Dose
Liver: Basophilic Foci[b]	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	7/9 (77)[e]
P Values[c]		--	--	--	--	P = 0.001
Relative Risk[d]						Infinite
Lower Limit						2.590
Upper Limit						Infinite
Weeks to First Observed Lesion	--	--	--	--	--	5
Liver: Foci of Cellular Alteration or Basophilic Foci[b]	0/10 (0)	0/10 (0)	0/10 (0)	9/10 (90)	10/10 (100)	7/9 (77)[e]
P Values[c]		--	--	P < 0.001	P < 0.001	P = 0.001
Relative Risk[d]				Infinite	Infinite	Infinite
Lower Limit				3.265	3.968	2.590
Upper Limit				Infinite	Infinite	Infinite
Weeks to First Observed Lesion	--	--	--	13	13	5

[a]Dosed groups received 190, 375, 750, 1,500, or 3,000 ppm.

[b]Number of lesion-bearing animals/number of animals examined at site (percent).

Table C2. Analyses of the Incidence of Tumors or Foci Alterations in the Liver of Female Rats Fed Direct Blue 6 in the Diet[a]

(continued)

[c]Beneath the incidence of lesions in a dosed group is the probability level for the Fisher exact test for the comparison of that dosed group with the matched-control group when $P < 0.05$; otherwise, not significant (N.S.) is indicated.

[d]The 95% confidence interval of the relative risk between each dosed group and the control group.

[e]Five of these animals were also reported to have liver tumors.

Table C3. Analyses of the Incidence of Tumors or Foci Alterations in the Liver of Male Rats Fed Direct Black 38 in the Diet[a]

Compound	Matched Control	190 ppm Dose	375 ppm Dose	750 ppm Dose	1,500 ppm Dose	3,000 ppm Dose
Liver: Hepatocellular Carcinoma[b]	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	4/9 (44)	0/9 (0)
P Values[c]		--	--	--	P = 0.033	--
Relative Risk[d]					Infinite	
Lower Limit					1.183	
Upper Limit					Infinite	
Weeks to First Observed Tumor	--	--	--	--	5	--
Liver: Hepatocellular Carcinoma or Neoplastic Nodule[b]	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	9/9 (100)	0/9 (0)
P Values[c]		--	--	--	P < 0.001	--
Relative Risk[d]					Infinite	
Lower Limit					3.895	
Upper Limit					Infinite	
Weeks to First Observed Tumor	--	--	--	--	4	--

Table C3. Analyses of the Incidence of Tumors or Foci Alterations in the Liver of Male Rats Fed Direct Black 38 in the Diet[a]

(continued)

Compound	Matched Control	190 ppm Dose	375 ppm Dose	750 ppm Dose	1,500 ppm Dose	3,000 ppm Dose
Liver: Basophilic Foci[b]	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	3/9 (33)[e]	0/9 (0)
P Values[c]	--	--	--	--	N.S.	--
Relative Risk[d]					Infinite	
Lower Limit					0.759	
Upper Limit					Infinite	
Weeks to First Observed Lesion	--	--	--	--	5	--
Liver: Foci of Cellular Alteration or Basophilic Foci[b]	0/10 (0)	0/10 (0)	7/10 (70)	9/10 (90)	5/9 (55)[e]	0/9 (0)
P Values[c]		--	P = 0.002	P < 0.001	P = 0.011	--
Relative Risk[d]			Infinite	Infinite	Infinite	
Lower Limit			2.291	3.265	1.628	
Upper Limit			Infinite	Infinite	Infinite	
Weeks to First Observed Lesion	--	--	13	13	5	--

[a]Dosed groups received 190, 375, 750, 1,500, or 3,000 ppm.

[b]Number of lesion-bearing animals/number of animals examined at site (percent).

Table C3. Analyses of the Incidence of Tumors or Foci Alterations in the Liver of Male Rats Fed Direct Black 38 in the Diet[a]

(continued)

[c]Beneath the incidence of lesions in a dosed group is the probability level for the Fisher exact test for the comparison of that dosed group with the matched-control group when $P < 0.05$; otherwise, not significant (N.S.) is indicated.

[d]The 95% confidence interval of the relative risk between each dosed group and the control group.

[e]All of these animals were also reported to have liver tumors.

Table C4. Analyses of the Incidence of Tumors or Foci Alterations in the Liver of Female Rats Fed Direct Black 38 in the Diet[a]

Compound	Matched Control	190 ppm Dose	375 ppm Dose	750 ppm Dose	1,500 ppm Dose	3,000 ppm Dose
Liver: Neoplastic Nodule[b]	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	5/10 (50)	0/8 (0)
P Values[c]		--	--	--	P = 0.016	--
Relative Risk[d]					Infinite	
Lower Limit					1.454	
Upper Limit					Infinite	
Weeks to First Observed Tumor	--	--	--	--	13	--
Liver: Basophilic Foci[b]	0/10 (0)	0/10 (0)	0/9 (0)	0/10 (0)	0/10 (0)	1/8 (13)
P Values[c]	--	--	--	--	--	N.S.
Relative Risk[d]						Infinite
Lower Limit						0.072
Upper Limit						Infinite
Weeks to First Observed Lesion	--	--	--	--	--	5

Table C4. Analyses of the Incidence of Tumors or Foci Alterations in the Liver of Female Rats Fed Direct Black 38 in the Diet[a]

(continued)

Compound	Matched Control	190 ppm Dose	375 ppm Dose	750 ppm Dose	1,500 ppm Dose	3,000 ppm Dose
Liver: Foci of Cellular Alteration or Basophilic Foci[b]	0/10 (0)	0/10 (0)	5/9 (56)	10/10 (100)	10/10 (100)	1/8 (13)
P Values[c]		--	P = 0.011	P < 0.001	P < 0.001	N.S.
Relative Risk[d]			Infinite	Infinite	Infinite	Infinite
Lower Limit			1.628	3.968	3.968	0.072
Upper Limit			Infinite	Infinite	Infinite	Infinite
Weeks to First Observed Lesion		--	13	13	13	5

[a]Dosed groups received 190, 375, 750, 1,500, or 3,000 ppm.

[b]Number of lesion-bearing animals/number of animals examined at site (percent).

[c]Beneath the incidence of lesions in a dosed group is the probability level for the Fisher exact test for the comparison of that dosed group with the matched-control group when P < 0.05; otherwise, not significant (N.S.) is indicated.

[d]The 95% confidence interval of the relative risk between each dosed group and the control group.

Table C5. Analyses of the Incidence of Tumors or Foci Alterations in the Liver of Male Rats Fed Direct Brown 95 in the Diet[a]

Compound	Matched Control	190 ppm Dose	375 ppm Dose	750 ppm Dose	1,500 ppm Dose	3,000 ppm Dose
Liver: Basophilic Foci[b]	0/10 (0)	0/10 (0)	0/10 (0)	3/10 (30)	7/8 (88)	2/9 (22)
P Values[c]	--	--	--	N.S.	P < 0.001	N.S.
Relative Risk[d]				Infinite	Infinite	Infinite
Lower Limit				0.681	3.007	0.368
Upper Limit				Infinite	Infinite	Infinite
Weeks to First Observed Lesion	--	--	--	4	4	4
Liver: Foci of Cellular Alteration or Basophilic Foci[b]	0/10 (0)	0/10 (0)	10/10 (100)	5/10 (50)	7/8 (88)	2/9 (22)
P Values[c]		--	P < 0.001	P = 0.016	P < 0.001	N.S.
Relative Risk[d]			Infinite	Infinite	Infinite	Infinite
Lower Limit			3.968	1.454	3.007	0.368
Upper Limit			Infinite	Infinite	Infinite	Infinite
Weeks to First Observed Lesion	--	--	14	4	4	4

[a]Dosed groups received 190, 375, 750, 1,500, or 3,000 ppm.

[b]Number of lesion-bearing animals/number of animals examined at site (percent).

Table C5. Analyses of the Incidence of Tumors or Foci Alterations in the Liver of Male Rats Fed Direct Brown 95 in the Diet[a]

(continued)

[c]Beneath the incidence of lesions in a dosed group is the probability level for the Fisher exact test for the comparison of that dosed group with the matched-control group when $P < 0.05$; otherwise, not significant (N.S.) is indicated.

[d]The 95% confidence interval of the relative risk between each dosed group and the control group.

Table C6. Analyses of the Incidence of Tumors or Foci Alterations in the Liver of Female Rats Fed Direct Brown 95 in the Diet[a]

Compound	Matched Control	190 ppm Dose	375 ppm Dose	750 ppm Dose	1,500 ppm Dose	3,000 ppm Dose
Liver: Hepatocellular Carcinoma[b]	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	1/8 (13)	0/8 (0)
P Values[c]		--	--	--	N.S.	--
Relative Risk[d]					Infinite	
Lower Limit					0.072	
Upper Limit					Infinite	
Weeks to First Observed Tumor	--	--	--	--	5	--
Liver: Hepatocellular Carcinoma or Neoplastic Nodule[b]	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	5/8 (63)	0/8 (0)
P Values[c]		--	--	--	P = 0.007	--
Relative Risk[d]					Infinite	
Lower Limit					1.851	
Upper Limit					Infinite	
Weeks to First Observed Tumor		--	--	--	5	--

Table C6. Analyses of the Incidence of Tumors or Foci Alterations in the Liver of Female Rats Fed Direct Brown 95 in the Diet[a]

(continued)

Compound	Matched Control	190 ppm Dose	375 ppm Dose	750 ppm Dose	1,500 ppm Dose	3,000 ppm Dose
Liver: Basophilic Foci[b]	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	3/8 (38)[e]	3/8 (38)
P Values[c]	--	--	--	--	N.S.	N.S.
Relative Risk[d]					Infinite	Infinite
Lower Limit					0.858	0.858
Upper Limit					Infinite	Infinite
Weeks to First Observed Lesion	--	--	--	--	5	5
Liver: Foci of Cellular Alteration or Basophilic Foci[b]	0/10 (0)	0/10 (0)	3/10 (30)	3/10 (30)	6/8 (75)[f]	3/8 (38)
P Values[c]		--	N.S.	N.S.	P = 0.002	N.S.
Relative Risk[d]			Infinite	Infinite	Infinite	Infinite
Lower Limit			0.681	0.681	2.397	0.858
Upper Limit			Infinite	Infinite	Infinite	Infinite
Weeks to First Observed Lesion	--	--	13	13	5	5

[a]Dosed groups received 190, 375, 750, 1,500, or 3,000 ppm.

[b]Number of lesion-bearing animals/number of animals examined at site (percent).

Table C6. Analyses of the Incidence of Tumors or Foci Alterations in the Liver of Female Rats Fed Direct Brown 95 in the Diet[a]

(continued)

[c]Beneath the incidence of lesions in a dosed group is the probability level for the Fisher exact test for the comparison of that dosed group with the matched-control group when $P < 0.05$; otherwise, not significant (N.S.) is indicated.

[d]The 95% confidence interval of the relative risk between each dosed group and the control group.

[e]Two of these animals were also reported to have liver tumors.

[f]Four of these animals were also reported to have liver tumors.

APPENDIX D

BENZIDINE IN THE URINE OF RATS AND MICE FED DIRECT DYES

APPENDIX D

Benzidine in the Urine of Rats and Mice Fed Direct Dyes

The purpose of this study was to determine whether mice and rats fed diets containing direct blue 6, direct black 38, or direct brown 95 metabolically reduced these compounds to benzidine. These azo dyes are benzidine derivatives and have been shown to be reduced to benzidine in rhesus monkeys (Rinde and Troll, 1975).

Materials and Methods

Urine was collected from three rats of each sex and three mice of each sex at each of three dose levels of each of the three dyes and from associated untreated controls during weeks 4 and 12 (rats) and weeks 3 and 11 (mice) of the 13-week subchronic toxicity studies. The intent was to collect urine from the three highest dose groups; however, lower dose groups, as indicated in table D1, had to be used because of mortality in the higher dose groups. Animals were placed for 24 hours in metabolism cages. The collection vessels for these cages each contained 2.0 ml of 0.1 N HCl added as a preservative. Animals were fasted during urine collection, but water was available *ad libitum*. Volumes of urine collected were recorded, and samples were stored frozen at -20°C in the dark until analysis.

The benzidine assay procedure was a modification of the method of Rinde and Troll (1975, 1976). To 5.0 ml of urine was added 0.5 ml of sodium citrate buffer, 1.0M, pH 5.0. To each tube 2 ml of chloroform was added, and the mixture was shaken by hand for 1 minute. After complete phase separation, 1.7 ml of the organic layer was transfered to another test tube containing 2.0 ml of 0.01 N HCl. This mixture was shaken for 1 minute by hand. A 1.6-ml aliquot of the aqueous layer was transfered to another test tube containing 0.5 ml of 1.0 M citrate buffer, pH 5.0. A 0.2-ml volume of chloroform was added to each test tube. The mixture was shaken, and the upper (aqueous) layer was withdrawn and discarded.

A 100-μl portion of the chloroform layer was then spotted onto silica gel thin-layer chromatography (tlc) plates (Quantum Industries, LQ6DF plates). The plates were developed in chloroform-ethanol (9:1). After drying in a fume hood in the dark, each plate was sprayed with Fluram reagent (1.0 mg/ml fluorescamine in glacial acetic acid). Yellow spots were scraped from the plates, and the yellow color eluted with methanol (1.0 ml).

Each sample was centrifuged, transfered to cuvettes, and read at 415 nanometers, using methanol as blank. Benzidine standards (Nanogen, Watsonville, California) were routinely used as a

reference for R_f values and for determining and validating standard curves.

Mass spectral analyses were run on pooled samples, both from rats and mice. For these samples, spots on tlc plates were visualized by ultraviolet light (rather than spray reagent), scraped, and extracted in chloroform. Each sample was then subjected to direct probe mass spectral analysis, on a Finnigan 4000 Mass Spectrometer, using the chemical ionization technique.

Results and Discussion

A benzidine standard curve (absorbance vs. μgs of benzidine spotted) was constructed by spotting benzidine (0.2 - 8.0 μg) in duplicate onto tlc plates and proceeding as described in the previous section.

All urine samples from dosed animals and all standards contained sufficient benzidine so that Fluram spraying produced a yellow spot detectable to the naked eye at R_f 0.82. Most samples from dosed animals also produced a second spot on the tlc plates at R_f 0.68. None of the samples from untreated-control rats or mice contained substances which produced color on tlc plates following Fluram spraying.

Tables D1 and D2 present the 24-hour excretion of benzidine for

rats and mice, respectively. Each value represents a mean of three samples, except where indicated, with one rat or three mice per sample. Standard deviations are presented in parentheses. There are large standard deviations associated with many of the dosed groups. For the lowest dose groups, the lower limits of sensitivity for the quantitation of benzidine were approached. The tlc spot with R_f 0.68 was not quantitated for lack of standard.

The data in tables D1 and D2 clearly demonstrate that benzidine is excreted in the urine by rats and mice dosed with direct blue 6, direct black 38, or direct brown 95. The quantity of benzidine excreted generally increases with increasing dietary concentration of the direct dye, although the values for rats at high doses are unexpectedly low at 12 weeks. Mice dosed with direct black 38 excreted somewhat more benzidine than mice dosed with the other dyes, but no difference among the dyes was apparent in rats. There are no consistent differences between males and females in either rats or mice.

These data cannot be explained by possible residual benzidine in the dyes. No benzidine was detected in any of the dyes, with a detection limit of 0.004%. Even if it is assumed that the dyes did contain 0.004% benzidine and that all of it is excreted in the urine (Rinde and Troll, 1975, report finding 1.45% of an oral

dose of benzidine in the urine in rhesus monkeys), the levels found in this study in most cases exceed the maximum possible from residual benzidine in the dyes. In addition, the portion of benzidine excreted as the monoacetyl derivative was not quantitated and would increase the values reported in tables 1 and 2.

The identities of the R_f 0.82 and 0.68 tlc spots were confirmed by chemical ionization mass spectrometry. From mice and from rats, the chemical at R_f 0.82, reported above as benzidine, was verified as benzidine (parent and base peak at m/e 185, corresponding to protonated benzidine). The chemical at R_f 0.68 was identified as monoacetylbenzidine (base peak at m/e 227, corresponding to the protonated form; correct fragmentation pattern); Rinde and Troll (1975) identified this derivative as a second metabolite of these same three dyes in rhesus monkeys.

Table D1. Benzidine Excretion (μg/24 hr) Per Rat[a]

Dye Dietary Concentration, ppm	Weeks on Diet			
	4		12	
	Male	Female	Male	Female
Direct Blue 6				
3,000 or 1,500[b]	5.8 (0.9)[c]	8.0 (6.7)	0.77 (0.65)	0.55 (0.29)
750	1.4 (0.8)	0.94 (0.27)	0.32 (0.10)	0.29 (0.18)
190	0.85 (0.18)	0.62 (0.17)	0.44 (0.41)	0.16 (0.10)
Direct Black 38				
1,500	3.6 (4.8)	16.8 (n=2)	0.16 (0.03)	0.31 (0.16)
750	1.7 (n=2)	2.1 (0.06)	0.46 (0.09)	1.4 (0.35)
190	0.55 (0.31)	0.44 (0.13)	0.49 (0.39)	0.43 (0.32)
Direct Brown 95				
750	4.2 (1.3)	3.7 (2.9)	0.44 (0.12)	1.1 (n=1)
375	1.0 (0.77)	4.2 (1.3)	—	5.8 (9.7)
190	0.80 (n=2)	0.66 (0.24)	0.29 (0.11)	0.27 (0.05)

[a]Samples from untreated controls taken at weeks 4 and 12 showed no benzidine when spotted on tlc plates.

[b]Female rats at week 4 were from the 3,000-ppm group, male rats at week 4 and both males and females at week 12 were from the 1,500-ppm group.

[c]Numbers in parentheses are standard deviations. If fewer than three samples were averaged, the number of samples is given in parentheses instead.

Table D2. Benzidine Excretion (μg/24 hr) Per Mouse[a]

Dye Dietary Concentration, ppm	Weeks on Diet: 3 Male	3 Female	11 Male	11 Female
Direct Blue 6				
12,500	5.2 (0.85)[b]	5.1 (n=2)	2.4 (1.3)	5.5 (1.0)
3,000	0.97 (0.32)	1.1 (0.35)	1.7 (0.62)	3.1 (0.94)
750	0.55 (0.65)	0.31 (0.023)	1.1 (0.72)	0.52 (0.17)
Direct Black 38				
12,500	12.8 (2.8)	6.08 (1.8)	14.4 (2.7)	8.6 (1.0)
3,000	3.5 (2.1)	7.3 (n=2)	7.3 (2.2)	7.4 (1.7)
750	3.6 (3.4)	3.0 (2.7)	2.8 (3.2)	2.0 (1.8)
Direct Brown 95				
12,500	9.4 (n=2)		7.5 (0.90)	
3,000	4.7 (0.93)		1.1 (0.20)	
750	0.39 (0.09)		0.49 (0.23)	
6,000		3.5 (1.8)		3.2 (0.59)
1,500		2.8 (0.85)		0.35 (0.12)
375		0.56 (0.19)		0.19 (0.12)

[a]Samples from untreated controls taken at weeks 3 and 11 showed no benzidine when spotted on tlc plates.

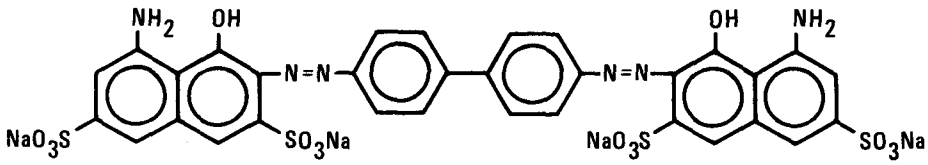
[b]Numbers in parentheses are standard deviations. If fewer than three samples were averaged, the number of samples is given in parentheses instead.

APPENDIX E

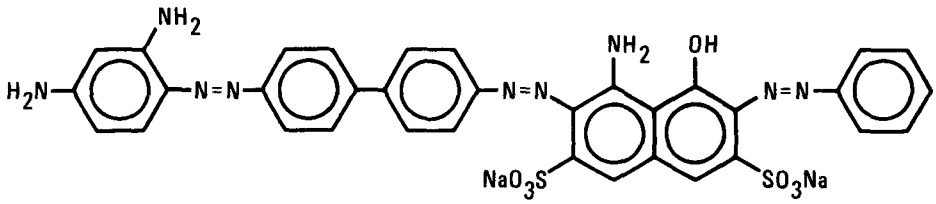
MOLECULAR STRUCTURES OF DIRECT BLUE 6, DIRECT BLACK 38, AND DIRECT BROWN 95

APPENDIX E

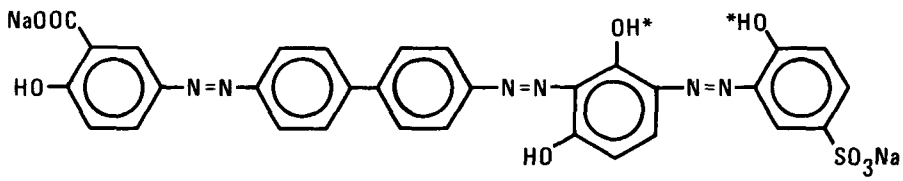
MOLECULAR STRUCTURES OF DIRECT BLUE 6, DIRECT BLACK 38, AND DIRECT BROWN 95



DIRECT BLUE 6 $C_{32}H_{20}O_{14}N_6Na_4S_4$



DIRECT BLACK 38 $C_{34}H_{25}N_9O_7S_2Na_2$



DIRECT BROWN 95 $C_{31}H_2N_6O_9SNa_2$ •Cu complex

Copper complexed at *

Review of the Bioassay of Direct Blue 6, Direct Black 38, and Direct Brown 95 Dyes* for Carcinogenicity by the Data Evaluation/Risk Assessment Subgroup of the Clearinghouse on Environmental Carcinogens

March 6, 1978

The Clearinghouse on Environmental Carcinogens was established in May, 1976, in compliance with DHEW Committee Regulations and the Provisions of the Federal Advisory Committee Act. The purpose of the Clearinghouse is to advise the Director of the National Cancer Institute (NCI) on its bioassay program to identify and to evaluate chemical carcinogens in the environment to which humans may be exposed. The members of the Clearinghouse have been drawn from academia, industry, organized labor, public interest groups, State health officials, and quasi-public health and research organizations. Members have been selected on the basis of their experience in carcinogenesis or related fields and, collectively, provide expertise in laboratory animal sciences, chemistry, biochemistry, biostatistics, toxicology, pathology, and epidemiology. Representatives of various Governmental agencies participate as ad hoc members. The Data Evaluation/Risk Assessment Subgroup of the Clearinghouse is charged with the responsibility of providing a peer review of reports prepared on NCI-sponsored bioassays of chemicals studied for carcinogenicity. It is in this context that the below critique is given on the bioassay of Direct Blue 6, Direct Black 38, and Direct Brown 95 dyes for carcinogenicity.

The primary reviewer agreed with the conclusions given in the report that Direct Blue 6 and Direct Black 38 were carcinogenic in both sexes of the Fischer 344 rat and Direct Brown 95 was carcinogenic in the female rat, under the conditions of test. He described the experimental design employed during the 13-week subchronic study and noted that the technical grade dyes contained unidentified impurities. Despite the fact that only 10 animals of each sex were used in each treatment group, given the demonstrated carcinogenicity of the tested materials, it did not compromise the study. Determinations of methemoglobin and urinary benzidine levels and the well-conducted pathology supported the confidence that could be given to the study. The primary reviewer concluded that the tested materials posed a potential carcinogenic risk to humans.

The secondary reviewer expressed concern regarding the high percent of impurities contained in the tested materials. He agreed with the primary reviewer, however, that the dyes were strong hepatic carcinogens in the rat.

With respect to the impurities, a Program staff member noted that each batch of the tested material was analyzed for free benzidine and benzidine salt. Since none was found, it was concluded that the urinary benzidine in the treated animals was a metabolic product of the dyes. A Subgroup member pointed out that the majority of the impurity was probably sodium chloride, based on analyses given in the report. The secondary reviewer agreed that the significance of the impurity was diminished if, in fact, most of it was salt.

A Program staff member said that there were no plans to initiate a 2-year chronic study on the dye materials, since all of them already have been shown to be carcinogenic. A Subgroup member commented that he would expect a chronic study to last no more than six or nine months based on the demonstrated carcinogenicity of the dyes in the 13-week test. He added that a smaller number than the standard 50 animals per test group could be used and suggested that parallel studies be conducted using dyes of different purities. Given the Program's mission and the fact that benzidine was likely the carcinogenic metabolite, it was felt that an additional study was not necessary.

A staff pathologist showed slides of the liver tumors induced in the treated rats. He described the histological changes and indicated the nomenclature applied to the pathology. Although some tumors appeared to invade or push into the wall of veins, no metastases were observed. He attributed the lack of metastases to the short study period.

A motion was made that the report on the bioassay of the direct dyes be accepted as written. The motion was seconded and approved unanimously.

Members present were

Gerald N. Wogan (Chairman), Massachusetts Institute of Technology
Arnold Brown, Mayo Clinic
Lawrence Garfinkel, American Cancer Society
E. Cuyler Hammond, American Cancer Society
Joseph Highland, Environmental Defense Fund

Henry Pitot, University of Wisconsin Medical Center
George Roush, Jr., Monsanto Company
Sheldon Samuels, Industrial Union Department, AFL-CIO
Michael Shimkin, University of California at San Diego
John Weisburger, American Health Foundation
Sidney Wolfe, Health Research Group

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* Subsequent to this review, changes may have been made in the bioassay report either as a result of the review or other reasons. Thus, certain comments and criticisms reflected in the review may no longer be appropriate.

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