NATIONAL TOXICOLOGY PROGRAM

EXECUTIVE SUMMARY OF SAFETY AND TOXICITY INFORMATION

METHYLENE BLUE

CAS Number 61-73-4/7220-79-3

November 30, 1990

Submitted to:

NATIONAL TOXICOLOGY PROGRAM

Submitted by:

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OVERVIEW[1]

Nomination History: *Methylene blue was nominated for carcinogenicity testing by the National Cancer Institute (NCI) in 1989. The nomination was based on the numerous uses of this compound and the potential for high exposure in animals and humans. In addition, the NCI noted the lack of long-term toxicity data, including epidemiological studies on methylene blue, as well as the inadequate animal data on this compound.*

Chemical and Physical Properties: *Methylene blue is a dark green powder or crystalline solid. This odorless compound decomposes at 100°-110° C. Methylene blue is incompatible with strong oxidizing and reducing agents.*

Production/Uses/Exposure: *Methylene blue is used therapeutically in the treatment of methemoglobinemia and cyanide poisoning. Other medicinal uses of methylene blue include the management of chronic urolithiasis and treatment of cutaneous viral infections as well as the treatment of manic-depressive psychosis. As a dye/stain, methylene blue is used in surgical and medical marking and as an indicator dye, a bacteriologic stain, ~~a food colorant~~ (see note) and a dye for cotton and wool. Data from the National Occupational Exposure Survey (NOES) indicate that 69,563 workers, including 42,026 female employees were potentially exposed to methylene blue between 1981 and 1983. No data were found on environmental exposure to methylene blue. In addition, there is no OSHA permissible exposure limit, ACGIH-recommended threshold limit value or NIOSH-recommended exposure limit for methylene blue.*

Toxicological Effects:

Human: *Acute exposure to methylene blue has been found to cause increased heart rate, cyanosis, vomiting, shock, Heinz body formation, jaundice, quadriplegia and tissue necrosis in humans. In addition, corneal and conjunctival injury has been reported following acute exposure to this compound. Intravenous administration of methylene blue has been found to cause bluish discoloration of the urine and stool. Numerous case reports were found in the literature describing the effects of methylene blue on the newborn, following the injection of this compound into the amniotic fluid before delivery. At birth, many of the infants reportedly had deep blue stained skin and voided blue urine. Other symptoms including respiratory distress, hyperbilirubinemia, methemoglobinemia, Heinz body formation and increased heart rate occurred after birth. No data were found on the prechronic effects of methylene blue in humans. Chronic application of methylene blue-containing eye drops has been found to result in staining of the bulbar and palpebral conjunctiva, the lid margins and slight staining of the corneal epithilium. No other data were found on the chronic/carcinogenic effects of methylene blue in humans. Methylene blue has been found to cause an elevation in follicle stimulating hormone and estradiol in fallopian tube secretions, and uterine and peritoneal fluids in vitro. In addition, methylene blue was found to significantly inhibit sperm motility in vitro in samples prepared from human semen. No other studies were found on the reproductive effects of methylene blue in humans.*

Animal: *Numerous reports were found in the literature describing the association between methylene blue and the formation of Heinz bodies in animals. Intraperitoneal administration of methylene blue has been found to induce Heinz body formation in cats, dogs, mice and rabbits. In cats, methylene blue was observed to cause bluish stained skin, anemia, discolored urine, dyspnea, depression, respiratory stimulation and increased blood pressure. In addition, methylene blue has been found to cause corneal and conjunctival injury in rabbits. No data were available on the prechronic, effects of methylene blue in animals. Administration of this compound at a concentration of 4% in the diet for 2 years was not found to induce tumor formation. Methylene blue has been found to inhibit the ability of mouse embryos to grow and cleave in vitro. This compound also caused an increase in implantations and resorptions in litters born to rats fed diet containing methylene blue.*

Genetic Toxicology: *Methylene blue has been found to be mutagenic to Micrococcus aureus. In addition, methylene blue was mutagenic to Salmonella typhimurium in the presence and absence of metabolic activation. This compound was mutagenic to Escherichia coli in the microsuspension and DNA-cell binding assays. There are conflicting reports concerning the mutagenicity of methylene blue in Bacillus subtilis. One author reported that methylene blue was mutagenic to B. subtilis in the rec-assay. However, methylene blue was non-mutagenic to this bacteria in another study. Several reports were found concerning mutation induction via irradiation with visible light, in the presence of methylene blue. Photodynamic mutagenesis in the presence of methylene blue has been induced in bacteriophage Serratiaphage X, Escherichia coli and Salmonella typhimurium. Methylene blue was non-mutagenic to Chinese hamsters, in vivo. In addition, this compound was non-mutagenic in cultured Chinese hamster ovary cells and lung fibroblasts. Methylene blue did not induce mutagenic effects in Drosophila melanogaster.*

Structure Activity Relationships: *No information was found on structure activity relationships for methylene blue.*

I. NOMINATION HISTORY AND REVIEW

A. Nomination History

1. Source: National Cancer Institute [NCI, 1989, a, b]
2. Date: March, 1989
3. Recommendations: Carcinogenicity
4. Priority: High
5. Rationale/Remarks:
    - Numerous uses: treatment of manic-depressive illness and nitrate poisoning, anti-tumorigenic activity, *in vivo* detection of bladder tumors
    - Potential for high exposure in humans
    - No FDA long-term studies on methylene blue
    - Lack of epidemiological studies
    - Available animal studies inadequate by current standards
    - Numerous short-term tests with varying results

B. Chemical Evaluation Committee Review

1. Date of Review: September 12, 1990
2. Recommendation:
    - Carcinogenicity
    - Reproductive and teratogenicity studies
    - Determine whether the chemical crosses the blood/brain barrier
3. Priority: High
4. NTP Chemical Selection Principle(s): 3, 4, 6
5. Rationale/Remarks:
    - Widespread uses
    - Some medical and veterinary applications of the chemical have not been approved by FDA
    - Potential for exposure
    - Lack of carcinogenicity data

C. Board of Scientific Counselors Review

1. Date of Review: October 15, 1990

2. Recommendations:
    - Carcinogenicity
    - Reproductive Studies
3. Priority:
    - High for carcinogenicity
    - Moderate for reproductive studies
4. Rationale/Remarks:
    - Widely used compound
    - Potential for exposure
    - Lack of carcinogenicity data
    - Previous neurotoxicity studies are sufficient

D. **Executive Committee Review**

1. Date of Review:
2. Decision:

II. CHEMICAL AND PHYSICAL DATA

A. Chemical Identifiers

METHYLENE BLUE

Executive Committee Draft Report

CAS No. 61-73-4 (anhydrous)
7220-79-3 (trihydrate)

RTECS No. SO5600000M

Molecular formula: **$C_{16}H_{18}H_3SCl$ (anhydrous)** Molecular weight: **319.85**
$C_{16}H_{18}H_3SCl.3H_2O$ (trihydrate) **373.90**

- Synonyms and Trade Names

Synonyms: Phenothiazine-5-ium, 3,7-bis(dimethylamino)-, chloride (9CI); C.I. Basic Blue 9 (8CI); methylthionine chloride; methylthioninium chloride; tetramethylthionine chloride; swiss blue; aizen methylene blue; C.I. 52015

Trade Names: Desmoid piller, Desmoidpillen, Methylene Blue, Panatone, Urolene Blue, Vitableu

- Chemical and Physical Properties

Description:	Dark green powder or crystals [Weast, 1988] with bronze luster [Budavari, 1989]/ Odorless [Budavari, 1989]
Melting Point:	Decomposes at 100°-110° C [Kirk-Othmer, 1978]
Boiling Point:	No data was found
Density/Specific Gravity:	No data was found
Refractive Index:	No data was found
Solubility in Water:	Water (1 gram in 25 ml of water [Budavari, 1989]); crystallizes with 3 and 5 mols of water [Budavari, 1989]
Solubility in other Solvents:	Chloroform, alcohol [Weast, 1988] (1 gram in 65 ml alcohol) [Budavari, 1989], glycerol, glacial acetic acid, 0.5% soluble in acetone [Kirk-Othmer, 1978]
Log Octanol/Water Partition Coefficient:	No data was found
Reactive Chemical Hazards:	• Incompatible with caustic alkali, dichromates, alkali iodides, reducing agents [Budavari, 1989] • Incompatible with strong oxidizing agents [Lenga, 1988] • Decomposition products include toxic fumes of carbon monoxide, nitrogen oxides, sulfur oxides, and hydrogen chloride gas [Lenga, 1988; Sax, 1989]
Flammability Hazards:	No data was found (non-combustible under normal conditions)

PRODUCTION/USE

A. Production

1. Manufacturing Process

Methylene blue is manufactured by the oxidation of p-aminodimethyl-aniline with ferric chloride in the presence of hydrogen sulfide [Budavari, 1989; Sax, 1987]. The principal steps in the manufacture of methylene blue include (1) nitrosation of dimethylaniline, (2) reduction to p-aminodimethylaniline, (3) oxidation in the presence of an excess of sodium thiosulfate to form 4-dimethylamino-1-amino-2-benzenethiosulfonic acid, (4) oxidation in the presence of dimethylaniline to form indamine thiosulfonic acid, and (5) ring closure to form methylene blue.

The nitrosation and reduction steps described above are carried out at 0°-2°C. The reduction step is carried out at high acidity with finely ground iron powder, and the two oxidation steps are performed at 0°-5°C. Ring closure is carried out at the boil, using copper sulfate as the catalyst. The dye must be separated from the large amount of sludges, which result from the use of iron and dichromate, using large volumes of water. Methylene blue is isolated by adding sodium chloride to make zinc-free methylene blue, or if the zinc salt is needed, zinc chloride is added to form the double salt [Kirk-Othmer, 1978].

2. Producers and Importers

U.S. Producers :

- Aldrich Chemical Company, Inc.

  Milwaukee, Wisconsin [SRI, 1989]

- American Cyanamid Company, Chemical Products Division

  Marietta, Ohio [SRI, 1989; USITC, 1989]

- Amersco (American Research Products Company)

  Solon, Ohio [OPD Chemical Buyer's Directory, 1989]

- Arrow Chemical Company

  Westwood, New Jersey [OPD Chemical Buyer's Directory, 1989]

- Atlantic Industries, Inc.

  Nutley, New Jersey [SRI, 1989]

- BASF Corporation, Chemicals Division

  Rensselaer, New York [SRI, 1989; USITC, 1989]

- Berncolors-Poughkeepsie, Inc.

  Poughkeepsie, New York [SRI, 1989]

- Burlington Bio-Medical Corporation

  Farmingdale, New York [Chemical Week Buyer's Guide, 1989]
- C. Lever Company, Inc.

  Philadelphia, Pennsylvania [SRI, 1989]
- Ciba-Geigy Corporation, Dyestuffs and Chemicals Division

  Greensboro, North Carolina [SRI, 1989]
- Crompton & Knowles Corporation, Dyes and Chemicals Division

  Charlotte, North Carolina [SRI, 1989]
- Industrial Products Division

  Newark, New Jersey [SRI, 1989; USITC, 1989]
- Curtis Labs, Inc.

  Bensalem, Pennsylvania [Chemical Week Buyer's Guide, 1989]
- Eastman Kodak Co., Laboratory and Research Products Division

  Rochester, New York [Chemical Week Buyer's Guide, 1989]
- EM Industries, Inc.

  Hawthorne, New York [Chemical Week Buyer's Guide, 1989]
- Fabricolor Incorporated

  Paterson, New Jersey [SRI, 1989]
- GAF Corporation

  New York, New York [USEPA, 1990]
- Gallard-Schlesinger Industries, Inc.

  Carle Place, New York [OPD Chemical Buyer's Directory, 1989]
- Haven Chemical

  Philadelphia, Pennsylvania [USEPA, 1990]
- Jonas Chemical Corporation, Specialty Chemical Division

  Brooklyn, New York [SRI, 1989]
- Keystone Aniline Corporation

Chicago, Illinois [Chemical Week Buyer's Guide, 1989]

- Mobay Corporation, subsidiary of Bayer USA, Inc.

  Pittsburgh, Pennsylvania [SRI, 1989]

- Nachem, Inc.

  Braintree, Massachusetts [Chemical Week Buyer's Guide, 1989]

- Pylam Products Company, Inc.

  Garden City, New York [Chemical Week Buyer's Guide, 1989]

- Roussel Corporation

  Englewood Cliffs, New Jersey [OPD Chemical Buyer's Directory, 1989]

- Royce Associates

  East Rutherford, New Jersey [SRI, 1989]

- Passaic Color & Chemical Company, subsidiary of Royce Associates Paterson, New Jersey [SRI, 1989]

- Sandoz Chemicals Corp.

  Charlotte, North Carolina [SRI, 1989]

European Producers :

- Crompton & Knowles Tertre SA

  Villerot (Hainaut) [SRI, 1989]

- SA Ciba-Geigy,

  Rueil-Malmaison (Hauts de Seine) [SRI, 1989]

Importers:

The following importers of methylene blue are listed in the EPA TSCA Inventory:

- American Cyanamid Company

  Bound Brook, New Jersey [USEPA, 1990]

- American Hoechst Corporation

  Bridgewater, New Jersey [USEPA, 1989]

- American Research Products Company

South Euclid, Ohio [USEPA, 1990]

- Atlantic Chemical Corporation

  Nutley, New Jersey [USEPA, 1990]

- Carey Industries, Inc.

  Danbury, Connecticut [USEPA, 1990]

- Dainichiseika Color and Chemical

  New York, New York [USEPA, 1990]

- Degussa Corporation

  Teterboro, New Jersey [USEPA, 1990]

- EM Laboratories, Inc.

  Elmsford, New York [USEPA, 1990]

- L and R Dyestuffs Corporation

  Clifton, New Jersey [USEPA, 1990]

- Rohm and Haas Company

  Philadelphia, Pennsylvania [USEPA, 1990]

- Ugine Kuhlmann of America, Inc

  Paramus, New Jersey [USEPA, 1990]

3. Volume

   The production volume of methylene blue has been reported in the public file of the EPA Toxic Substances Control Act (TSCA) Inventory. In 1977, six manufacturers listed as producers of methylene blue reported a total production volume ranging from 12,000 to 120,000 pounds.

   Methylene blue is not listed in SRI's Chemical Economics Handbook. No specific data on production of methylene blue were available from the U.S. International Trade Commission. However, the USITC reported an annual production of 1,462,000-2,062,000 pounds for basic blue dyes for the years 1985-1988 [USITC, 1986-1989].

4. Technical Product Composition

   Methylene blue is available in technical and USP grades ($\geq$98% contains no less than 98% and no greater than 103% of C16H18ClN3S, calculated on a dried basis) [U.S. Pharmacopeial Convention, 1984]. Commercial methylene blue may contain the double chloride of tetramethylthionine and zinc [Reynolds and Prasad, 1989].

B. Use

Therapeutic Uses:

- Treatment of methemoglobinemia [McEvoy, 1989; U.S. Pharmacopeial Convention, 1984]; dosage is usually 1 to 2 mg/kg over a 5 minute period as a 1% solution intravenously [Clayton, 1981].
- Antidote for cyanide poisoning [McEvoy, 1989].
- Management of chronic urolithiasis [McEvoy, 1989].
- Treatment of cutaneous viral infections (herpes simplex)[McEvoy, 1989].
- Treatment of glutaricaciduria [Reynolds and Prasad, 1989].
- Treatment for manic-depressive psychosis [Reynolds and Prasad, 1989].
- Formerly used as a urinary antiseptic [Gennaro, 1985] and stimulant to mucous surfaces in the treatment of cystitis and urethritis; currently more effective agents are used [McEvoy, 1989].
- Formerly used as an analgesic, antipyretic and antiparasitic [Gennaro, 1985; McEvoy, 1989].

Use as a dye/stain:

- Bacteriologic stain [Gennaro, 1985; McEvoy, 1989]- tubercle and lepra bacilli in mammalian tissue [Aldrich, 1988], stains plasmodia [Kirk-Othmer, 1979].
- Indicator dye [McEvoy, 1989].
- Surgical and medical marking [McEvoy, 1989]-stain for elastic fibers and connective tissue, component of Tetrachrome Stain for differentiation of blood corpuscles [Aldrich, 1988], staining of corneal nerves [Grant,1986], intramniotic injection used to detect subtle rupture of the membranes and to differentiate the sacs of twins [Spahr et al., 1980].
- Diagnostic agent in renal function tests and in vital nerve staining [McEvoy, 1989].
- Coloring paper [Kirk-Othmer, 1978].
- Temporary hair colorant [Kirk-Othmer, 1980].
- Dyeing cotton, wool [Sax, 1987] and leather [Kirk-Othmer, 1978].

Miscellaneous uses:

- Coating for paper stock [Kirk-Othmer, 1978].
- Reagent in oxidation-reduction titrations in volumetric analysis [Sax, 1987].

- **EXPOSURE/REGULATORY STATUS**

A. Consumer Exposure

Consumer exposure to methylene blue results from the medicinal and therapeutic uses of this compound. In addition, consumer exposure may occur from the use of methylene blue in ~~food and~~ (See note) and hair colorants. No quantitative data were found on consumer exposure to this compound.

B. Occupational Exposure

Occupational exposure to methylene blue may occur in industry, laboratories and health care facilities. Data from the National Occupational Exposure Survey (NOES), conducted by the National Institute for Occupational Safety and Health (NIOSH) between 1981 and 1983, indicate that 69, 563 workers, including 42,026 female employees, were potentially exposed to methylene blue in the workplace. The NOES data base does not contain information on the frequency, level or duration of exposure to workers of any chemicals listed therein [NIOSH, 1990].

C. Environmental Exposure

No data were found on environmental exposure to methylene blue.

D. Regulatory Status

- Methylene blue is listed in the Canadian Workplace Hazardous Materials Information System (WHMIS) ingredient disclosure list [Roytech, 1989].
- OSHA has not established a permissible exposure limit (PEL) for methylene blue.

E. Exposure Recommendations

- ACGIH has not recommended a threshold limit value (TLV) for methylene blue.
- NIOSH has not recommended an exposure limit (REL) for methylene blue.

- **TOXICOLOGICAL EFFECTS**

A. Chemical Disposition

1. Human Data

- Seven adult male volunteers were administered 10 mg of methylene blue orally in a gelatin capsule to determine the pharmacokinetics of the chemical. The subjects fasted overnight and for four hours post-dosing. Urine samples were collected quantitatively. After oral administration of methylene blue, an average of 74% (range 53%-97%) of the dose was recovered in the urine. An average of 78% (range 65%-85%) of the chemical recovered was excreted as leucomethylene blue stabilized in some salt complex or combination form. Diurnal cycling of the excretion rates was demonstrated after oral administration of methylene blue [DiSanto and Wagner, 1972].

2. Animal Data

- The concentration of methylene blue in whole blood, urine, feces, and milk was

determined in 6 lactating cows, 6 lactating goats, and 6 steers. Methylene blue was administered intravenously at a concentration of 10 mg/kg. Three, six, and nine days after treatment, the cows and steers were sacrificed and organ blood levels were measured.

In addition, methylene blue was administered by intravenous drip for 2-3 hours to six other lactating goats and infused into the mammary glands of six additional lactating goats as a 10% aqueous solution at 10 mg/kg.

Methylene blue was detected in the milk of goats 5 minutes after beginning the IV drip. At equilibrium, drug concentrations in the milk were 5 to 7 times higher than in the blood. Methylene blue was detected in the blood of the goats 30 minutes to 12 hours following intramammary infusion.

Urine and fecal recovery of methylene blue in cows and steers was less than 2% and 1%, respectively. Low concentrations (quantity not specified) of methylene blue were found in the kidneys of these animals by day three post-treatment, and no residual levels were detected in any body organ or fluid by day six [Ziv, et al., 1982].

- One male beagle dog was administered 15 mg/kg of methylene blue orally in a gelatin capsule and blood samples were taken post-administration to determine the pharmacokinetics of this compound. In addition, one female mongrel dog was given one 15 mg/kg dose and one 10 mg/kg dose of methylene blue, three weeks apart, administered orally in a gelatin capsule. Urine samples were collected every hour for 10 hours after the 15 mg/kg dose in female dogs, and blood samples were taken at 2.5 and 3.5 hours after methylene blue administration.

  Urine collected from the female mongrel dog after administration of the 15 mg/kg and the 10 mg/kg doses of methylene blue contained 2.4% and 3.8% of the administered dose, respectively. Blood samples in the female yielded no methylene blue 2.5 and 3.5 hours after administration. Blood concentrations from the male beagle dog indicated values below the assay sensitivity level (0.02 mg/ml) 0-6.9 hours after dosing. The authors report that these results indicate poor absorption of methylene blue in the dog [DiSanto and Wagner, 1972].

- *In situ* recirculation techniques have been used to determine the extent of small intestine absorption of methylene blue in male Wistar rats, male Hartley guinea pigs, and male albino rabbits (number of animals not specified). For the recirculation experiments, a 2.4 x 10-5 M solution of methylene blue in isotonic saline buffer was prepared, and the small intestines of the animals were excised and added to the buffer solution.

  After 120 minutes, the amount of methylene blue absorbed in rats, guinea pigs, and rabbits was 54%, 36%, and 47% respectively. The surface areas of the small intestines of the three animals were calculated, and absorption ratios per unit surface area of the small intestine were determined to be 1.0:0.77:1.2 (rabbit:guinea pig:rat).

  Eight percent, 46%, and 30% of the original methylene blue was found to be metabolized to leucomethylene blue in rats, guinea pigs and rabbits respectively. The remaining ratio of leucomethylene blue per unit surface area of the small intestine was determined to be 1.0:0.25:1.5 (rabbit:rat:guinea pig).

  Urinary and biliary excretion of total methylene blue (methylene blue + leucothylene blue)

was determined in rabbits. After 180 minutes, 12.8% of the methylene blue was excreted in urine and 8.0% was excreted in the bile.

Rat and guinea pig small intestines were incubated with methylene blue solution (quantity not specified) *in vitro*. Methylene blue in the mucosal fluid was found to be reduced to leucomethylene blue and nearly all of the methylene blue disappeared in approximately 90 minutes in both rats and guinea pigs. Leucomethylene blue was detected in the serosal fluid in rats, guinea pigs and rabbits, and the amount increased with time. The authors report that leucomethylene blue was able to penetrate through the small intestine barrier [Watanabe and Mori, 1977].

- Eight sheep of mixed breeding were administered methylene blue to evaluate the disposition and urinary excretion pharmacokinetics of this compound. Sheep were divided into two groups each receiving 15 mg/kg of methylene blue via jugular catheterization, 20 minutes after administration of either 100 ml of saline solution (4 sheep), or 50 mg/kg sodium nitrate[2] (4 sheep). Blood samples were taken, and urine samples were collected 0, 15, 30, 60, 120, 180 and 240 minutes after methylene blue administration. In the four sheep administered methylene blue and saline, an additional intravenous dose of methylene blue was given.

  Six percent of the administered dose of methylene blue was excreted after four hours. Most of the methylene blue was eliminated as leucomethylene blue. There was a significant increase ($p=0.05$) in urinary excretion of methylene blue at 1, 2, and 3 hours in the group receiving sodium nitrate, although for both groups, excretion rates were low. The half-life of methylene blue was 101.6 ± 16.2 minutes and was not influenced by the presence of sodium nitrate.

  The overall elimination rate constant of methylene blue (0.0076 ± 0.0016) was not found to be influenced by the administration of sodium nitrite in sheep given 15 mg/kg of methylene blue. Only the kinetics of the distribution phase (distribution rate, alpha) were significantly changed ($p=0.05$) by the administration of sodium nitrate.

  The authors suggest that methylene blue is eliminated by metabolism and/or biliary excretion, or is sequestered in tissues with slow release into the peripheral circulation. Elimination in urine occurs in very low concentrations over a long period, and the presence of sodium nitrate has little influence on either the rate of elimination or the metabolic pathway [Burrows, 1984].

- A male beagle dog was administered 2.0, 5.0, 7.5, 10.0, and 15.0 mg/kg of methylene blue intravenously, with two to three weeks between each dose, in order to examine whole blood concentrations of methylene blue after administration.

  From non-linear (one fluid-one tissue) model analysis, it was determined that the average volume of distribution of the five sets of blood samples was 0.222 L/kg, or 22.2% of body weight. The authors suggested that the linear volume of distribution obtained by the non-linear model may have physiological significance. Using this non-linear model, no systemic trends in the estimated parameters in relation to dose were found. The concentration data correlated well with the classical linear, two-compartment open model, systemic trends in the estimated parameters in relation to dose were observed. By this method, the average volume of distribution was found to be 87.6% of the body weight.

In order to investigate the uptake of methylene blue by the tissues, and to obtain data on the amount of methylene blue bound as a fraction of dose, methylene blue at doses of 2.0, 5.0, 7.5, 10.0, 15.0, and 25.0 mg/kg was administered intravenously into the penis vein of adult male Sprague-Dawley rats. The rats were sacrificed 3 minutes post-injection, blood was collected, and the heart, lungs, liver, and kidneys were examined for tissue uptake of methylene blue.

The amount of methylene blue found in the blood was negligible compared to the amount detected in the tissues studied. These organs accounted for an average of 29.8% of the administered dose of methylene blue (See Table 1). The authors reported that uptake of methylene blue by rat tissue was rapid supporting the non-linear heterogenous, one-compartment, one tissue-type model observed in the beagle dog study [DiSanto and Wagner, 1972].

3. Human/Animal Data

- Heparinized blood samples were collected from dogs and cats of mixed breeding and from laboratory personnel in order to examine methylene blue uptake in red blood cells. One milliliter of erythrocyte suspension was added to 10 ml of buffer containing 11.1 mM glucose, 154 mM NaCl and 4 mM phosphate. One milliliter of methylene blue (0.006% in buffer) was added to the suspension.

  Methylene blue uptake was significantly higher ($p<0.001$) in human erythrocytes than in cat or dog erythrocytes. In humans, virtually all of methylene blue uptake occurred within 20 minutes. Feline methylene blue uptake was significantly greater (p value not reported) than uptake by the dog. However, the authors report that this result may not be significant due to varying degrees of hemolysis in feline blood samples [Harvey and Kaneko, 1974].

B. Acute

1. Human Data

Gosselin et al. have given methylene blue a toxicity rating of 4 [Gosselin, et al., 1984]. Acute exposure to methylene blue by intravenous injection has been found to cause hypertension, sweating, chest pain, confusion, nausea, vomiting, dizziness, and cyanosis, as well as urine and stool discoloration [Arena, 1986; Gennaro, 1985].

Large, oral doses of methylene blue may cause fever [McEvoy, 1989]. Methylene blue has also been reported to cause quadriplegia after intrathecal injection [Driesbach, 1980]. Subcutaneous injection of methylene blue may cause necrotic abscesses [Reynolds and Prasad, 1989].

Methylene blue induces hemolytic anemia in individuals with glucose-6-phosphate dehydrogenase (G-6-PD) enzyme deficiency. Dark-skinned races have a higher incidence of methylene blue-induced hemolytic anemia than lighter skinned races, but the disease has not been noted in North American Indians or Eskimos [Thienes and Haley, 1972].

Methylene blue is a severe eye irritant [Lenga, 1988]. High concentrations of methylene blue have been found to induce corneal and conjunctival injury [Grant, 1986].

2. Case Reports

- The ocular toxicity of a non-prescription topical medication containing methylene blue,

naphazoline hydrochloride, or nitrate and amylocaine hydrochloride has been reported for six patients. The medication is used to relieve ocular redness and discomfort.

Two of the patients experienced a blue staining of the conjunctiva, either in one or both eyes, which was believed to be caused by methylene blue. Four patients were diagnosed with follicular conjunctivitis, with three of the four showing symptoms in both eyes. One patient with follicular conjunctivitis in both eyes also demonstrated a concurrent blue discoloration of the limbus in one eye. It was reported that the follicular conjunctivitis observed may have been caused by the naphazoline or methylene blue [Brownstein, et al, 1989].

- Methylene blue toxicity was described in a case-report of a 62 year-old, former dye worker diagnosed with carcinoma of the bladder. Two milliliters of 1% methylene blue were injected into the web spaces of the feet to examine the pelvic lymphatics during surgery.

  Eight days later both feet became swollen, and five weeks following the injection of methylene blue, ulcers formed on the left foot at the injection sites. Three days later, additional ulcers appeared on the patient's right foot at the sites of methylene blue injection. The ulcers were necrotic and extended to the deep fascial layer of the skin, which later required grafting [Perry and Meinhard, 1974].

- Tissue necrosis occurred in a 35-year-old male after intravenous injection of 70 mg of methylene blue in order to treat a suspected case of methemoglobinemia. The patient showed no improvement following treatment, and was subsequently found to be in vascular collapse and shock due to effects of an acute gastroenteritis on previously unsuspected severe adrenal insufficiency, and diabetes secondary to remote head trauma.

  Ninety minutes after injection of methylene blue, a zone of skin pallor with surrounding traces of blue color was noted at the injection site. During the following 48 hours, necrosis of the skin developed and became progressively larger during the next five days. In addition, an incomplete left median nerve palsy developed. A debridement procedure revealed complete necrosis of the skin, subcutaneous tissue and underlying fatty tissue of the left antecubital fossa. When the superficial fascia of the arm was excised, "dark blue coloration oozed from the wound."

  It was determined that the patient had suffered an accidental injection of methylene blue into the subcutaneous tissues of the left antecubital fossa. The authors report that although the patient made a complete recovery from his presenting life-threatening acute illness, his course was complicated by severe tissue necrosis of the arm which added substantially to his morbidity and resulted in diminished function of the arm 18-months post-injury [Ruhlen, 1982].

- Spinal cord necrosis was reported in a 59-year-old male who underwent testing for unilateral cerebrospinal fluid rhinorrhea. Six milliliters of 1% methylene blue was injected into the lumbar subarachnoid space in order to identify the cause of the rhinorrhea. Two hours later, the patient went into shock, vomited, and within 24 hours developed paraparesis with urinary retention, but without sensory loss.

  Four months after the injection, paraparesis had progressed, urinary retention persisted, and sensory loss had occurred. Eighteen months after the injection, there was complete loss of strength in the right lower limb. Three and one half years after the injection, total

paraplegia was observed, which persisted until the patient died five years later.

The authors reported three phases of spinal cord and nerve damage which may have resulted following the injection of methylene blue. The first phase occurred shortly after injection and resulted in direct damage to the surface of the cord and its surface blood vessels, probably accentuated by poor perfusion during a period of hypotension. The second stage was a temporary recovery possibly related to a regression of edema around the areas of infarction in the cord, and third was progression to total paraplegia. This last phase may have resulted from progressive ischemia of the cord accompanying fibrosis and scarring of the meninges [Sharr, et al.,1978].

- Gross (1974) reported on a patient who became paraplegic after injection of 1 cc of methylene blue into the lumbar subarachnoid space to localize a cerebrospinal fluid fistula [Gross, 1974]. No other information was provided.

- Eighteen milliliters of 0.2% aqueous methylene blue solution was injected into the uterus of a 20-year-old woman during a laparoscopy. Since the woman was believed to have primary sterility, the dye was injected in order to examine the fallopian tubes. Methylene blue passed through both tubes freely. Two weeks later, it was determined that at the time of the laparoscopy the woman was pregnant. The baby was delivered spontaneously at 40 weeks without complications. Examination of the infant did not reveal any abnormalities. No anemia of any type was detected. The purpose of this report, as stated by the authors, was to assure obstetricians that injection of methylene blue very early in pregnancy may not affect the embryo [Katz and Lancet, 1981].

- One milliliter of methylene blue was injected into the amniotic fluid of a 33-year-old woman during week 26 of gestation, to confirm the obstetrician's suspicion of membrane rupture. The infant was delivered vaginally 18 hours later with a deep blue staining of the skin and mucous membranes. Apgar scores ranging from 4 to 7 were reported, although values were believed to be underestimated due to the extreme discoloration of the infant's skin. No hemolysis was detected and indirect hyperbilirubinemia was successfully treated with phototherapy. The intense skin discoloration was reported to have interfered with the treatment of the newborn and persisted for more than two weeks despite numerous baths [Troche, 1989].

- As part of an amniocentesis procedure three days before scheduled delivery, 10 mg of methylene blue was injected intra-amniotically in a 32-year-old pregnant woman. Twin girls were delivered vaginally nine hours later, following spontaneous rupture of the membranes. One twin was born with a bluish tinge of the skin and voided blue urine. Twenty-four hours after birth, jaundice and mild retractions were noted in the same twin. Respiratory distress worsened and further examination revealed substernal retractions, basilar rales, the liver edge 2 cm below the right costal margin, and a palpable spleen tip. Heinz bodies, burr cells, fragmented red blood cells and target cells were identified in the peripheral blood smear. The reticulocyte count was 30%. The methemoglobin level on the second day after birth was 20% of the total hemoglobin (normal <1%). However, methemoglobin could not be detected by the fourth day. Glucose 6-phosphate dehydrogenase concentration was normal and the direct Coombs test was negative. The twin was treated with phototherapy and exposed to an oxygen-rich environment, and was subsequently discharged with normal color and functioning when she was 9 days old.

  The other twin and mother voided bluish-tinged urine. However, no other signs of

methylene blue toxicity were observed. [Spahr, et al., 1980].

- Two milliliters of a sterile methylene blue solution were injected intra-amniotically into a 17-year-old adolescent girl at 34 weeks gestation to document membrane rupture. Shortly after amniocentesis, the patient went into labor. Immediately after injection of the methylene blue, the fetal heart rate rose from 146 beats/minute at baseline to 220 beats/minute for 40 minutes. Fourteen hours after the injection of methylene blue, a male infant was delivered vaginally.

  Low Apgar scores and blue tracheal secretions were reported after delivery. In addition, a chest x-ray revealed mild hyaline membrane disease. The primary toxic effect observed in the infant was hyperbilirubinemia secondary to hemolysis. On day three of life, the infant's methemoglobin level was elevated at 4.1 g/dl, (normal value < 1 g/dl), and a blood transfusion was required. The direct fraction of the bilirubin was elevated after day 7, secondary to inspissated bile syndrome caused by hepatic cellular injury from the hemolysis. Additional tests revealed low thyroid function and blood in the urine. The infant was discharged from the hospital on day 48 of life and was reportedly "neurologically and developmentally" healthy [McEnerney, 1983].

- During amniocentesis, 7 ml of 1% methylene blue was injected into the amniotic cavity of a 30-year-old mother at 37 weeks gestation. Three days following amniocentesis, a female infant was delivered. At birth the baby was stained bright blue, required nasopharyngeal suction and facial oxygen. Mild respiratory distress occurred at age 3 hours. At 23 hours of age, the baby was noted to be jaundiced. Clinical laboratory tests revealed acute hemolysis and hyperbilirubinemia. Phototherapy was initiated and two exchange transfusions were performed. Although the baby was slow to progress, she was discharged at age 17 days in good health [Crooks, 1982].

- A 27 year-old woman underwent amniocentesis at 32 weeks gestation to determine fetal lung maturity of her developing twins. Less than 50 mg of methylene blue was injected into the amniotic sac of the first twin. Twenty-six hours later, the infants were delivered by cesarean section. At birth, the first twin's skin was stained blue and Apgar scores of 7 and 8 were determined at 1 and 5 minutes, respectively. Mild respiratory distress and two brief spells of bradycardia were noted during the first 36 hours after birth. Marked hyperbilirubinemia and Heinz-body anemia were also diagnosed. The twin was treated with phototherapy and received packed erythrocyte transfusions for the anemia. At 24 days of age, the infant was discharged. The second twin sustained none of these complications, although she was born with a severe defect of both legs, having only femoral stumps [Vincer, et al., 1987].

- Methylene blue (1% solution) was injected into the amniotic cavity during week 41 of gestation to detect premature membrane rupture. Because a decelerating fetal heart rate was detected after methylene blue administration, a cesarean section was performed.

  At birth, the infant's skin, oral mucosa and urine were stained a deep blue. The infant became jaundiced and developed hemolytic anemia and hyperbilirubinemia. The infant was treated with phototherapy and by three weeks of age, symptoms of methylene blue toxicity had subsided [Serota, et al., 1979].

- Cases concerning four newborns delivered to mothers who received an intra-amniotic injection of 3.5 ml of 1% methylene blue solution from 24 hours to 5 weeks before

delivery have been described in a letter to the editor. The infants developed hemolytic anemia and hyperbilirubinemia. Two of the infants developed sepsis and one died from gram-negative septicemia [Plunkett, 1973].

- Methylene blue (1% solution) was injected into the amniotic cavity of a 29-year-old woman during week 36 of pregnancy. Ruptured membranes were confirmed by this procedure, and labor was subsequently induced. After delivery the infant appeared depressed. The Apgar score was 6 at 1 minute and the infant required oropharyngeal suction and administration of oxygen. The infant had a bluish tinge and developed significant respiratory distress. He proceeded to develop hemolysis and hyperbilirubinemia.

  Following 2 exchange transfusions and phototherapy, his symptoms gradually diminished. The infant's skin color was noted to gradually lighten, and the bluish discoloration completely disappeared 72 hours after delivery [Cowett, et al., 1976].

3. Animal Data

Adverse effects resulting from acute toxicity in animals exposed to methylene blue by parenteral administration include hemoconcentration, hypothermia, acidosis, hypercapnia, hypoxia, increased blood pressure and increased frequency and amplitude of respiration [Gosselin, et al, 1984].

Methylene blue has been found to cause corneal and conjunctival injury in rabbits. Intracameral injection of methylene blue has reportedly caused loss of cells from the anterior polar region of the lens, with temporary opacity. This condition reportedly cleared as repopulation of cells from the periphery occurred [Grant, 1986].

LD_{50} animal studies on methylene blue found in the literature are summarized in Table 2.

- The short-term toxicity of methylene blue was studied in two species of catfish, *Heteropneustes fossilis* and *Mystus vittatus*. Ten groups of 20 *Heteropneustes fossilis* were exposed to methylene blue at concentrations of 140, 150, 160, 170, 180, 190, 200, 210, 220, and 230 ppm for 96 hours. A control group of 20 *H. fossilis* were exposed to tap water only. Ten groups of 20 *Mystus vittatus* were exposed to methylene blue at concentrations of 5, 10, 20, 25, 30, 35, 40, 45, and 50 ppm. A control group was used as described above.

  The LC_{50} of methylene blue in *Heteropneustes fossilis* was 188.5, 181.5, 172.0. and 165.5 ppm for 24, 48, 72, and 96 hours, respectively. LC_{50} values for *Mystus vittatus* were 27.5, 24.0, 22.0, and 18.5 ppm for 24, 48, 72, and 96 hours, respectively. The no-effect concentration of methylene blue for *Herteropneustes fossilis* was 50.48 ppm and 5.48 ppm for *Mystus vittatus*. Toxic effects observed for both species of catfish included irritation as evidenced by erratic movements and violent actions of the pelvic fins. At high concentrations, signs of restlessness, muscle spasm, body torsion and massive mucous secretion from the general body surface of the fish were observed, and the fish became sluggish and lethargic [Ahmed, et al., 1983].

  Numerous reports have indicated an association between methylene blue and the formation of Heinz bodies in animals, caused by the irreversible oxidative denaturation of hemoglobin [Christiansen, 1980; Rentsch and Wittekind, 1967; Ridder and Oehme, 1974].

Studies concerning the effects of methylene blue on Heinz body formation and other hematological parameters are summarized in Table 3.

C. Human/Animal Data

- Heparinized blood samples from dogs and cats of mixed breeding and from human volunteers were used to measure *in vitro* Heinz body production in the presence of methylene blue. Erythrocytes were suspended in a buffer in which methylene blue was present at a final concentration of 6.7 μM.

  In the erythrocytes of the cat, small Heinz bodies began to appear after two hours of incubation and were present at high concentrations after four hours. In contrast, dog and human erythrocytes did not contain Heinz bodies until six hours after incubation, and the Heinz bodies were smaller than those seen in the feline erythrocytes. Feline erythrocytes incubated under identical conditions without methylene blue did not develop Heinz bodies [Harvey and Kaneko, 1974].

- Prechronic

1. Human Data

   No data were found on the prechronic effects of methylene blue in humans.

2. Animal Data

   No data were found on the prechronic effects of methylene blue in animals.

- Chronic/Carcinogenicity

1. Human Data

   Chronic application of methylene blue-containing eye drops has been found to result in strong staining of bulbar and palpebral conjunctiva, the lid margins and slight staining of the corneal epithelium [Grant, 1986]. No other data were found on the chronic effects of methylene blue in humans. In addition, no data were found on the carcinogenic effects of methylene blue in humans.

2. Animal Data

   - In a study concerning dietary carcinogenesis, twenty-seven dyes, including methylene blue, were fed to Wistar rats at a concentration of 4 percent in the diet for a duration of two years. Each dye was tested in five male rats and 5 female rats. A total of fifty rats were used in the control group. Five of the dyes tested were found to cause malignant tumors of the lymphatic system, and three other dyes induced cirrhotic changes in the liver accompanied by numerous hyperplastic foci. However, methylene blue was not found to induce carcinogenesis or hepatic cirrhotic changes [Willheim and Ivy, 1953].

   - The effects of chronic exposure to methylene blue were studied in freshwater catfish, *Mystus vittatus*. Fifty catfish were exposed for 56 days to methylene blue at a concentration of 7 ppm. Fish were sacrificed after 1, 2, 3, 4, 5, 6, 7, 14, 21, 28, and 56 days. A control group with 15 fish was exposed to tap water only. Chronic effects from exposure to methylene blue after 21 days included swollen and raised epithelium with sloughing. After 28 days, the epithelium was heavily necrotized and degenerated. After 56 days of exposure, necrosis and sloughing of the respiratory epithelium left the pillar cell systems exposed [Ahmed, 1984].

- Fifty freshwater fish, *Heteropneustes fossilis*, were exposed to a sublethal concentration (100 ppm) of methylene blue for 84 days. (A control group with 15 fish was exposed to tap water only.) Fish were sacrificed after 1, 2, 3, 4, 5, 6, 7, 14, 21, 28, 56 and 84 days. After 56 days of exposure to methylene blue, vacuolation in the cytoplasm of the hepatocytes and pyknosis was observed. After 84 days of exposure, the cellular organization had become disturbed and karyorrhexis and karolysis in nuclei, liver cord disarray, and bizarre and eccentric nuclei had appeared.

  After 28 days of exposure to methylene blue, hypertrophy and hyperplasia in the mucous cells of the skin had led to distortion of these cells and to massive mucous secretion. The maximum degree of severity was observed after 84 days of exposure, at which time complete loss of cellular organization was observed [Ahmed and Shrivastava, 1985].

- Reproductive Effects and Teratogenicity

1. Case Reports

   - The effect of methylene blue on reproductive tract fluids was examined using the fallopian tube secretions and uterine and peritoneal fluids from female volunteers. The women were fertile, between the ages of 18 and 40, and had undergone laparotomy for tubal reanastomosis. Methylene blue and normal saline were added to the aliquots of the reproductive tract fluids of the experimental and control groups respectively. Final concentrations of 0.0005%, 0.001%, 0.005%, 0.01%, and 0.05% methylene blue by volume were used.

     Methylene blue induced alterations in the electrophoretic profile of proteins or polypeptides in all reproductive tract fluids examined. Artificially high levels of follicle-stimulating hormone (FSH) and estradiol (E2), directly measured by radioimmunoassay, were identified. Methylene blue at concentrations of 0.005%, 0.01%, and 0.05% increased levels of FSH and E2 by 20% ($p<0.05$), 60% ($p<0.05$), and 160% ($p<0.05$), respectively.

     In this same study, the effect of methylene blue on sperm motility was examined. Semen from men believed to have normal fertility (sperm motility > 85%, sperm concentration at least 40 x 106) was used to prepare aliquots. The aliquots were incubated, assessed for initial motility, and added to a medium containing methylene blue at concentrations described above. Sperm motility was measured after 5, 15, 30, 45, and 60 minutes.

     Sperm motility was found to be significantly inhibited by methylene blue in a time- and dose-dependent manner ($p<0.0001$). At the lowest concentration of methylene blue (0.0005%), less than 50% were motile after 15 minutes, less than 25% were motile after 30 minutes and less than 15% were motile after 45 minutes. Results of sperm motility measurements were similar at concentrations of 0.001%, while at higher concentrations of methylene blue, inhibition of sperm motility was increased. At concentrations of 0.01% methylene blue, less than 5% of the sperm were motile after 5 minutes. After 45 and 60 minutes, sperm were unable to recover motility after exposure to methylene blue at concentrations greater than 0.005% [Coddington, et al., 1989].

2. Animal Data

   - The effect of methylene blue on embryo cleavage and growth was examined using embryos (2-cell) collected from mice (strain not specified) that were superovulated. The embryos were cultured in medium containing methylene blue at concentrations of 0.0005%, 0.001%, 0.005%,

0.01%, or 0.05%. Cultures were examined daily for growth, and embryos reaching each developmental stage were scored after three days in culture. Methylene blue had no effect on pH, osmolarity, or conductivity in the medium, regardless of dose. Addition of methylene blue completely inhibited the ability of 2-cell mouse embryos to continue to grow and cleave. In contrast, in the cultures containing no methylene blue, greater than 90% of the embryos reached the morula or blastocyst stage within three days [Coddington, et al., 1989].

- Walter Reed Carworth Farm strain rats in their first gestation were randomly assigned to experimental and control groups (number of rats per group not specified). The experimental group was given 0.5 grams of methylene blue in their diet. Twenty-two days after positive mating, the pregnant rats were sacrificed and the young were delivered by cesarean section. Macroscopic examination of the uterus was made to identify resorption sites.

  Methylene blue was found to cause maternal toxicity, as poor pregnancy weight gain was observed. No other information on maternal effects, including reference to a maximum tolerated dose, was provided.

  From the 10 litters born to rats exposed to methylene blue, 100 implantations were identified. Sixty-two normal fetuses were delivered. Ninety percent of the litters had one or more resorptions and 38.0% of all recognizable implantations had terminated in resorptions. In the control group, 40.8% of the litters had one or more resorptions and 10.6 % of all implantations reportedly terminated in resorptions (p values not reported) [Telford, et al., 1962].

- Genetic Toxicology

1. Human Data

   No data were found on the genetic effects of methylene blue in humans.

2. Prokaryotic Data

   - The mutagenicity of methylene blue in *Micrococcus pyogenes var, aureus* strain FDA209 was tested *in vitro* using penicillin and streptomycin resistance as genetic markers. Methylene blue, at a concentration of 0.002% in nutrient broth, was found to be mutagenic to *Micrococcus aureus*, based on the number of penicillin and streptomycin- resistant cells observed [Clark, 1953].

   - Methylene blue, at a concentration of 20 μg/plate, was tested *in vitro* in *Salmonella typhimurium* strain TA 100, in the presence and absence of rat S-9 metabolic activation or EDTA-Fe according to the Ames Test (with modifications).

     Methylene blue was found to be mutagenic under all test conditions. However, the mutagenicity of methylene blue was not enhanced by the addition of S9 or EDTA-Fe. Further, the addition of superoxide dismutase (100 μg/ml) inhibited the mutagenicity of methylene blue by 40%, although complete inhibition was not obtained. 2,5-Dimethyl furan (1x10-3 M), D-alpha-tocopherol (5x10-4 M), and hydroquinone (5x10-5 M) markedly inhibited the mutagenicity of methylene blue at a concentration of 8 μg/plate, while ethanol (0.1 M), D-mannitol (10 mM), formic acid (2 mM), and 1,1-dimethoxyethane (0.5 M) did not significantly affect the mutagenicity of methylene blue. The authors reported that singlet oxygen recoverers inhibited the mutagenicity of methylene blue. However, hydroxy radical scavengers did not significantly affect the mutagenicity of this compound [Yamaguchi, 1981].

   - Methylene blue was tested at concentrations of 5, 10, 25, 50, 100, 250, 500, and 1,000 μg in

Salmonella typhimurium strain TA 98 in the *Salmonella*-mammalian microsome mutagenicity test. Methylene blue yielded positive results with and without metabolic activation, but was found to be a more potent mutagen in the presence of metabolic activation [Chung, et al., 1981].

- Methylene blue was tested *in vitro* in the microsuspension assay on seven strains of *Escherichia coli* (WP2, WP2 uvr A, WP67 uvrA- polA-, CM611 uvr- lexA-, WP100 uvrA-recA-, W3110 polA+, and p3478 polA-) in the absence of metabolic activation. The specific dose of methylene blue was not reported, although it was determined in preliminary solubility and toxicity studies. Methylene blue was found to be mutagenic to three strains of *E. coli* (WP100, W3110, and p3478) [McCarroll, et al., 1981].

- Methylene blue was tested at concentrations of 70 μM and 700 μM *in vitro* in the DNA-cell binding (DCB) assay. Methylene blue was mutagenic to *Escherichia coli* in the presence of metabolic activation [Kubinski, et al., 1981].

- Methylene blue (concentration not specified) was tested in vitro in *Bacillus subtilis*, strains H17 (rec+) and M45 (rec-), to determine its ability to induce DNA damage. Methylene blue was found to be non-mutagenic to both stains of *Bacillus subtilis* [Kada, 1973].

  Conflicting results were found in an abstract describing a Japanese study which reported that methylene blue was mutagenic to *Bacillus subtilis* (strain not specified) in the rec-assay. In addition, these authors report that methylene blue was also mutagenic to *Escherichia coli* (strain not specified) and *Salmonella typhimurium* (strain not specified). These latter studies were conducted both in the light and in the dark [Fujita et al., 1976].

- Mutation induction via irradiation with visible light in the presence of methylene blue has been tested in bacteriophage (*Serratiaphage x*). Based on the detection of plaques (clear or lightly turbid) among the turbid, wild-type plaques, methylene blue (concentration not reported) was found to be mutagenic in this test system. Randomly picked mutants were tested for their allocation to one of the four genome regions. Irradiation of *Serratiaphage x* that had been sensitized with methylene blue induced mutations nearly exclusively in the cIII region of the phage DNA [Brendel, 1973].

- Photodynamic mutagenesis has been studied in chemostat cultures of *Escherichia coli*, strain B/r (T1R trp)in the presence of 1-2 μM methylene blue.

  Methylene blue was found to be an "effective" sensitizing agent for mutagenesis. Methylene blue mutation rates appeared to be proportional to fluence rate and dye concentration. In continuous cultures, rates of photomutagenesis of methylene blue were independent of growth rate [Webb, et al., 1979].

- The mutagenicity of methylene blue has been tested in repair-deficient strains of *E. coli* B/r (T1R trp) in the dark. In addition to *E. coli* B/r(T1R trp) and K12 AB1157 (repair-proficient strains), the following strains were used: WP2 (trp, wild-type for repair), WP2s (trp uvrA), WP10 (trp recA) and WP6 (trp pol A1). Methylene blue, at a concentration of 2 μM, was found to increase the mutation rate in the dark 2.9-fold in WP2s, 2.8- fold in WP10, 2.2-fold in WP6, 2.0 fold in WP2 and 3.2 fold in K12 AB1157. Based on this data, methylene blue was reported to be a moderate mutagen in strain WP6 and the other repair-deficient strains tested [Webb, et al., 1984].

- Methylene blue has been found to cause frameshift mutations and base-pair substitutions in several strains of *Salmonella typhimurium* exposed to photodynamic treatment.

Three strains of *Salmonella typhimurium* containing the base pair substitution mutation his G46 (DG2657, DG2678, TA1530) were exposed to a visible light source for various times (20 to 90 seconds) following sensitization for 30 minutes with 30 μg/ml of methylene blue. Methylene blue was found to cause photodynamic-induced base-pair substitution in the three strains tested. The frequency of induced revertants was significantly higher in strain TA1530 (uvrB) than in DG2657 (wild-type), and was significantly lower in strain DG2678 (rec) than in either of the other two strains (p values not reported).

Three strains of *S. typhimurium* containing the frameshift mutation hisD3052 were exposed to a visible light source for 30 to 120 seconds, following sensitization for 30 minutes with 30 μg/ml of methylene blue. Frameshift mutations were induced in all of the strains tested. The frequency of induced revertants was significantly higher in strain DG2659 (wild type), than in both DG2666 (rec) and DG2594 (uvrB) [Imray and MacPhee, 1975].

- The mutagenic effect of visible light in the presence of methylene blue on *Salmonella typhimurium* strains TA1535, TA100, TA2638, and TA104 has been reported. Methylene blue was added at a concentration of 10 μg/ml, and cells were illuminated with visible light for 0-5 minutes.

  The combined treatment of methylene blue and visible light was found to induce DNA damage (primarily base modifications) in all strains tested. *Salmonella typhimurium* strains treated with methylene blue in the absence of visible light were not mutated. In addition, visible light in the absence of methylene blue reportedly caused insignificant (TA100, TA 2638) or very low (TA104) mutation rates. The authors reported that singlet oxygen is the reactive species in *S. typhimurium* DNA damage induced by methylene blue/visible light [Epe, et al., 1989].

3. Eukaryotic Data

   - Methylene blue was tested *in vivo* at concentrations of 1, 3, 6, and 12 mg/kg in the sister chromatid exchange (SCE) test by intraperitoneal injection into the bone marrow of Chinese hamsters. Methylene blue did not cause a significant induction of SCEs compared to a control group receiving no test compound [Speit, 1982].

   - Methylene blue was tested *in vitro* at a concentration of 20 μM for 5 hours in a Chinese hamster ovary cell line (line not specified). Methylene blue did not induce significant chromosome damage in this cell line compared to controls treated with water or ethanol [Au and Hsu, 1979].

   - Methylene blue at a concentration of 1.0 μg/ml was not found to induce SCEs or structural chromosome aberrations in cultured V79-4 Chinese hamster lung fibroblasts [Popescu, et al., 1977].

   - Methylene blue was tested at a concentration of 0.1% in standard food medium in *Drosophila melanogaster*, wild-type stock +S50. Methylene blue was non-mutagenic in this test system, as demonstrated by its failure to significantly increase the frequency of sex-linked recessive lethal mutations [Clark, 1953].

- Other Toxicological Effects

1. Immunotoxicity

   No data were found on the immunotoxic effects of methylene blue.

2. Neurotoxicity

    - In order to examine the neurotoxic effects of methylene blue, slices of young rat (strain not identified) cerebellum were incubated with methylene blue for one hour. Ten μM of methylene blue caused damage to the edges of the incubated cells, while 100 μM of methylene blue resulted in complete destruction of the differentiating cell layers [Garthwaite, 1988].

    - One cat (strain not specified) was administered a single epidural injection of 1% methylene blue (pH 3.2). Another cat was injected five times with 1% methylene blue (pH3.2), and three additional cats received five injections of 1% methylene blue (pH 7.0). The four cats that received multiple injections became agitated immediately following the second injection. Other abnormalities in these four cats included reluctance to move, varying degrees of paraplegia, dragging of hind legs and inability to stand. The fifth cat developed flaccid paralysis of the hind limbs five minutes after the first injection.

      Gross examination of the spinal cords of all five cats revealed blue discoloration of the dura, leptomeninges and spinal cord. Microscopic examination of the spinal cord showed acute inflammation of the leptomeninges and nerve roots, deposition of fibrin, localized necrosis of the dura, swelling and inflammation of myelin sheaths and axonal swelling in the anterior and posterior roots of the grey matter.

      Eight cats in this same study that received epidural injections of either physiologic saline or prilocaine solution exhibited no significant gross or microscopic changes [Poppers, et al., 1970].

    - Application of 0.25% and 0.5% methylene blue in Ringer's solution to frog sciatic nerves resulted in no alteration in the form or amplitude of the compound action potential, or change in the conduction velocity of the nerve. Microscopic examination of four sciatic-peroneal nerves immersed in 1% methylene blue for 24 hours revealed no abnormalities in the myelin sheaths or Schwann and endoneurial cells, and most axons were well preserved. The control sciatic-peroneal nerve exposed to physiologic saline solution also showed no pathologic changes [Poppers, et al. 1970].

3. Biochemical Toxicology

    Methylene blue acts as an electron acceptor in the transfer of electrons from reduced pyridine nucleotides (NADPH and NATPH) to methemoglobin, facilitating reduction of ferric to ferrous iron. Methylene blue is readily reduced to leucomethylene blue, which is readily reoxidized to methylene blue [Gennaro, 1985].

    After the administration of methylene blue in the treatment of methemo-globinemia, methylene blue is converted to a leuco base by the coenzyme diphosphopyridine nucleotide (DPN). This leuco base rapidly reduces ferric iron (Fe3+) to ferrous iron (Fe2+) [Dreisbach, 1980]. If the dose of methylene blue is high, the oxidation potential favors the formation of methemoglobin from hemoglobin [Gennaro, 1985].

    The leucomethylene blue formed reduces oxygen to hydrogen peroxide. The hydrogen peroxide formed is initially detoxified through the hexose monophosphate shunt. When the capacity of the shunt is exhausted, reduced glutathione is depleted. The excess hydrogen peroxide then oxidizes both membrane lipid components and hemoglobin. The oxidation of the hemoglobin leads to the formation of sulfhemoglobin and consequently Heinz bodies. Hemolysis is thought to be secondary to the damage to the lipid membrane and the pitting of the Heinz bodies by the reticuloendothelial cells (See Figure 1,

Heinz Body Formation From Excess Methylene Blue) [Kirsch and Cohen, 1980].

- In an article describing the use of methylene blue in the treatment of acute nitrate toxicity, it was postulated that the methylene blue opens a new pathway for reducing methemoglobin which requires TPNH (triphosphopyridine nucleotide methomemoglobin reductase) and coenzyme factor [Ridder, 1974].

- It has been proposed that an enzymatic process occurs in the decomposition of methylene blue. Demethylation in the liver may be the first step in the decomposition of methylene blue to the Heinz body producing agent. According to this theory, decomposition of methylene blue must continue to occur after demethylation, although the mechanism of this second step is still in question [Rentsch and Wittekind, 1967].

- Heparinized blood samples from dogs and cats of mixed breeding and from human volunteers were used to measure glutathione (GSH) stability after exposure to methylene blue. Erythrocytes were suspended in buffer and incubated with and without methylene blue (6.7 μM), in the presence and absence of glucose.

  In the absence of methylene blue, there was a significant decrease in GSH ($p<0.01$) in feline erythrocytes incubated with glucose. Percent of preincubation glucose remaining was 85% in feline erythrocytes and 99%-100% in dog and human erythrocytes.

  When methylene blue was included in the media, there was a significant decrease in GSH in the red blood cells of the dog and cat ($p<0.01$ and <0.001, respectively). This decrease was not seen in the human red blood cells. Ninety-five percent of the GSH was still present at the end of the incubation period in the human red blood cells, compared to 64% in the cat and 76% in the dog. When glucose was not included in the media, there was no significant difference between any of the species in the percent of GSH remaining in the red blood cells. This was true whether methylene blue was present or not [Harvey and Kaneko 1974].

- **STRUCTURE ACTIVITY RELATIONSHIPS**

No data were found on structure activity relationships for methylene blue.

FAD = Flavin Adenine Dinucleotide
NAD = Nicotinamide Adenine Dinucleotide
GSH = reduced Glutathione
GSSG = oxidized Glutathione
NADP = Nicotinamide Adenine Dinucleotide Phosphate
[Kirsch and Cohen, 1980]

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Table 1. Tissue Uptake of Methylene Blue After Intravenous Administration to the Rat

	Methylene Blue Bound (mcg/g tissue)					
	Dose, mg/kg					
Tissue	2	5	7.5	10	15	25
Heart	15.8	40.7	45.5	46.8	97.9	114.0
Lung	9.6	31.0	28.5	23.5	49.1	80.9
Liver	13.7	45.9	37.2	41.1	107.0	77.6
Kidney	18.2	52.3	78.6	93.2	124.0	386.0

TABLE 2. Studies on Acute Exposure to Methylene Blue in Animals

Species/ Strain	Number of Animals/ Sex	Doses	Route of Administration	Comments	Reference
Rat (strain not specified)	Not Available	LD_{50}=1250 mg/kg	Intravenous	Effects not reported	Schmidt and Haym, 1968
Sheep (mixed breed)	20/Female	LD_{50}=42.3 mg/kg	Intravenous	Effects not reported	Burrows, 1984
Cats (strain not specified)	Not Available	LD_{LO}=41 mg/kg	Intravenous	Toxic effects: transient rise in blood pressure, respiratory stimulation, hyperpyrexia, methemoglobi-nemia	Macht and Harden, 1933

Table 3. Studies on the Effects of Methylene Blue on Hematological Parameters and the Formation of Heinz Bodies.

Species/Sex/Strain/Number	Route of Administration	Dose of Methylene Blue	Heinz Body Formation (% Erythrocytes)	Other Adverse Effects	Reference
Dog/M/Swiss Beagles/6	Intraperitoneal	100mg/kg	(100)	NA	
Mice/UN[a.]/Fullinsdorf Albino/45	Intraperitoneal	200mg/kg	(100)	NA	
Rats/UN/Fullinsdorf Albino/12	Intraperitoneal	200 mg/kg	(12)	NA	
Guinea Pig/UN/Fullinsdorf Himalayan/10	Intraperitoneal	100 mg/kg	(5)	NA	
Cat/M&F/Commercial/ 6	Intraperitoneal	100 mg/kg	(100)	NA	
Rabbits/M&F/Commercial/9	Intraperitoneal	200 mg/kg	(70)	NA	[Rentsch and Wittekind, 1967]
Dog/UN/UN/24	Intravenous	1,3,5 mg/kg	Dose Dependent(UN)	None	[Fingeroth et al., 1987]
Cat/UN/Domestic/1	Oral (in prescription of 50 mg Amoxicillin, Urised)	UN	(100)	bluish hue to skin, increased total & direct bilirubin	[Quinley, 1987]
Cat/UN/UN/6	Stomach Tube	50 mg/kg[b.]	5 Fold Increase[c.] (90)[d.]	-intravascular hemolysis[e.] -methemoglobin (0.0-5.6%)	[Spicer and Thompson, 1949]
Dog/UN/Mongrel Greyhound/4	Subcutaneous injection into web spaces	2 ml of 1%	NA	-inflamed ulcer w/psuedoepithelioma tous hyperplasia in 1 dog -1 μM particles in macrophages	[Perry and Meinhard, 1974]
Rat/F/Sprague-Dawley/14	Intraperitoneal	65 mg/kg	NA	Dec. blood pH	
Rat/M/Sprague-Dawley/11	Intraperitoneal	65 mg/kg	NA	Dec. blood pH Dec. O_2 Inc. CO_2	
Dog/F/UN/3	Infused via catheter into foreleg vein	20 mg/kg	NA	Inc. hematocrit value (6%±2%) Inc. respiratory rate (12 beats/min to 30 beats/min)	
Rat/22F-19M/Sprague-Dawley/41	Intraperitoneal	65 mg/kg[f.] 65 mg/kg[g.]	NA	Inc. hematocrit (7% in unsplenectomized)	[Stossel, 1969]

	65 mg/kg[g]		Inc. hematocrit (2% in splenectomized) Dec. hemocrit (pretreated) Inc. 12% hematocrit (not pretreated)

a. Unspecified
b. In 3 cats, following administration of 30 mg/kg aniline
c. Seen in cats treated with methylene blue and aniline
d. Seen in cats treated with methylene blue only
e. Four cats, 2 from each treatment with and without aniline died due to intravascular hemolysis within 3-4 days
f. Ten female rats were splenectomized.
g. Eleven male rats received 15 mg/kg phenoxybenzamine by gavage prior to methylene blue administration

Inc. Increase
Dec. Decrease

APPENDIX I. ON-LINE DATABASES SEARCHED

	DATE OF SEARCH	TIME PERIOD
BRS:		
HZDB	May, 1990	
DIALOG:		
Agricola	May, 1990	1970-1990
Agris International	May, 1990	1974-1990
Aquatic Sciences Abstracts	May, 1990	1974-1990
Biosis Previews	May, 1990	1969-1990
CAB Abstracts	May, 1990	1972-1990
Cancerlit	May, 1990	1963-1990
Chem Bus Newsbase	March, 1990	1984-1990
Chemical Exposure	April,1990	1974-1987
Compendex Plus	February, 1990	1970-1990
CRIS USDA	February, 1990	
Embase	February, 1990	1974-1990
Enviroline	May, 1990	1970-1990
Environmental Bibliography	May, 1990	1974-1990
Federal Register	May, 1990	1977-1990
Foods Adlibra	February, 1990	1974-1990
FSTA	May, 1990	1969-1990
Life Sciences Collection	February, 1990	1978-1990
Medline	May, 1990	1966-1990
NTIS	May, 1990	1964-1990
Occupational Safety and Health	May, 1990	1973-1990
PTS Newsletter	May, 1990	1987-1990
PTS Prompt	February, 1990	1972-1990
Pollution Abstracts	May, 1990	1970-1990
Trade and Industry ASAP	February, 1990	1983-1990
MEAD:		
Nexis/Lexis-BNA ENV	May, 1990	
NLM:		
Chemline	May, 1990	
HSDB	May, 1990	
RTECS	May, 1990	
Toxline 65	May, 1990	1965-1980
Toxline	May, 1990	1981-1990
Toxlit	May, 1990	1981-1990
Toxlit 65	May, 1990	1965-1980
STN:		
CA	May, 1990	1967-1990
Chemlist	May, 1990	

APPENDIX II. SAFETY INFORMATION

- **HANDLING AND STORAGE**

  Methylene blue is stable under normal laboratory conditions.

- **EMERGENCY FIRST AID PROCEDURES**

  Eye: First check the victim for contact lenses and remove if present. Flush victim's eyes with water or normal saline solution for 20 to 30 minutes while simultaneously calling a hospital or poison control center. Do not put any ointments, oils, or medication in the victim's eyes without specific instructions from a physician. Immediately transport the victim to a hospital even if no symptoms (such as redness or irritation) develop.

  Skin: IMMEDIATELY flood affected skin with water while removing and isolating all contaminated clothing. Gently was affected skin areas thoroughly with soap and water. If symptoms such as inflammation or irritation develop, IMMEDIATELY call a physician or go to a hospital for treatment.

  Inhalation: IMMEDIATELY leave the contaminated area and take deep breaths of fresh air. If symptoms (such as wheezing, coughing, shortness of breath, or burning in the mouth, throat, or chest) develop, call a physician and be prepared to transport the victim to a hospital.

  Provide proper respiratory protection to rescuers entering an unknown atmosphere. Whenever possible, Self-Contained Breathing Apparatus (SCBA) should be used.

  Ingestion: If the victim is conscious and not convulsing, give 1 or 2 glasses of water to dilute the chemical and IMMEDIATELY call a hospital or poison control center. Be prepared to transport the victim to a hospital if advised by a physician.

  If the victim is convulsing or unconscious, do not give anything by mouth, ensure that the victim's airway is open and lay the victim on his/her side with the head lower than the body. DO NOT INDUCE VOMITING. IMMEDIATELY TRANSPORT THE VICTIM TO A HOSPITAL.

- **PROTECTIVE EQUIPMENT**

  Eye: Safety glasses

  Gloves: Two pairs of dissimilar protective gloves shall be worn when handling the neat chemical, otherwise one pair. When contact with this chemical has been known to occur, change gloves immediately.

  Clothing: Minimally, a disposable laboratory suit (e.g. Tyvek ®) shall be worn, as specified in the most current NTP Statement of Work or the NTP Health and Safety Minimum Requirements.

  Respiratory Protection: A NIOSH-approved chemical cartridge respirator with an organic vapor and high-efficiency particulate filter cartridge.

- **EXTINGUISHANT**

  Dry chemical, carbon dioxide or halon extinguisher.

- **MONITORING PROCEDURES**

  There is no NIOSH analytical method reported in the NIOSH Manual of Analytical Methods for methylene blue.

- **SPILLS AND LEAKAGE**

Persons not wearing the appropriate protective equipment and clothing shall be restricted from areas of spills until cleanup has been completed. When exposure to unknown concentrations may occur, air-purifying respirators may not be used. Chemical cartridge respirators with organic vapor cartridges may not be used when airborne concentrations exceed 1000 ppm.

If methylene blue is spilled the following steps shall be taken:

1. In order to prevent dust formation, use moistened paper towels to clean up a solid spill. Avoid dry sweeping.

2. If a liquid solution is spilled, use vermiculite, sodium bicarbonate, sand, or paper towels to contain and absorb the spill.

3. Clean the spill area with dilute alcohol (approximately 60-70%) followed by a strong soap and warm water washing.

4. Dispose of all absorbed material as hazardous waste.

- **DECONTAMINATION OF LABORATORY EQUIPMENT**

TDMS Terminal:

Whenever feasible, a protective covering (e.g.,plastic wrap) shall be placed over the keyboard when in use.

General Equipment:

Before removing general laboratory equipment (i.e., lab carts, portable hoods and balances) from animal dosing rooms and/or chemical preparation areas, a decontamination process shall be conducted in addition to routine housekeeping procedures.

- **WASTE MANAGEMENT AND DISPOSAL PROCEDURES**

Waste Management:

If an inhalation study is to be conducted, all exhaust air from the inhalation chamber must be cleaned with appropriate air cleaning devices unless the laboratory has informed local and state air pollution regulatory agencies of both the laboratory's operating practices and the potential hazards of the chemical's in use. Compliance with all federal, state and local air pollution laws and regulations is required. A specific air cleaning system design must consider the specific conditions of the laboratory (eg., air flow rates and volumes, mixing of exhaust streams, size of inhalation chamber, etc.) and the dosing regimen selected. Air cleaning systems designs must be described by the laboratory and approved by the NTP Office of Laboratory Health and Safety.

Waste Disposal:

Securely package and label, in double bags, all waste material. All potentially contaminated material (i.e., carcasses, bedding, disposable cages, labware) shall be disposed of by incineration in a manner consistent with federal (EPA), state, and local regulations or disposed of in a

licensed hazardous waste landfill.

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1. The information contained in this Executive Summary of Safety and Toxicity Information (ESSTI) is based on data from current published literature. The summary represents information provided in selected sources and is not claimed to be exhaustive.

2. Because methylene blue is used therapeutically as a treatment for nitrate/nitrite intoxication in ruminants, the effect of sodium nitrate on metnylene blue disposition was also investigated.

Note: No documentation for use as food colorant (March, 1998)