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Toxicological Reviews of Cyanobacterial Toxins: Cylindrospermopsin

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LIST OF ABBREVIATIONS

AIC	Aikake's Information Criteria
BMD	Benchmark dose
BMDL	Statistical lower confidence limit on the benchmark dose
BMDS	Benchmark dose software
BMR	Benchmark response
CYP450	Cytochrome P-450
DMSO	Dimethyl sulfoxide
EPA	Environmental Protection Agency
GFR	Glomerular filtration rate
HPLC	High performance liquid chromatography
i.p.	Intraperitoneal
LD_{50}	Dose lethal to 50% of the population
LOAEL	Lowest-observed-adverse-effect level
NOAEL	No-observed-adverse-effect level
PBPK	Physiologically based pharmacokinetic
POD	Point of departure
RfC	Reference concentration
RfD	Reference dose
ROS	Reactive oxygen species
THP	Tamm-Harsfall protein
TPA	O-Tetradecanoylphorbol 13-acetate
UF	Uncertainty factor

PREFACE

The Safe Drinking Water Act (SDWA), as amended in 1996, requires the Environmental Protection Agency (EPA) to publish a list of contaminants that, at the time of publication, are not subject to any proposed or promulgated national primary drinking water regulations, are known or anticipated to occur in public water systems, and may require regulations under SDWA. This list, known as the Contaminant Candidate List (CCL), was first published in 1998 and then again in 2005. The 1998 and 2005 CCLs include "cyanobacteria (blue-green algae), other freshwater algae, and their toxins" as microbial contaminants.

In 2001, a meeting was held among EPA, researchers from the drinking water industry, academia and government agencies with expertise in the area of fresh water algae and their toxins. The goal of this meeting was to convene a panel of scientists to assist in identifying a target list of algal toxins that are likely to pose a health risk in source and finished waters of the drinking water utilities in the U.S. Toxin selection was based on four criteria: health effects, occurrence in the United States, susceptibility to drinking water treatment and toxin stability. Cylindrospermopsin was identified at this meeting as being a toxin of high priority based on those criteria.

The National Center for Environmental Assessment has prepared this Toxicological Review of Cyanobacterial Toxins: Cylindrospermopsin as one in a series of dose-response assessments to support the health assessment of unregulated contaminants on the CCL. The purpose of this document is to compile and evaluate the available data regarding cylindrospermopsin toxicity to aid the Office of Water in regulatory decision making. It is not intended to be a comprehensive treatise on the chemical or toxicological nature of cylindrospermopsin.

In Section 6, *Major Conclusions in the Characterization of Hazard and Dose Response*, EPA has characterized its overall confidence in the quantitative and qualitative aspects of the hazard and dose response by addressing knowledge gaps, uncertainties, quality of data and scientific controversies. The discussion is intended to convey the limitations of the assessment and to aid and guide the Office of Water in the ensuing steps of the human health risk assessment of cylindrospermopsin.

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1. INTRODUCTION

This toxicological review presents background and justification for hazard and dose-response assessments of cylindrospermopsin. U.S. Environmental Protection Agency (EPA) toxicological reviews may include oral reference doses (RfD) and inhalation reference concentrations (RfC) for chronic and less-than-lifetime exposure durations and a carcinogenicity assessment.

The RfD and RfC provide quantitative information for use in risk assessments for health effects known or assumed to be produced through a nonlinear (possibly threshold) mode of action. These reference values are defined as an estimate of an exposure, designated by duration and route, to the human population (including susceptible subgroups), that is likely to be without an appreciable risk of adverse effects. Reference values may be derived for acute ($\leq$24 hours), short-term (up to 30 days), subchronic (up to 10% of average lifespan) and chronic (up to lifetime) exposures, all considered to be continuous exposures throughout the duration specified. A reference value is derived from a BMDL (a statistical lower confidence limit on the benchmark dose), a no-observed-adverse-effect level (NOAEL), lowest-observed-adverse-effect level (LOAEL) or other suitable point of departure with uncertainty/variability factors applied to reflect limitations of the data used. The RfD is expressed in units of mg/kg-day, and the RfC in units of mg/m^3.

The carcinogenicity assessment provides information on the carcinogenic hazard potential of the substance in question and quantitative estimates of risk from oral exposure and inhalation exposure. The information includes a weight-of-evidence judgment of the likelihood that the agent is a human carcinogen and the conditions under which the carcinogenic effects may be expressed. Quantitative risk estimates are presented in three ways. The *slope factor* is the result of application of a low-dose extrapolation procedure and is presented as the risk per mg/kg-day. The *unit risk* is the quantitative estimate in terms of either risk per µg/L drinking water or risk per $\mu g/m^3$ air breathed. Another form in which risk is presented is a drinking water or air concentration providing cancer risks of 1 in 10,000; 1 in 100,000; or 1 in 1,000,000.

Development of these hazard identification and dose-response assessments for cylindrospermopsin has followed the general guidelines for risk assessment as set forth by the National Research Council (NRC, 1983). EPA guidelines that were used in the development of this assessment include the following: *Guidelines for the Health Risk Assessment of Chemical Mixtures* (U.S. EPA, 1986a), *Guidelines for Mutagenicity Risk Assessment* (U.S. EPA, 1986b), *Guidelines for Developmental Toxicity Risk Assessment* (U.S. EPA, 1991), *Guidelines for Reproductive Toxicity Risk Assessment* (U.S. EPA, 1996), *Guidelines for Neurotoxicity Risk Assessment* (U.S. EPA, 1998a), *Guidelines for Carcinogen Assessment* (U.S. EPA, 2005a), *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens* (U.S. EPA, 2005b), *Recommendations for and Documentation of Biological Values for Use in Risk Assessment* (U.S. EPA, 1988), (proposed) *Interim Policy for Particle Size and Limit Concentration Issues in Inhalation Toxicity* (U.S. EPA, 1994a), *Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry* (U.S. EPA, 1994b), *Use of the Benchmark Dose Approach in Health Risk Assessment* (U.S. EPA, 1995),

Science Policy Council Handbook: Peer Review (U.S. EPA, 1998b, 2000a), *Science Policy Council Handbook*: *Risk Characterization* (U.S. EPA, 2000b), *Benchmark Dose Technical Guidance Document* (U.S. EPA, 2000c) and *Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (U.S. EPA, 2000d) and *A Review of the Reference Dose and Reference Concentration Processes* (U.S. EPA, 2002).

Literature searches were conducted for studies relevant to the derivation of toxicity and carcinogenicity values for cylindrospermopsin. The following databases were searched: MEDLINE (PubMed), TOXLINE, BIOSIS, CANCERLIT, TSCATS, CCRIS, DART/ETIC, EMIC, GENETOX, HSDB and RTECS. The relevant literature was reviewed through May 2006.

2. CHEMICAL AND PHYSICAL INFORMATION

Cylindrospermopsin is a naturally occurring toxin produced by particular strains of *Cylindrospermopsis raciborskii* and at least four other freshwater cyanobacterial species, including *Umezakia natans*, *Aphanizomenon ovalisporum*, *Anabaena bergii* and *Raphidiopsis curvata* (Fastner et al., 2003). The chemical structure of cylindrospermopsin was not elucidated until 1992. It consists of a tricyclic guanidine moiety combined with hydroxymethyluracil (Figure 2-1) (Humpage and Falconer, 2003; Ohtani et al., 1992), has a molecular formula of $C_{15}H_{21}N_5O_7S$ and a molecular weight of 415.43 (Lewis, 2000). It is zwitterionic (i.e., a dipolar ion with localized positive and negative charges) (Ohtani et al., 1992). Deoxycylindrospermopsin, an analog of cylindrospermopsin in which the hydroxyl group on the uracil bridge (C-7) has been removed, has been isolated from *C. raciborskii* and *R. curvata* (Li et al., 2001; Norris et al., 1999). Another structural variant of cylindrospermopsin, 7-epicylindrospermopsin, was isolated from *A. ovalisporum* (Banker et al., 2000).

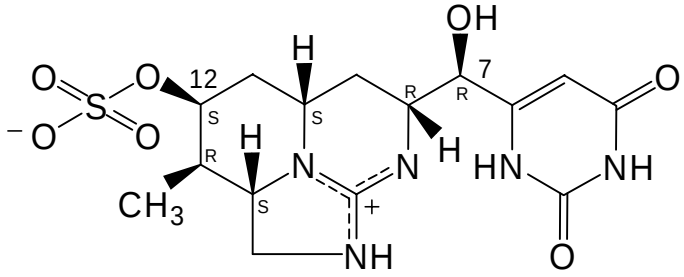

Figure 2-1. Chemical Structure of Cylindrospermopsin*

* Conformations of steriocenters within the structure are indicated as either R or S. The numbers 7 and 12 indicate carbon positions for identification purposes.

Cylindrospermopsin is a white powder that is highly soluble in water (Ohtani et al., 1992; Sigma, 2006). It is also soluble in dimethylsulfoxide (DMSO) and methanol (Sigma, 2006). Cylindrospermopsin is chemically stable in sunlight, at high temperatures and through a wide range of pH values (Chiswell et al., 1999). Additional chemical and physical property data are not available in the open literature for cylindrospermopsin (HSDB, 2006; Lewis, 2000; O'Neil, 2001). This substance is produced on a small scale for research purposes (Sigma, 2006).

3. TOXICOKINETICS

3.1. ABSORPTION

No quantitative data were located regarding the rate or extent of absorption of cylindrospermopsin in humans or animals following oral, inhalation or dermal exposure. Absorption of cylindrospermopsin from the gastrointestinal tract of mice is demonstrated by the induction of hepatic and other systemic effects in 14-day and 11-week oral toxicity studies of pure cylindrospermopsin (Humpage and Falconer, 2003; Shaw et al., 2000, 2001) (see Section 4.2.1).

3.2. DISTRIBUTION

No information was located regarding the tissue distribution of cylindrospermopsin following oral, inhalation or dermal exposure. The distribution and elimination of intraperitoneally (i.p.) administered ^{14}C-cylindrospermopsin (>95% pure; extracted and purified from lyophilized *C. raciborskii* cells) in normal saline was studied in male Quackenbush mice in a series of experiments using sublethal and lethal dose levels of the chemical (Norris et al., 2001). In one experiment, four mice were given a single sublethal dose of 0.1 mg/kg, and urine and feces were collected for the following 48 hours. Most of the ^{14}C was eliminated in the urine and feces, as discussed in Section 3.4. Analysis of liver, kidneys and spleen at 48 hours showed mean ^{14}C recovery of 13.1% of the dose in the liver and <1% in the other tissues. Total recovery of radiolabel was 85-90% of the administered dose in each of the four mice.

The second experiment reported by Norris et al. (2001) included 12 mice administered a single 0.2 mg/kg dose of ^{14}C-cylindrospermopsin, which is the approximate median lethal i.p. dose (Norris et al., 2001). ^{14}C content was determined in the urine and feces in all animals after 12 and 24 hours, and in the liver, kidneys and spleen in five mice that were euthanized after 5-6 days due to toxicity (effects not specified) and after 7 days in the surviving 7 mice that had no signs of toxicity. Most of the ^{14}C was eliminated in the urine and feces, as discussed in Section 3.4. The overall mean recoveries of ^{14}C in the liver, kidneys and spleen after 5-7 days were 2.1, 0.15 and <0.1% of the dose, respectively. Comparison of data from four mice with signs of toxicity and four mice without signs of toxicity showed no clear relationship between toxicity and patterns of tissue distribution, although a trend toward decreased liver retention in the surviving mice was suggested.

Norris et al. (2001) reported a third experiment, in which excretion and tissue distribution were assessed in four mice that were given a 0.2 mg/kg i.p. dose of ^{14}C-cylindrospermopsin and evaluated after 6 hours (Norris et al., 2001). ^{14}C was detected in all tissues that were examined (liver, kidney, heart, lung, spleen, blood and bile), but occurred predominantly in the liver and kidneys (20.6 and 4.3% of the dose, respectively). Approximately 60% of the administered dose of ^{14}C was eliminated in the urine and feces (see Section 3.4).

3.3. METABOLISM

The distribution and elimination of i.p administered ^{14}C-cylindrospermopsin (>95% pure; extracted and purified from lyophilized *C. raciborskii* cells) in saline was studied in a series of mouse experiments (Norris et al., 2001), as detailed in Sections 3.2 and 3.4. Urine, fecal, liver and kidney samples from these studies were extracted with methanol to precipitate proteins, and the ^{14}C in the supernatant was fractionated using high performance liquid chromatography (HPLC) for the detection of metabolites. No attempt was made to fractionate or otherwise identify the ^{14}C in the protein precipitate. Analysis of methanol extracts of urine samples collected for 12 hours following a single dose of 0.1 mg/kg (4 mice) or 0.2 mg/kg (12 mice) suggested that a large part (72%) of the excreted ^{14}C was present as cylindrospermopsin (as determined by retention times). Some (~23.5%) of the urinary ^{14}C was detected in protein precipitated by the methanol, suggesting the presence of a protein-bound metabolite. The authors did not indicate whether the level of protein in the urine was normal or abnormal. Most (94.3%) of the ^{14}C in an aqueous extract of the feces had the same retention time as cylindrospermopsin, but only one mouse dosed with 0.2 mg/kg was tested. Analysis of liver tissue showed the presence of ^{14}C in both methanol extract and protein precipitate. When fractionated by HLPC, the extracted ^{14}C had the same elution characteristics seen in some of the urine methanol extracts, suggesting the presence of the same metabolite. The authors could not rule out the possibility that the non-extractable ^{14}C in the liver was protein-bound cylindrospermopsin itself, although the evidence for metabolic activation of cylindrospermopsin in other studies (Runnegar et al., 1995; Shaw et al., 2000) suggested that it might also be a metabolite. The methanol-extractable metabolite was not found in kidney tissue. No identification of metabolites was performed.

There is evidence indicating that the hepatic cytochrome P-450 (CYP450) enzyme system is involved in the metabolism and toxicity of cylindrospermopsin. As discussed in Section 4.5.1, pretreatment of hepatocytes with known inhibitors of CYP450 diminished the *in vitro* cytotoxicity of cylindrospermopsin (Froscio et al., 2003; Runnegar et al., 1995). Similarly, pretreatment of mice with a CYP450 inhibitor protected against the acute lethality of cylindrospermopsin (Norris et al., 2002). Additionally, a main target of cylindrospermopsin toxicity is the periacinar region of the liver, which is where CYP450-mediated xenobiotic metabolism occurs (Shaw et al., 2000, 2001).

3.4. ELIMINATION

No information was located regarding the elimination of cylindrospermopsin following oral, inhalation or dermal exposure. The elimination of i.p administered ^{14}C-cylindrospermopsin (>95% pure; extracted and purified from lyophilized *C. raciborskii* cells) in saline was studied in male Quackenbush mice in a series of experiments using sublethal and lethal dose levels of the chemical (Norris et al., 2001). In one experiment, four mice were given a single sublethal dose of 0.1 mg/kg, and urine and feces were collected for the following 48 hours. The mean cumulative excretion of ^{14}C in the first 12 hours after dosing was 62.8% of the administered dose in the urine and 15.5% in the feces. There was little additional excretion of ^{14}C in either the urine or feces following 12 additional hours. The 15.5% mean fecal excretion value reflects a very high fecal excretion in one of the four animals (nearly 60% of the dose compared to <5% in

the other mice); the authors considered the possibility that the high value in the one animal resulted from the injection entering the upper gastrointestinal tract, but concluded that this possibility was unlikely given the injection technique used, the recovery of 4.7% of the injected dose in the liver after 48 hours and a similarly high fecal excretion of ^{14}C in another animal in the third experiment in this study (discussed below). Total mean recovery in the urine, feces, liver, kidneys and spleen was 85-90% of the ^{14}C dose in each of the four mice.

The second experiment reported by Norris et al. (2001) included 12 mice administered a single 0.2 mg/kg dose of ^{14}C-cylindrospermopsin, which is the approximate median lethal i.p. dose (Norris et al., 2001). Five of the 12 dosed animals died within 5-6 days (signs of toxicity not reported). ^{14}C content was determined in the urine and feces in all animals after 12 and 24 hours. Results were similar to those obtained with a sublethal dose (reported above), except that there was some continued urinary and fecal excretion over the second 12 hours of the monitoring period. The mean cumulative urinary and fecal excretion of ^{14}C was 66.0 and 5.7% of the dose within 12 hours, and 68.4 and 8.5% of the dose within 24 hours, respectively. The mean total recovery in the urine and feces after 24 hours was 76.9% of the administered dose. The overall mean recoveries of ^{14}C in the liver, kidneys and spleen after 5-7 days were 2.1, 0.15 and <0.1% of the administered dose, respectively. Comparison of data from four mice with signs of toxicity and four mice without signs of toxicity showed no clear relationship between toxicity and patterns of excretion, although trends toward increased urinary excretion and decreased fecal excretion in surviving mice were suggested.

Norris et al. (2001) reported a third experiment in which four mice were given a 0.2 mg/kg i.p. dose of ^{14}C-cylindrospermopsin and evaluated for 6 hours (Norris et al., 2001). The mean cumulative urinary and fecal excretion of ^{14}C after 6 hours was 48.2 and 11.9% of the administered dose, respectively. One of the four mice showed more than 40% of the dose in the feces (additional data not reported).

3.5. PHYSIOLOGICALLY BASED TOXICOKINETIC MODELS

No physiologically based toxicokinetic models have been developed for cylindrospermopsin.

4. HAZARD IDENTIFICATION

4.1. STUDIES IN HUMANS - EPIDEMIOLOGY, CASE REPORTS, CLINICAL CONTROLS

An outbreak of a hepatoenteritis-like illness occurred in 148 residents of aboriginal descent in the Palm Island community in Queensland, Australia, in 1979 (Blyth, 1980; Griffiths and Saker, 2003). The total number of people exposed was not reported. Of the 148 cases, 138 were children (mean age 8.4 years, range 2-16 years, 41% male and 59% female) and 10 were adults (sex and age not reported). The majority of the cases in the outbreak, called the "Palm Island mystery disease," required hospitalization. The clinical symptoms included fever, headache, vomiting, profuse bloody diarrhea, hepatomegaly and renal damage as indicated by loss of water, electrolytes, proteins, ketones and carbohydrates. Many of the individuals required intravenous therapy for electrolyte imbalance and, in some cases, for hypovolemic and acidotic shock. The findings may indicate increased susceptibility of children unless the 138 children were from the households of the 10 adults (not indicated) or if there was differential exposure between the children and the adults (not indicated); the child:adult ratio is approximately 14:1. A few days prior to the outbreak, the major drinking water supply for the island, Solomon Dam reservoir, had been treated with unreported levels of copper sulfate to control a dense algal bloom; only households connected to the reservoir were affected by the illness. Retrospective analyses, including epidemiological and ecological assessments, implicated the predominant cyanobacterial species in the reservoir, *C. raciborskii*, as the likely source of the illness (Griffiths and Saker, 2003; Hawkins et al., 1985). Intraperitoneal injection of cell extracts of *C. raciborskii* from the reservoir caused damage to the liver, kidneys and other organs in mice (Hawkins et al., 1985), and the toxin was later identified as cylindrospermopsin (Ohtani et al., 1992). Some symptoms of acute oral exposure to high concentrations of copper sulfate, including headache, nausea, vomiting and diarrhea (HSDB, 2006), are similar to those observed during the outbreak. The only information that was located regarding a potential role of the copper sulfate treatment in the outbreak is an indication that its algalcidal mode of action, cell lysis, could have contributed to the release of cylindrospermopsin and other cellular toxins into the water (Griffiths and Saker, 2003).

Hayman (1992) investigated reports of disease (sometimes called "Barcoo fever") in the Australian outback dating back as far as 1887. He concluded that the reported symptoms were similar to those of the Palm Island mystery disease and that they might have been caused by exposure to *C. raciborskii*. No additional information was located regarding effects in humans known or suspected to be associated with exposure to cylindrospermopsin.

An outbreak of acute liver failure occurred in patients at a renal dialysis clinic in Caruaru, Brazil (Carmichael et al., 2001). Following routine hemodialysis treatment during a week in February 1996, 116 of 131 patients experienced headache, eye pain, blurred vision, nausea and vomiting. Subsequently, 100 of the affected patients developed acute liver failure and, of these, 76 died. Analysis of the clinic's water treatment system (samples of carbon, sand and cation/anion exchange resin from in-house filters) for microcystins and cylindrospermopsin showed the presence of both cyanotoxins. Analyses of blood sera and liver samples revealed

microcystins, but not cylindrospermopsin, although the method used to extract cylindrospermopsin from these samples may have been inadequate. Based on a comparison of victims' symptoms and liver pathology using animal studies of microcystins and cylindrospermopsin, it was concluded that the major contributing factor to death of the dialysis patients was intravenous exposure to microcystins.

The skin irritant potential of cylindrospermosin was evaluated using skin-patch testing in humans (Pilotto et al., 2004). Both whole and lysed preparations of laboratory-grown *C. raciborskii* cells were applied to the skin of 50 adult volunteers using adhesive patches divided into 10 individual filter pad-containing chambers. Each volunteer was exposed to one patch for whole cells and one patch for lysed cells with each patch containing six cell concentrations, two positive controls (1 and 5% solutions of sodium lauryl sulfate), and two negative controls (culture media and an empty patch). The concentrations (densities) of cells were consistent with those found in *C. raciborskii*-containing water bodies used for recreational water activities. Patches were removed after 24 hours and erythematous reactions were graded as 0 (no reaction or erythema), 1 (minimal or very weak spotty erythema), 2 (mild diffuse erythema), 3 (moderate diffuse erythema) or 4 (severe diffuse erythema with edema) by a dermatologist blinded to the cell type and concentration. The distribution of clinical gradings by patch type (control/active), cell type and cell concentration was assessed using logistic regression modeling. Due to a relatively small number of high-level gradings, each observation was dichotomized into no reaction (grade 0) and a positive reaction (1, 2, 3 or 4) prior to modeling. The subjects were more likely to have skin reactions to the active patches than to the negative control patches for both whole cells (odds ratio (OR) = 2.13, 95% confidence interval (CI) 1.79-4.21, $p<0.001$) and lysed cells (OR = 3.41, 95% CI 2.00-5.84, $p<0.001$). The mean percentages of subjects having a reaction were 20% (95% CI 15-31%) for all subjects (n=50) and 11% (95% CI 6-18%) for subjects not reacting to negative controls (n=39). The irritation was mild and resolved within 24 to 72 hours. There was no evidence of a statistically significant increasing dose-response relationship between skin reactions and increasing cell concentrations for either whole or lysed cells, although there was a slight reduction in response with increasing cell concentration for the whole cells (OR = 0.966, 95% CI 0.936-0.997, $p = 0.03$). Additionally, there was no evidence for a threshold effect (i.e., a particular concentration above which there were frequent or strong reactions).

4.2. ACUTE, SHORT-TERM, SUBCHRONIC AND CHRONIC STUDIES AND CANCER BIOASSAYS IN ANIMALS - ORAL AND INHALATION

Toxicity studies in animals have been performed using pure cylindrospermopsin isolated and purified from cell extracts of *C. raciborskii* or other cylindrospermopsin-producing cyanobacteria. Studies have also been performed in which the administered material consisted of whole cell extracts, lyophilized (freeze-dried) cells in suspension and cell-free extracts of sonicated freeze-dried cells. These studies are included in this report because they contribute salient information to the overall toxicological database for cylindrospermopsin. However, due to confounding factors discussed below, the studies of cell extracts are not useful for dose-response assessment of cylindrospermopsin and are considered supplemental information for hazard identification.

Most of the cell extract studies were performed using laboratory cultures of cyanobacterial cells, but there is no clear means of predicting the cylindrospermopsin content in a particular extract. Studies with cylindrospermopsin and other cyanobacterial toxins indicate that growth conditions can significantly contribute to the level of toxin produced by a given species and strain, and that toxin concentration can also vary depending on the method used to produce a material for toxicological testing (Chiswell et al., 1999; WHO, 1999). The extracellular fraction of cylindrospermopsin can sometimes exceed the intracellular fraction (Griffiths and Saker, 2003). For example, at different stages of a *C. raciborskii* bloom, extracellular cylindrospermopsin ranged from 19 to 98% of the total amount in water (Chiswell et al., 1999). Similarly, during a bloom of *A. ovalisporum*, >85% of the cylindrospermopsin was extracellular (Shaw et al., 1999). In these studies, intracellular concentration of cylindrospermopsin was determined by taking the difference between the concentration in a sample of filtered water and the concentration in a sample of water that was frozen to release the toxin contained in the cells. Extracts obtained by removing intact cells may or may not contain toxin or may have variable amounts of toxin. For example, Falconer et al. (1999) found that the cylindrospermopsin content in four different batches of cell-free extracts of *C. raciborskii* varied from 1.3 to 5.4 mg/g extract. Additionally, cell extracts containing cylindrospermopsin can also contain other potentially toxic substances. The 24-hour i.p. LD_{50} (dose lethal to 50% of the population) of purified cylindrospermopsin in male CH3 mice was 2.1 mg/kg (Ohtani et al., 1992), whereas the value for a cell extract in male Swiss mice was 0.29 mg/kg (Hawkins et al., 1997), nearly an order of magnitude lower. Hawkins et al. (1997) proposed that the difference in potency could reflect the presence of other toxins in the cell extract that were not present in the purified cylindrospermopsin (see Section 4.4.1).

4.2.1. Oral Exposure

4.2.1.1. Acute Studies

4.2.1.1.1. Studies of Purified Cylindrospermopsin

No information regarding the acute oral toxicity of purified cylindrospermopsin was identified in the materials reviewed for this document.

4.2.1.1.2. Cell Extract Studies

Twelve male MF1 mice were administered a saline suspension of freeze-dried *C. raciborskii* cells (strains PHAWT/M or PHAWT/1) by gavage in single reported doses ranging from 4.4 to 8.3 mg/kg (cylindrospermopsin-equivalent), and observed for the following 8 days (Seawright et al., 1999). The following dose levels were tested (one mouse per level except as noted): 4.4, 5.3, 5.7 (two mice), 5.8, 6.2, 6.5, 6.7, 6.8, 6.9, 8.0 and 8.3 mg/kg; there was no control group. Eight of the 12 mice died. The lowest lethal dose was 4.4 mg/kg, the highest nonlethal dose was 6.9 mg/kg and the average lethal dose was approximately 6 mg/kg. Deaths occurred 2-6 days after treatment, and histological examinations showed effects that included fatty liver with periacinar coagulative necrosis, acute renal tubular necrosis, atrophy of the lymphoid tissue of the spleen and thymus, subepicardial and myocardial hemorrhages in the

heart and ulceration of the esophageal section of the gastric mucosa. Some of the animals also developed thrombohemorrhagic lesions in one or both eye orbits.

An aqueous suspension of a cell-free extract of freeze-dried and sonicated *C. raciborskii* cells (strain AWT 205) was administered to an unspecified number of male Swiss mice in a single gavage dose of 1400 mg extract/kg (Falconer et al., 1999). The cylindrospermopsin content of the extract was not specified, but ranged from 1.3 to 5.4 mg/g extract in concurrent i.p. experiments, indicating that the cylindrospermopsin-equivalent gavage dose was likely in the range of 1.8-7.6 mg/kg. This dose level was not fatal, but caused severe liver and kidney pathology. Histological changes were not detailed, but patterns of damage were reported to be similar to those observed following i.p. administration (see Section 4.4.1). Additional information on the design and results of the oral study were not provided.

Another gavage study reported that the minimum lethal dose of a saline extract of freeze-dried *C. raciborskii* cells (strain AWT 205) in Swiss mice was 2500 mg extract/kg (Falconer and Humpage, 2001). Based on a reported cylindrospermopsin content of 5.5 mg/g extract, the equivalent dose of cylindrospermopsin was 13.8 mg/kg.

Groups of four Quackenbush mice were administered a cell-free extract of freeze-dried and sonicated *C. raciborskii* cells (strain AWT 205) in water in a single gavage dose of 0, 1, 2, 4, 6 or 8 mg cylindrospermopsin/kg and observed for the following 7 days (Shaw et al., 2000, 2001). All animals were evaluated for gross pathological and histological (liver, kidney, spleen, heart, lungs and thymus) changes. Hepatic effects were observed at all dose levels, as shown by foamy hepatocellular cytoplasmic changes at 1 and 2 mg/kg, lipid infiltration with some hepatocyte necrosis in the periacinar region at 4 mg/kg, and uniformly pale and mottled livers with lipid infiltration throughout and cell necrosis mainly in the periacinar region at 6 mg/kg. Mortality occurred in 2/4 mice at 6 mg/kg (in 5 days) and 4/4 mice at 8 mg/kg (in 24-48 hours). Additional information on the experimental design and results was not reported.

4.2.1.2. Short-Term Studies

4.2.1.2.1. Studies of Purified Cylindrospermopsin

Groups of four Quackenbush mice were administered purified cylindrospermopsin by daily gavage for 14 days (Shaw et al., 2000, 2001). The cylindrospermopsin was purified (purity not reported) from an extract of freeze-dried *C. raciborskii* cells (strain AWT 205). All animals were evaluated for gross pathological and histological (liver, kidney, spleen, heart, lungs and thymus) changes. The authors identified the following effect levels: a NOAEL of 0.05 mg cylindrospermopsin/kg-day and a LOAEL of 0.15 mg cylindrospermopsin/kg-day for lipid infiltration in the liver, and a NOAEL of 0.3 mg cylindrospermopsin/kg-day (highest tested dose) for lymphophagocytosis in the spleen. Additional information on the experimental design and results was not reported.

4.2.1.2.2. Cell Extract Studies

Groups of four Quackenbush mice were administered an aqueous cell-free extract of freeze-dried and sonicated *C. raciborskii* cells (strain AWT 205) by daily gavage for 14 days (Shaw et al., 2000, 2001). All animals were evaluated for gross pathological and histological (liver, kidney, spleen, heart, lungs and thymus) changes. The authors identified the following effect levels: a NOAEL of 0.05 mg cylindrospermopsin/kg-day and a LOAEL of 0.15 mg cylindrospermopsin/kg-day for lipid infiltration in the liver, and a LOAEL of 0.05 mg cylindrospermopsin/kg-day for lymphophagocytosis in the spleen. Additional information on the experimental design and results was not reported.

4.2.1.2.3. Other Studies

Six Quackenbush mice and two Wistar rats were exposed for 21 days to drinking water containing 800 μg/L cylindrospermopsin (Shaw et al., 2000, 2001). The water was "sourced" from a dammed impoundment. The reported approximate daily dose based on water consumption was 0.2 mg cylindrospermopsin/kg-day in both species. Gross pathological and histological (liver, kidney, spleen, heart, lungs and thymus) examinations showed no effects, indicating that 0.2 mg/kg-day was a NOAEL in the rats and mice. Additional information on the experimental design and results was not reported.

4.2.1.3. Subchronic Studies

4.2.1.3.1. Studies of Purified Cylindrospermopsin

Groups of male Swiss albino mice (10 per dose, 6 in the highest dose group) were administered purified cylindrospermopsin in water by gavage in doses of 0, 30, 60, 120 or 240 μg/kg-day for 11 weeks (Humpage and Falconer, 2003). The cylindrospermopsin was purified (purity not reported) from an extract of freeze-dried *C. raciborskii* cells (strain AWT 205). Endpoints monitored throughout the study included food and water consumption and body weight. A clinical examination that focused on physiological and behavioral signs of toxicity was conducted after 9 weeks of exposure. Hematology (all animals; red cell counts, hemoglobin, packed cell volume, and white cell total and differential counts), serum chemistry (five mice/group except all six mice at the high dose; total protein, albumin, globulin, glucose, creatinine, urea, total bilirubin, total bile acids, cholesterol, triglycerides, sodium, potassium, calcium, bicarbonate, creatinine kinase, alanine and aspartate aminotransferases [ALT and AST, respectively], and alkaline phosphatase) and urine (five mice/group excluding high dose; specific gravity, protein, glucose, ketones, creatinine, sodium, potassium, chloride, calcium, bicarbonate, phosphate, pH, volume and presence of blood) evaluations were performed near or at the end of the treatment period. Postmortem examinations included organ weights (liver, spleen, kidneys, adrenal glands, heart, testis, epididymis and brain) and comprehensive histological evaluations. The histological examinations were conducted in accordance with Organization for Economic Cooperation and Development recommendations and performed on the following tissues: liver, kidney, heart, lungs, thymus, thyroid, trachea, salivary glands, adrenal glands, epididymis, testis, prostate, gall bladder, esophagus, stomach, duodenum/small intestine, large intestine, pancreas,

spleen, urinary bladder, eyes, lymph nodes, aorta, cerebrum, cerebellum, spinal cord (cervical, thoracic and lumbar) and peripheral nerve.

No deaths were reported. The mean final body weight was 7-15% higher than controls in all dose groups, but the increases were not dose-related and were statistically significant only at 30 and 60 μg/kg-day (Humpage and Falconer, 2003). There were no significant changes in food consumption; however, water intake was significantly reduced in all dose groups (data not reported). Relative kidney weight was increased in a significant, dose-related manner beginning at 60 μg/kg-day (12-23% greater than controls), while relative liver weight was significantly increased only at the high dose of 240 μg/kg-day (13% greater than controls). Information on absolute kidney and liver weights was not reported. Absolute testis weights were significantly increased at ≥60 μg/kg-day (data not reported), but these changes were not significant when normalized to body weight. The hematology, serum chemistry and urine evaluations showed no clear exposure-related changes in any endpoint (including serum indicators of liver injury), except for significant decreases in urine protein concentrations (g/mmol creatinine) at ≥120 μg/kg-day and urine specific gravity at 240 μg/kg-day (data presented graphically). The postmortem examinations showed "minor increases in histopathological damage to the liver" at ≥120 μg/kg-day and proximal renal tubular damage at 240 μg/kg-day, but additional information regarding the type, severity and incidences of the liver and kidney lesions was not reported.

Cylindrospermopsin is known to inhibit protein synthesis in the liver (see Section 4.5.1). Serum albumin, a major product of liver protein synthesis, was not decreased in this study (Humpage and Falconer, 2003), but the most sensitive effects, decreased urinary protein at ≥120 μg/kg-day and increased relative kidney weight at ≥60 μg/kg-day, are both potential indicators of suppressed protein synthesis. As hypothesized by the authors, the decrease in urinary protein is consistent with decreased availability of protein and the increase in kidney weight may reflect a compensatory hyperplasia, such that the kidney, as a protein-synthesizing organ, is stimulated to grow in an attempt to maintain homeostasis in response to a chemically-induced decrease in protein synthesis. Information supporting the hypothesis that the decrease in urinary protein excretion reflects a specific effect of cylindrospermopsin on protein synthesis, as well as the possibility that it reflects a functional change in the nephron, is discussed in Section 4.5.2. Because the renal effects observed by Humpage and Falconer (2003) are consistent with a known mode of action of cylindrospermopsin, and plausibly represent part of the progression of effects leading to toxicity (Section 4.5.2), they are considered to be adverse. This study, therefore, identifies a NOAEL and LOAEL of 30 and 60 μg/kg-day, respectively.

4.2.1.3.2. Cell Extract Studies

Groups of male Swiss albino mice (10 per dose except 12 controls and 5 at high-dose) were exposed to a cell-free extract of sonicated and frozen *C. raciborskii* cells (strain AWT 205) in the drinking water at reported cylindrospermopsin doses of 0, 216, 432 or 657 μg/kg-day for 10 weeks (doses based on actual water consumption) (Humpage and Falconer, 2003). Food and water consumption and body weight were measured throughout the study. Urinalyses (12 unspecified parameters) were performed after 5 and 10 weeks. Serum chemistry (15 unspecified parameters) evaluations and examinations of unspecified major organs (organ weight, gross

pathology and histopathology) were performed at the end of the exposure period. Hematology was not evaluated.

Final body weights were significantly reduced at 432 and 657 μg/kg-day (9 and 7% less than controls, respectively), and relative liver and kidney weights were significantly increased in a dose-related manner at 216-657 μg/kg-day (27-47 and 30-43% greater than controls, respectively). Other statistically significant effects included increased serum total bilirubin at ≥216 μg/kg-day, decreased serum total bile acids at ≥216 μg/kg-day and decreased urine protein concentration (g/mmol creatinine) at ≥432 μg/kg-day. There were no clear exposure-related changes in any other serum or urine endpoints and no additional indicators of liver or kidney injury. Results of the postmortem pathology examinations were not reported. The low dose of 216 μg/kg-day is a LOAEL for this study, based on increased relative liver and kidney weights, increased serum bilirubin and decreased serum bile acids. An increase in serum bilirubin is indicative of liver dysfunction or bile duct blockage as it reflects the ability of the liver to take up, process and secrete bilirubin into the bile. Serum bile acids can be decreased due to an inhibition of bile acid synthesis or an interference with bile acid resorption in the gastrointestinal tract; bile acids are synthesized from cholesterol in the liver, conjugated, excreted in the bile and resorbed in the ileum.

Quackenbush mice were administered drinking water containing a cell-free extract of freeze-dried and sonicated *C. raciborskii* cells (strain AWT 205) for 90 days (Shaw et al., 2000, 2001). Gross pathological and histological (liver, kidney, spleen, heart, lungs and thymus) examinations showed no effects at dose levels as high as 0.15 mg cylindrospermopsin/kg-day (the highest tested dose), indicating that a NOAEL of 0.15 mg/kg-day was identified. Additional information on the experimental design and results was not reported. The 0.15 mg/kg-day NOAEL in Quackenbush mice is only slightly below the 216 μg/kg-day (0.22 mg/kg-day) LOAEL for liver and kidney effects in the 10-week study with Swiss mice summarized above (Humpage and Falconer, 2003); however, the LOAEL is based on different measured endpoints (liver and kidney weights, serum bilirubin and serum bile acids) than the NOAEL (histopathology).

4.2.1.4. Chronic Studies

No information regarding the chronic oral toxicity of cylindrospermopsin was identified in the materials reviewed for this document.

4.2.2. Inhalation Exposure

No information regarding the inhalation toxicity of cylindrospermopsin was identified in the materials reviewed for this document.

4.3. REPRODUCTIVE/DEVELOPMENTAL STUDIES - ORAL AND INHALATION

No information regarding the reproductive or developmental toxicity of cylindrospermopsin was identified in the materials reviewed for this document.

4.4. OTHER STUDIES

4.4.1. Effects By Parenteral Exposure.

4.4.1.1. Studies of Purified Cylindrospermopsin

Acute lethality values have been determined for cylindrospermopsin purified from extracts of cultured *C. raciborskii* or *U. natans* cells (Ohtani et al., 1992; Shaw et al., 2000, 2001; Terao et al., 1994). In male CH3 mice, 24-hour and 5- to 6-day LD_{50} values of 2.1 and 0.2 mg/kg, respectively, have been reported for a single i.p. dose of purified cylindrospermopsin (purity not reported) (Ohtani et al., 1992). Another study found that a single 0.2 mg/kg i.p. dose of purified cylindrospermopsin (purity not reported) caused 50% moribundity after 31 hours in Quackenbush mice (Shaw et al., 2000, 2001). The main pathological findings in the moribund animals were lipid infiltration and cell necrosis in the liver. Terao et al. (1994) also found that the liver was the main target of toxicity in male ICR mice administered a single 0.2 mg/kg i.p. dose of purified cylindrospermopsin (purity not reported), although treatment-related lesions were additionally noted in the thymus, kidney and heart. A time series of ultrastructural tissue examinations indicated four sequential phases of liver changes: inhibition of protein synthesis, membrane proliferation, fat droplet accumulation and cell death.

4.4.1.2. Cell Extract Studies

The results of acute i.p. studies of extracts of freeze-dried and sonicated *C. raciborskii* cells are generally similar to those of the i.p. studies of purified cylindrospermopsin. A single 0.2 mg/kg cylindrospermopsin-equivalent dose caused 50% moribundity in Quackenbush mice after 98 hours (Shaw et al., 2000, 2001). Other single-dose LD_{50} values, expressed as cylindrospermopsin-equivalent doses, included 24-hour and 7-day values of 0.29 and 0.18 mg/kg, respectively, in male Swiss mice (Hawkins et al., 1997). This 24-hour LD_{50} was lower than the 24-hour i.p. LD_{50} of 2.1 mg/kg for purified cylindrospermopsin in mice (Ohtani et al., 1992), leading the authors to suggest that the extract contained more than one toxin. The liver was the main target organ in the extract studies, although lesions also occurred in other tissues, including kidney, adrenal gland, lung and intestine (Hawkins et al., 1985, 1997; Shaw et al., 2000, 2001).

A single dose i.p. LD_{50} value of 64 mg freeze-dried culture/kg was determined in mice observed for 24 hours (Hawkins et al., 1985). Falconer et al. (1999) assessed the acute lethality and liver and kidney effects of four different batches of cell-free extracts of sonicated freeze-dried *C. raciborskii* cells in male Swiss albino mice treated by single i.p. injection. Reported 24-hour and 7-day LD_{50} values for the four batches were 50-110 and 20-65 mg extract/kg, respectively. The cylindrospermopsin content in the four batches varied from 1.3 to 5.4 mg/g extract, indicating that the cylindrospermopsin-equivalent LD_{50} values were 0.07-0.6 mg/kg (24-hour) and 0.03-0.4 mg/kg (7-day). Liver damage was characterized by cellular vacuolation, intercellular spaces and darker nuclear and cytoplasmic staining. Kidney damage included proximal tubule epithelial necrosis and presence of proteinaceous material in the distal tubules. There was no clear correlation between cylindrospermopsin batch concentration and the LD_{50}

values or severity of liver or kidney lesions, leading the study authors to suggest that more than one toxin was present in the extract.

4.4.2. Immunotoxicity

No information was located regarding effects of cylindrospermopsin on immune function, although immune system tissues appear to be a target of short-term, high-level exposures. Massive necrosis of lymphocytes occurred in the cortical layer of the thymus of male ICR mice given a single 0.2 mg/kg i.p. dose of cylindrospermopsin purified (purity not reported) from cultured *U. natans* cells (Terao et al., 1994). Effects observed in MF1 mice administered a single gavage dose of a suspension of freeze-dried *C. raciborskii* cells, in the lethal dose range of 4.4-8.3 mg cylindrospermopsin/kg, included atrophy in lymphoid tissue of the spleen (follicular lymphocyte loss due to lymphophagocytosis) and thymus (degeneration and necrosis of cortical lymphocytes) (Seawright et al., 1999). These effects were considered by the study authors to be normal responses of the immune system to the stress of severe intoxication. Lympho-phagocytosis was observed in the spleen of Quackenbush mice exposed to a cell-free extract of freeze-dried and sonicated *C. raciborskii* cells by gavage at a nonlethal dose level of 0.05 mg cylindrospermopsin/kg-day for 14 days (Shaw et al., 2000, 2001).

4.4.3. Tumor Initiation

The tumor initiating activity of cylindrospermopsin was tested in male Swiss mice using O-tetradecanoylphorbol 13-acetate (TPA) as the promoter (Falconer and Humpage, 2001). Mice were administered a gavage dose of saline (27 mice) or 500 mg/kg of a saline extract of freeze-dried *C. raciborskii* cells (strain AWT 205) (34 mice) every other week for three doses. Other groups received a single dose of 1500 mg extract/kg (14 mice) or two doses of 1500 mg extract/kg separated by 2 weeks (17 mice). Most (70%) of the 2 x 1500 mg extract/kg group died within 1 week of the second dose, leaving five survivors for use in the rest of the study. Based on a reported cylindrospermopsin content of 5.5 mg/g extract, the cylindrospermopsin-equivalent doses in the 500 and 1500 mg extract/kg groups were 2.75 and 8.25 mg/kg, respectively. Two weeks after the final dose, the saline and 500 mg extract/kg groups were divided into subgroups of 13-18 mice that were fed liquid food containing TPA dissolved in DMSO, or food containing DMSO alone, for 24 hours twice weekly for 30 weeks. All of the mice in both 1500 mg extract/kg groups were similarly exposed to TPA-containing liquid food (no 1500 mg/kg mice were exposed to food containing DMSO alone). Histological examinations of the liver, kidneys, spleen and grossly abnormal organs were performed on all groups at the end of the 30-week promotion period. Neoplastic changes were found in none of the 27 control mice and in a total of 5 cylindrospermopsin-treated mice, a difference that was not statistically significant. There was no pattern to the neoplasic changes, as they occurred in different animals, target organs and treatment groups, as detailed in Table 4-1.

Table 4-1. Tumor Initiating Activity of *C. raciborskii* Extracts		
Oral Treatment (mg extract/kg)	Number of Mice	Histological Finding*
Saline/DMSO	14	No neoplasia observed
Saline/TPA	13	No neoplasia observed
3 x 500/DMSO	18	1 hepatocellular carcinoma 1 lymphoma
3 x 500/TPA	16	No neoplasia observed
1 x 1500/TPA	14	2 hepatocellular dysplastic foci 1 fibroblastic osteosarcoma
2 x 1500/TPA	5	No neoplasia observed

* All findings were in different animals
Source: Falconer and Humpage (2001)

4.4.4. Genotoxicity

Purified cylindrospermopsin caused an increase in the frequency of micronuclei in the human lymphoblastoid cell line WIL2-NS (Humpage et al., 2000). Both centromere-positive and centromere-negative micronuclei were induced, suggesting that whole chromosome loss, as well as DNA strand breaks, contributed to the *in vitro* cytogenetic damage. DNA strand breakage was also observed in the liver of Balb/c mice following a single 0.2 mg/kg i.p. dose of purified cylindrospermopsin (Shen et al., 2002). Covalent binding of cylindrospermopsin or a metabolite to DNA (adduct not identified) was detected in the liver of Quackenbush mice given a single i.p. injection of a cell-free extract of *C. raciborskii* (dose levels not reported) (Shaw et al., 2000). Purified cylindrospermopsin caused cell growth inhibition and altered cell morphology, but no apoptosis or DNA strand breaks, in Chinese hamster ovary K1 cells *in vitro* (Fessard and Bernard, 2003).

4.5. MECHANISTIC DATA AND OTHER STUDIES IN SUPPORT OF THE MODE OF ACTION

4.5.1. Liver Toxicity

The liver is widely regarded as the main target of cylindrospermopsin toxicity, and consequently, most mechanistic studies have assessed hepatic endpoints. The specific mechanism for the liver toxicity is not clearly understood, although it has generally been considered to involve cylindrospermopsin-induced inhibition of protein synthesis.

Cylindrospermopsin was shown to be a potent inhibitor of protein synthesis in an *in vitro* rabbit reticulocyte globin synthesis assay (Terao et al., 1994). Ultrastructural liver changes in mice treated with a single 0.2 mg/kg i.p. dose of purified cylindrospermopsin had features in common with those dosed with the protein synthesis inhibitor cycloheximide, particularly detachment of ribosomes from the rough endoplasmic reticulum, suggesting that protein synthesis inhibition plays a role in cylindrospermopsin hepatotoxicity *in vivo* (Terao et al., 1994). However, unlike the liver in the cycloheximide-dosed mice, the liver of those treated with cylindrospermopsin showed membrane proliferation, fat droplet accumulation and reduced amount of total P450 in microsomes, indicating that mechanisms other than protein synthesis inhibition must also contribute to cylindrospermopsin toxicity.

Cylindrospermopsin-induced depletion of mouse hepatic glutathione was demonstrated *in vivo* (Norris et al., 2002), although the study authors did not consider the effect to be of sufficient magnitude to represent the primary mechanism of cylindrospermopsin toxicity. Cylindrospermopsin also caused decreased glutathione levels, as well as decreased synthesis of glutathione and protein, in cultured rat hepatocytes (Runnegar et al., 1994, 1995, 2002). Inhibition of glutathione synthesis was the predominant mechanism for the reduction in glutathione; other mechanisms, including increased consumption of glutathione, increased formation of oxidized glutathione, increased glutathione efflux, hidden forms of glutathione, decreased glutathione precursor availability and decreased cellular ATP were effectively ruled out (Runnegar et al., 1995). Glutathione depletion occurred at non-toxic cylindrospermopsin concentrations and preceded the onset of observable toxicity at higher concentrations (Runnegar et al., 1994). Pretreatment with the CYP450 inhibitor, α-naphthoflavone, partially protected against cytotoxicity and cellular glutathione depletion, indicating involvement of the CYP450 enzyme system in cylindrospermopsin metabolism and that one or more metabolites might be more active than the parent compound in inhibiting glutathione synthesis (Runnegar et al., 1995). *In vitro* studies in mouse hepatocytes provided no indication that reductions in glutathione levels by cylindrospermopsin led to increased levels of reactive oxygen species (ROS) (Humpage et al., 2005).

Cylindrospermopsin induced time- and concentration-dependent toxicity and inhibition of protein synthesis in hepatocytes isolated from male Swiss mice (Froscio et al., 2003). The broad-spectrum CYP450 inhibitors proadifen (SKF525A) and ketoconazole diminished the induction of cytotoxicity by cylindrospermopsin, but did not diminish the inhibition of protein synthesis. These findings suggest that the cytotoxic effects of cylindrospermopsin might be linked more to CYP450-mediated bioactivation than to inhibition of protein synthesis by the parent compound. Similarly, pretreatment of male Quackenbush mice with the broad-spectrum CYP450 inhibitor piperonyl butoxide protected against the acute lethality of cylindrospermopsin (Norris et al., 2002). In a study using inhibitors of specific CYP450 isoforms, furafylline (CYP1A2) and omeprazole (CYP3A4 and CYP2C19) protected against cylindrospermopsin cytotoxicity in an *in vitro* mouse hepatocyte system; unspecified inhibitors of CYPs 2A6, 2D6 and 2E1 were not found to be cytoprotective (Humpage et al., 2005). Additional support for the involvement of CYP450 in the hepatotoxicity of cylindrospermopsin is the finding that liver histopathology is mainly induced in the region (periacinar) where CYP450-catalyzed xenobiotic metabolism occurs (Shaw et al., 2000, 2001).

4.5.2. Kidney Toxicity

No studies were located that specifically investigated the involvement of protein synthesis inhibition or other modes of action in cylindrospermopsin-induced toxicity in the kidney or other non-hepatic target tissues (e.g., spleen and thymus). As detailed in Section 4.2.1.3.1, the kidney was the most sensitive target in mice that were exposed to cylindrospermopsin by daily gavage for 11 weeks (Humpage and Falconer, 2003). Renal effects in the mice included increased relative kidney weight at ≥60 μg/kg-day, decreased urinary protein at ≥120 μg/kg-day and decreased urine specific gravity and proximal renal tubular lesions at 240 μg/kg-day. The authors hypothesized that the decrease in urinary protein is consistent with decreased availability of protein and that the increase in kidney weight may reflect a compensatory hyperplasia, such that the kidney, as a protein-synthesizing organ, is stimulated to grow in an attempt to maintain homeostasis in response to a cylindrospermopsin-related decrease in protein synthesis. Information supporting the hypothesis that the decrease in urinary protein excretion reflects a specific effect of cylindrospermopsin on protein synthesis, as well as the possibility that it reflects a functional change in the nephron, is discussed below. Also discussed is evidence suggesting a dose-severity progression of kidney effects.

Potential mechanisms for a decrease in urinary protein include a decrease in glomerular filtration (i.e., filtered load) of protein, an increase in resorption of filtered protein and a decrease in secretion of nephrogenic protein. A decrease in glomerular filtration of protein (e.g., μg protein/day) could result from a decrease in serum protein concentration or a decrease in glomerular filtration rate (mL/day, GFR). The predominant serum protein in urine of healthy animals (e.g., mice, rats and humans) is albumin (~50% of serum proteins in urine). In the Humpage and Falconer (2003) study, serum albumin concentration increased in mice exposed to cylindrospermopsin, and serum creatinine (a marker of GFR) apparently was unchanged; it was measured but not discussed in the results. Therefore, it is unlikely that glomerular filtration of serum proteins decreased in response to cylindrospermopsin (if a change occurred, it is likely to have been an increase in the rate of filtration of albumin). Furthermore, serum proteins normally account for approximately 15% of total urinary protein (Pesce and First, 1979). The decrease in urinary excretion of protein observed in Humpage and Falconer (2003) was substantially larger than this (~50%), indicating that the decrease in urinary protein cannot derive solely from a decrease in excretion (i.e., glomerular filtration) of serum proteins.

No information is presented in Humage and Falconer (2003) that would allow an assessment of tubular resorption of filtered protein (e.g., plasma-to-urine clearance of protein, excretion of low-molecular weight proteins such as $\beta_{2\mu}$globulin or retinal binding protein).

In healthy mammals, the dominant protein in urine (~50%) is the nephrogenic Tamm-Horsfall protein (THP, uromucoid) (Bachmann et al., 1991, 2005). In the absence of a decrease in filtration or increased resorption of filtered serum protein, the substantial decrease in urinary protein (i.e., ~50%) observed by Humpage and Falconer (2003) would almost certainly have to involve decreased excretion of THP, since it is the predominant protein in urine. Although there are numerous possible mechanisms for an acute change in THP excretion (Bachman et al., 1991), long-term maintenance of lower (i.e., steady-state) rate of urinary excretion of THP requires a decreased rate of synthesis of THP (Bachman et al., 1991, 2005; Schoel and Pfleiderer, 1987).

THP is synthesized exclusively in the thick ascending limb of the loop of Henle (TAL); therefore, a sustained change in THP excretion is likely to reflect a functional change in this region of the nephron. Increases and decreases in THP have been observed in various kidney diseases, and in association with experimental treatments that induce hypertrophy of the TAL, including increased dietary protein (Bachmann et al., 1991). Depletion of THP from the kidney may, in itself, be adverse. Mice deficient in THP (i.e., THP knockout mice) display impaired urine concentrating ability, up-regulation of distal nephron electrolyte transport proteins and increased susceptibility to urinary tract infections (Bachmann et al., 2005; Bates et al., 2004). The decrease in urine specific gravity in animals exposed to cylindrospermopsin in the Humpage and Falconer (2003) study may be indicative of impaired urine concentrating ability and, possibly, related to impaired function of the TAL (i.e., impairment of transport activity in this region of the nephron impairs urine concentrating ability) and/or decreased synthesis of THP.

Additional kidney effects in the Humpage and Falconer (2003) mouse study included proximal renal tubular damage (type and severity of lesions not reported) at the high dose. Clinical effects in the Palm Island outbreak in which humans apparently ingested drinking water containing elevated levels of cylindrospermopsin included renal damage, as indicated by loss of water, electrolytes, proteins, ketones and carbohydrates (Blyth, 1980; Griffiths and Saker, 2003) (Section 4.1). Proteinuria would be expected with proximal tubular damage, as this is the site of resorption of filtered protein. Proteinuria was not observed by Humpage and Falconer (2003), but information on the type and severity of the tubular damage was not reported. Proteinuria did occur in the humans, although other mechanisms could have caused it (e.g., glomerular injury will produce high molecular weight proteinuria). The evidence for proximal tubular damage and functional impairment (e.g., proteinuria, glucosuria) together strengthen the argument that the kidney is a target of cylindrospermopsin and, when considered with decreased protein excretion at lower doses, suggests a dose-severity progression.

4.5.3. Interactions with DNA and RNA

Based on structural characteristics of cylindrospermopsin (its nucleoside structure and potentially reactive guanidine and sulfate groups), it has been speculated that cylindrospermopsin may exert its toxic effects via pathways that include reactions with DNA and/or RNA (see Humpage et al., 2000; Shen et al., 2002). Covalent binding between DNA and cylindrospermopsin, or a metabolite, occurred in mouse liver *in vivo* (Shaw et al., 2000). DNA adducts were detected, but not identified, using the ^{32}P-postlabeling assay; this involved extraction of the DNA, hydrolysis into individual nucleotides, labeling of the nucleotides using ^{32}P-ATP, separation of adducted nucleotides using two-dimensional thin layer chromatography and visualization of adduct spots by autoradiography. Cylindrospermopsin also induced DNA strand breakage in mouse liver *in vivo* (Shen et al., 2002) and increases in micronuclei occurred in treated binucleated cells of the WIL2-NS lymphoblastoid cell-line (Humpage et al., 2000). Two mechanisms were suggested for causing the cytogenetic damage: one at the level of DNA to induce strand breaks and the other at the level of kinetochore/spindle function to induce loss of whole chromosomes (Humpage et al., 2000; Shen et al., 2002). The broad-spectrum CYP450 inhibitors omeprazole and SKF525A inhibited cylindrospermopsin-induced DNA damage in primary cultured mouse hepatocytes at subcytotoxic concentrations, suggesting that CYP-derived

metabolites are responsible for cylindrospermopsin genotoxicity and that genotoxicity is a primary effect of the chemical (Humpage et al., 2005).

Cylindrospermopsin-induced up-regulation of the tissue transglutaminase (tTGase) gene was demonstrated in liver RNA of Balb/c mice following i.p. injection of a single 100 μg/kg dose of cylindrospermopsin (Shen et al., 2003). tTGase is a unique member of the TGase (EC 2.3.2.13) family that catalyzes the post-translational modification of proteins via Ca^{2+}-dependent cross-linking reactions (Shen et al., 2003). The up-regulation of tTGase can lead to liver injury (Grenard et al., 2001; Mirza et al., 1997), and has been implicated in diverse biological processes, such as induction of apoptosis (Piacentini et al., 2002; Zhang et al., 1995), cell death and differentiation (Shen et al., 2003; Fesus et al., 1987) and adhesion and morphological changes of cells (Shen et al., 2003; Akimov and Belkin, 2001).

4.5.4. Structure-Activity Relationships

Natural cylindrospermopsin, synthetic (racemic) cylindrospermopsin and selected synthetically-produced cylindrospermopsin structural analogues were assessed for effects on protein synthesis in both the rabbit reticulocyte lysate system and cultured rat hepatocytes (Runnegar et al., 2002). No significant differences were observed in levels of protein synthesis inhibition elicited by natural cylindrospermopsin and its diol analogue, indicating that the sulfate group might not be a necessary component of cylindrospermopsin-induced protein synthesis inhibition. Additionally, the orientation of the hydroxyl group at C7 in the carbon bridge does not appear to be important, since the C7 epimer of cylindrospermopsin and its corresponding diol exhibited protein synthesis inhibition similar to that elicited by synthetic (racemic) cylindrospermopsin. The cyclopentyl ring and the methyl and hydroxyl groups on the adjacent hexyl ring may be important structural features, because the analogue lacking these features was 500-1000-fold less effective in the inhibition of protein synthesis.

The uracil portion of cylindrospermopsin appears to play an important role in cylindrospermopsin toxicity. Banker et al. (2001) found that the acute lethality of cylindrospermopsin to mice was eliminated by chlorination or partial cleavage of the uracil moiety (resulting in 5-chloro-cylindrospermopsin and cylindrospermic acid, respectively), as shown by a 5-day i.p. LD_{50} value of 0.2 mg/kg for cylindrospermopsin and 10-day i.p. LD_{50} values of >10 mg/kg for 5-chloro-cylindrospermopsin and >10 mg/kg for cylindrospermic acid.

Deoxycylindrospermopsin, an analogue of cylindrospermopsin isolated and purified from *C. raciborskii*, was tested for toxicity in male white Quackenbush mice treated by i.p. injection (Norris et al., 1999). Deoxycylindrospermopsin did not appear to be toxic during 5 days following administration of a 0.8 mg/kg dose, whereas Ohtani et al. (1992) reported a 5- to 6-day i.p. LD_{50} value of 0.2 mg/kg for cylindrospermopsin in male CD3 mice. Although this comparison suggests that deoxycylindrospermopsin is significantly less toxic than cylindrospermopsin, differences in study designs (e.g., the use of different strains of mice) could have contributed to the difference in toxicity.

4.6. SYNTHESIS AND EVALUATION OF MAJOR NONCANCER EFFECTS

4.6.1. Oral

Information on the health effects of cylindrospermopsin in humans is limited to observations on the Australian Palm Island poisoning incident that involved acute and/or short-term drinking water exposure to *C. raciborskii*, a non-infectious cyanobacterium (Blyth, 1980; Griffiths and Saker, 2003). The clinical picture of the illness is well-defined and includes fever, headache, vomiting, bloody diarrhea, hepatomegaly and kidney damage with loss of water, electrolytes and protein, but no data are available on exposure levels of cylindrospermopsin that induced these effects.

The preponderance of information on noncancer effects of cylindrospermopsin in animals is available from oral and i.p. administration studies in mice that were exposed to purified compound or extracts of *C. raciborskii* cells. These studies indicate that the liver and kidneys are main targets of toxicity and that cylindrospermopsin also causes significant lesions in other organs, particularly the spleen and thymus. Considering both animal and human kidney data, the evidence suggests a dose-severity progression of renal effects ranging from decreased protein synthesis at low doses to functional impairment at high doses. The cell extract studies provide limited dose-response information for cylindrospermopsin because concentrations vary between cultures and strains and, in some cases, may contain other toxins, as discussed in the introduction to Section 4.2. The available oral toxicity studies of purified cylindrospermopsin are summarized in Table 4-2.

No studies have been performed assessing the acute oral toxicity of purified cylindrospermopsin. Studies in which mice were administered single gavage doses of suspensions or cell-free extracts of *C. raciborskii* cells at near-lethal to lethal levels found severe damage to the liver (fatty and necrotic changes), kidneys (acute tubular necrosis), spleen and thymus (atrophy of lymphoid tissue), heart (hemorrhages) and gastric mucosa (ulceration of the esophageal section) (Falconer et al., 1999; Seawright et al., 1999; Shaw et al., 2000, 2001).

A limited amount of information on the short-term oral toxicity of cylindrospermopsin is available from inadequately reported 14- and 21-day studies.

Histological examinations of small numbers of mice that were administered daily gavage doses of purified cylindrospermopsin for 14 days showed effects in the liver (fatty infiltration) and spleen (lymphophagocytosis) (Shaw et al., 2000, 2001). Fatty infiltration in the liver was the more sensitive effect based on a reported NOAEL of 0.05 mg/kg-day and LOAEL of 0.15 mg/kg-day. Small numbers of mice and rats were exposed to cylindrospermopsin for 21 days in drinking water from a dammed impoundment at a reported approximate dose of 0.2 mg/kg-day (Shaw et al., 2000, 2001). No histopathological changes were noted, indicating a NOAEL of 0.2 mg/kg-day in drinking water. The adequacy of the 14- and 21-day effect levels cannot be assessed due to a lack of any additional reported information on the design and results of these studies.

Table 4-2. Summary Results of Oral Toxicity Studies of Pure Cylindrospermopsin in Experimental Animals*

Species	Sex	Average Daily Dose (mg/kg-day)	Exposure Duration	NOAEL (mg/kg-day)	LOAEL (mg/kg-day)	Responses	Comments	Reference
Acute Exposure								
No suitable acute studies are available								
Short-Term Exposure								
Mouse	NR	0.05, 0.15, 0.3 (gavage)	14 days	0.05	0.15	Lipid infiltration in liver.	Low confidence in NOAEL and LOAEL. A full report of this study has not been published; this table provides essentially all available information on experimental design and results.	Shaw et al., 2000, 2001
Subchronic Exposure								
Mouse	M	0, 0.03, 0.06, 0.12, 0.24 (drinking water)	11 weeks	0.03	0.06	Increased relative kidney weight with decreased urinary protein at $\geq$0.12 mg/kg-day.	Well-designed study with endpoints that included food and water consumption, body weight, clinical signs, hematology, serum chemistry, urinalysis, organ weights (eight organs) and histology (comprehensive). Ten mice/level (six in high dose group).	Humpage and Falconer, 2003
Chronic Exposure								
No suitable chronic studies are available.								

* Oral studies using suspensions or cell-free extracts of *C. raciborskii* cells are discussed in Section 4.2.1.
NR = Not reported

A comprehensive subchronic toxicity study was conducted in which mice were exposed to five dose levels of purified cylindrospermopsin (0, 30, 60, 120 or 240 μg/kg) by daily gavage for 11 weeks (Humpage and Falconer, 2003). Histopathological effects were observed in the liver at ≥120 μg/kg-day ("minor increases in histopathological damage") and kidneys at 240 μg/kg-day (proximal tubular damage), but no other information on the lesions, including incidence data, was reported. There were no changes in liver weight at doses below 240 μg/kg-day or serum indices of liver damage (e.g., serum ALT, AST and alkaline phosphatase) in any of the dose groups. Relative kidney weight was increased at ≥60 μg/kg-day and urine protein was decreased at ≥120 μg/kg-day. These effects are considered to be adverse because they are consistent with a known mode of action of cylindrospermopsin (inhibition of protein synthesis) and represent part of the spectrum of effects leading to toxicity, as discussed in Section 4.5.2. Based on the increase in kidney weight, the subchronic NOAEL and LOAEL values are 30 and 60 μg/kg-day, respectively.

No information was located regarding the chronic toxicity, neurotoxicity or developmental/reproductive toxicity of cylindrospermopsin.

4.6.2. Inhalation

No information was located regarding the inhalation toxicity of cylindrospermopsin.

4.6.3. Mode of Action Information

The liver and kidneys appear to be the main targets of cylindrospermopsin toxicity. The mechanism for liver toxicity is incompletely characterized, but involves inhibition of protein synthesis (Froscio et al., 2003; Terao et al., 1994). Available evidence indicates that the protein synthesis inhibition is not decreased by broad-spectrum CYP450 inhibitors, suggesting that it is mediated by the parent compound (Froscio et al., 2003). Hepatocytotoxicity occurs at higher levels of cylindrospermopsin and appears to be CYP450-dependent, indicating the involvement of metabolites and other mechanisms (Froscio et al., 2003; Humpage et al., 2005; Norris et al., 2002). Studies specifically investigating the inhibition of protein synthesis in the kidneys are not available, although the results of the 11-week oral toxicity study in mice (Humpage and Falconer, 2003) are consistent with an inhibition of protein synthesis. Effects in this study included decreased urinary protein and, at a higher dose, proximal renal tubular lesions. As discussed in Section 4.5.2, the decrease in urinary protein excretion at low doses could reflect a specific effect of cylindrospermopsin on protein synthesis or, possibly, a functional change in the nephron. The proximal renal tubular damage in mice (Humpage and Falconer, 2003), as well as the clinical findings of renal insufficiency in the Palm Island human poisoning incident (Blyth, 1980; Griffiths and Saker, 2003), suggest that cytotoxic mechanisms may predominate in the kidney at higher doses.

Genotoxic effects of cylindrospermopsin include DNA adduction and strand breakage in mouse liver (Shaw et al., 2000; Shen et al., 2002) and micronuclei formation in a lymphoblastoid cell line (Humpage et al., 2000). Broad spectrum CYP450 inhibitors inhibited cylindrospermopsin-induced DNA damage in mouse hepatocytes at sub-cytotoxic concentrations

(Humpage et al., 2005), suggesting that metabolites are responsible for cylindrospermopsin genotoxicity and that genotoxicity is a primary effect of the chemical.

4.7. WEIGHT-OF-EVIDENCE EVALUATION AND CANCER CHARACTERIZATION

4.7.1. Summary of Overall Weight-of-Evidence

No information is available on the carcinogenicity of cylindrospermopsin in humans, and no cancer studies of purified cylindrospermopsin have been conducted in animals. A test of an extract of *C. raciborskii* cells suggests that cylindrospermopsin has no tumor initiating activity in mice (Falconer and Humpage, 2001). A limited amount of data indicate that cylindrospermopsin or a metabolite can covalently bind to DNA (Shaw et al., 2000) and cause cytogenetic damage, as shown by induction of micronuclei (Humpage et al., 2000) and DNA strand breakage (Shen et al., 2002). In accordance with the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005a), the weight of evidence descriptor for the carcinogenic hazard potential of cylindrospermopsin is "*Inadequate Information to Assess Carcinogenic Potential*."

4.7.2. Synthesis of Human, Animal and Other Supporting Evidence

No information was located regarding the carcinogenicity of purified cylindrospermopsin in humans or animals. There was no indication that cylindrospermopsin had tumor initiating activity in a test in which mice were administered a cell-free extract of freeze-dried *C. raciborskii* cells by gavage followed by oral exposure to the tumor promoter TPA (Falconer and Humpage, 2001).

The nucleotide structure of cylindrospermopsin, as well as the presence of potentially reactive guanidine and sulfate groups, suggests the possibility of interference with DNA and/or RNA synthesis and induction of mutations. Covalent binding between DNA and cylindrospermopsin (or a metabolite) (Shaw et al., 2000, 2001) and DNA strand breakage (Shen et al., 2002) have been demonstrated in mouse liver, and micronuclei were induced in human WIL2-NS lymphoblasts (Humpage et al., 2000). Although the available data indicate that DNA strand breakage could be a key mechanism for cylindrospermopsin-induced cytogenetic damage (Humpage et al., 2000; Shen et al., 2002), insufficient data are available to speculate on the carcinogenic potential of cylindrospermopsin.

4.8. SUSCEPTIBLE POPULATIONS AND LIFE STAGES

4.8.1. Possible Childhood Susceptibility

As discussed in Section 4.1, cylindrospermopsin has been implicated in the Palm Island outbreak of a hepatoenteritis-like illness in 148 Australians (Blyth, 1980; Griffiths and Saker, 2003). Of the 148 cases, 138 were children (mean age 8.4 years, range 2-16 years, 41% male and 59% female) and 10 were adults (sex and age not reported). There are no reported indications that the 138 children were from the households of the 10 adults or that the children

and adults received different exposures, suggesting a possible increased sensitivity of children (the child:adult ratio is approximately 14:1).

4.8.2. Possible Gender Differences

There is no information on possible gender differences in the disposition of, or response to, cylindrospermopsin.

4.8.3. Other Possible Susceptible Populations

No data were located regarding populations that might be unusually susceptible to cylindrospermopsin. It is conceivable that individuals with liver and/or kidney disease might be more susceptible than the general population because of compromised detoxification mechanisms in the liver and impaired excretory mechanisms in the kidney.

5. DOSE-RESPONSE ASSESSMENTS

5.1. NARRATIVE DESCRIPTION OF THE EXTENT OF THE DATABASE

Studies on the absorption, tissue distribution, metabolism and elimination of cylindrospermopsin following oral, inhalation or dermal exposure have not been performed. Gastrointestinal absorption of cylindrospermopsin is indicated by the induction of systemic effects in oral toxicity studies. Studies in which cylindrospermopsin was administered to mice by acute i.p. injection indicate that it is largely distributed to the liver and rapidly eliminated in the urine as unmetabolized compound. The liver and kidneys are the main targets of cylindrospermopsin toxicity. Possible modes of action include inhibition of protein synthesis, CYP450-mediated bioactivation to a reactive intermediate and covalent binding between parent compound or a metabolite and DNA and/or RNA.

The only information on the toxicity of cylindrospermopsin in humans is from reports of a poisoning outbreak that is attributed to the consumption of drinking water containing toxin-producing *C. raciborskii*. Although the clinical picture of this hepatoenteritis-like illness is well defined and includes bloody diarrhea, swollen liver and impaired kidney function, there are no data on exposure levels that induced these effects.

Most of the available data on the toxicity of cylindrospermopsin in animals are available from oral and i.p. studies that tested purified compound or extracts of *C. raciborskii* cells. These studies are generally consistent in indicating that cylindrospermopsin causes lesions in the liver and other organs, particularly the kidneys, spleen and thymus. The cell extract studies are not useful for dose-response assessment of cylindrospermopsin due to the confounding factors discussed in the introduction to Section 4.2. The database on oral toxicity of pure cylindrospermopsin is limited by a small number of studies and insufficient reporting. No studies have been performed assessing the acute oral toxicity of pure cylindrospermopsin. Data on the short-term oral toxicity of pure compound are available from inadequately reported 14-day gavage and 21-day drinking water studies in mice and rats. The reports of these studies identify NOAELs and LOAELs for histopathology, but the adequacy of these effect levels cannot be verified due to a virtual lack of any additional information on the experimental designs and results. Data on the subchronic oral toxicity of pure cylindrospermopsin are available from a well-designed and reported 11-week study in mice that provides a suitable basis for derivation of a subchronic oral RfD value. No chronic toxicity, reproductive toxicity, developmental toxicity or carcinogenicity studies of pure cylindrospermopsin have been performed.

No information is available on the inhalation toxicity of cylindrospermopsin.

5.2. ORAL REFERENCE DOSE (RfD)

5.2.1. Data Considered in Deriving Reference Values

Data considered in deriving oral RfDs for each duration of exposure are summarized in Table 4-1 (Section 4.6.1).

5.2.2. Acute Duration

5.2.2.1. Choice of Principal Study and Critical Effect - with Rationale and Justification

Derivation of an acute oral RfD for cylindrospermopsin is precluded by insufficient data. The only information on the toxicity of cylindrospermopsin in humans is the outbreak of a hepatoenteritis-like illness that is attributed to the consumption of drinking water containing *C. raciborskii* (Blyth, 1980; Griffiths and Saker, 2003; Hawkins et al., 1985; Ohtani et al., 1992). Although the clinical picture of the illness is well defined, measured or estimated exposure levels have not been reported. No acute oral toxicity studies of purified cylindrospermopsin have been performed in animals. Single-dose studies of suspensions or cell-free extracts of *C. raciborskii* cells were conducted in mice, but only near-lethal to lethal dose levels were tested (Falconer et al., 1999; Seawright et al., 1999; Shaw et al., 2000, 2001).

5.2.3. Short-Term Duration

5.2.3.1. Choice of Principal Study and Critical Effect - with Rationale and Justification

Derivation of a short-term oral RfD for cylindrospermopsin is precluded by insufficient data. The only information relevant to the short-term toxicity of cylindrospermopsin in humans is qualitative data on the outbreak of the hepatoenteritis-like illness that is attributed to the consumption of drinking water containing *C. raciborskii* (Blyth, 1980; Griffiths and Saker, 2003; Hawkins et al., 1985; Ohtani et al., 1992). As discussed in Sections 4.2.1.2 and 4.5.1, a limited amount of information is available on the short-term oral toxicity of cylindrospermopsin from poorly reported 14-day gavage and 21-day drinking water studies (Shaw et al., 2000, 2001). The 14-day study reported a NOAEL of 0.05 mg/kg-day and LOAEL of 0.15 mg/kg-day for liver fatty infiltration in mice, and the 21-day study reported a free-standing NOAEL of 0.2 mg/kg-day for histopathology in mice and rats. The appropriateness of these effect levels cannot be assessed due to inadequate information on the design and results of the studies.

5.2.4. Subchronic Duration

5.2.4.1. Choice of Principal Study and Critical Effect - with Rationale and Justification

The comprehensive 11-week subchronic study in mice (Humpage and Falconer, 2003), detailed in Section 4.2.1.3, is the only subchronic study of purified cylindrospermopsin and provides a suitable basis for RfD derivation. The LOAEL was 60 μg/kg-day for increased relative kidney weight. At 120 μg/kg-day, there was a significant decrease in urinary protein concentration and minor histopathological changes in the liver. Decreased urinary protein and increased relative kidney weight are both potential indicators of suppressed protein synthesis, a known mode of action of cylindrospermopsin. The decrease in urinary protein is consistent with decreased availability of protein and the increase in kidney weight may reflect a compensatory hyperplasia, such that the kidney, as a protein-synthesizing organ, is stimulated to grow in an

attempt to maintain homeostasis in face of a chemical-related decrease in protein synthesis (Humpage and Falconer, 2003). Information supporting this hypothesis, as well as the possibility that the decrease in urinary protein excretion reflects a functional change in the nephron, is discussed in Section 4.5.2. Because the changes are consistent with a known mode of action and represent part of the progression of effects leading to toxicity, they are considered to be adverse and indicate that the LOAEL and NOAEL are 60 and 30 μg/kg-day, respectively.

5.2.4.2. Methods of Analysis - Including Models (PBPK, BMD, etc.)

A point of departure (POD) can be determined using the kidney weight data and BMD modeling, but BMD analysis of the urinary protein and histopathology data is precluded by insufficient data. In particular, the urinary protein data are limited by inadequate reporting (mean concentrations and errors are conveyed in a bar graph with no numerical values specifically reported, no indication if the error bars represent standard deviation or standard error, and no indication of numbers of animals) and the pathology findings are limited by a lack of incidence data.

In accordance with current BMD technical guidance (U.S. EPA, 2000c), available continuous-variable models in the EPA Benchmark Dose Software (BMDS version 1.3.2; linear, polynomial, power and Hill models) were fit to the data for changes in mean relative kidney weight shown in Table 5-1. Statistical tests in the BMDS showed that variance was homogeneous across dose groups. BMDs and BMDLs were calculated using 1 standard deviation above the control mean as the benchmark response level (BMR), while assuming homogenous variance across groups. Using data from all dose groups, an adequate fit to the data was obtained with the Hill model (Table 5-2), but the BMDS was not able to compute a BMDL. After dropping the high dose group, there were insufficient degrees of freedom remaining to fit the Hill model, but the linear model adequately fit the data and produced an estimated BMD of 43.1 μg/kg-day and BMDL of 33.1 μg/kg-day. The two-degree polynomial and power models defaulted to the same linear model, albeit with lower p-value and/or higher Aikake's Information Criteria (AIC) due to the greater number of parameters in these models. The BMD modeling results are summarized in Table 5-2 and detailed in Appendix A, and the fit of the linear model to the data is shown in Figure 5-1. The BMDL of 33.1 μg/kg-day is similar to the 30 μg/kg-day NOAEL for increased kidney weight and is used as the POD for the RfD.

5.2.4.3. RfD Derivation - Including Application of UFs

The BMDL of 33.1 μg/kg-day for increased relative kidney weight was used as the point of departure (POD) for the subchronic RfD. Dividing the BMDL of 33.1 μg/kg-day by a composite uncertainty factor (UF) of 1000 results in a subchronic RfD for cylindrospermopsin of $3x10^{-5}$ mg/kg-day.

Table 5-1. Relative Kidney Weights in Mice Exposed to Purified Cylindrospermopsin for 11 Weeks

	Oral Dose (μg/kg-day)				
	0	30	60	120	240
Relative kidney weight (mean ± standard deviation)	1.48 ± 0.10 (10)[a]	1.57 ± 0.14 (10)	1.66 ± 0.16[b] (9)	1.82 ± 0.12[c] (9)	1.78 ± 0.17[c] (6)

[a] Values in parentheses are the number of animals evaluated in each group
[b] Statistically significant difference from controls ($p<0.05$)
[c] Statistically significant difference from controls ($p<0.001$)
Source: Humpage and Falconer (2003)

Table 5-2. Summary of Benchmark Dose Modeling (Relative Kidney Weight)[a]

Model Fit to Means	df	p-Value for Model Fit	AIC for Fitted Model	BMD (μg/kg-day)	BMDL (μg/kg-day)
Relative kidney weight, all dose groups (p=0.59 for test of homogenous variance, indicating assumption of homogenous variance is appropriate)					
Linear	3	0.01	-120.85	106.07	76.56
2-Degree polynomial (pos betas)	2	0.003	-120.85	106.07	76.56
Power (power >=1)	2	0.003	-116.85	106.07	76.56
Hill (power >=1)	1	0.21	-124.74	43.19[b]	NA[c]
Relative kidney weight, high dose group dropped (p=0.52 for test of homogenous variance, indicating assumption of homogenous variance is appropriate)					
Linear	2	0.98	-116.47	43.90	33.07
2-Degree polynomial (pos betas)	1	0.84	-116.47	43.90	33.07
Power (power >=1)	1	0.84	-112.47	43.90	33.07
Hill (power >=1)	0	NA[d]	-110.51	41.20	21.72

[a] Modeling conducted assuming homogenous variance and using BMR of 1 standard deviation
[b] Optimum BMD may not have been found (i.e. bad completion code in the BMDS optimization routine)
[c] BMDL computation failed
[d] The Chi-Square test for fit is not valid due to insufficient degrees of freedom (df)
NA = not available

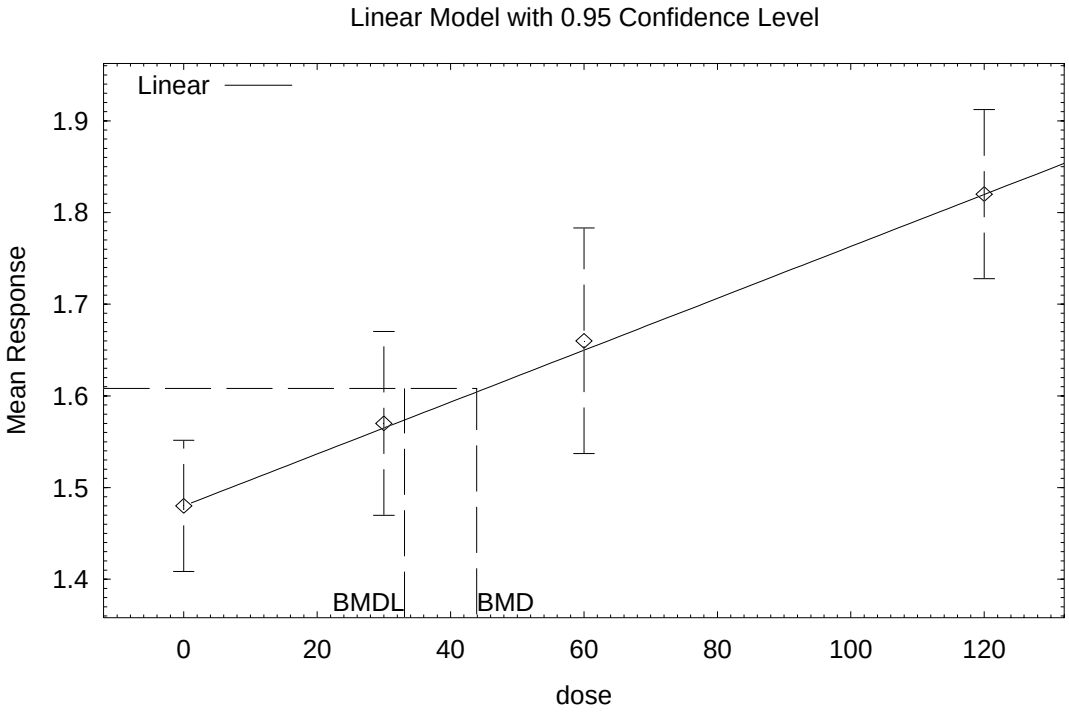

Figure 5-1. Linear Model Fit to Relative Kidney Weight Data (High Dose Group Dropped)
Source: Humpage and Falconer (2003)

Subchronic RfD = BMDL ÷ UF
= 33.1 μg/kg-day ÷ 1000
= **0.00003 mg/kg-day or $3x10^{-5}$ mg/kg-day**

The composite UF of 1000 includes a factor of 10 for interspecies extrapolation, a factor of 10 to account for interindividual variability in the human population and a factor of 10 for database limitations, as follows.

- A default 10-fold UF is used to account for the interspecies variability in extrapolating from laboratory animals to humans. No information is available on the toxicity of purified cylindrospermopsin in humans, and no data on toxicokinetic differences between animals and humans in the disposition of ingested cylindrospermopsin are available.

- A 10-fold UF is used to account for variation in sensitivity within human populations because there is insufficient information on the degree to which humans of varying gender, age, health status or genetic makeup might vary in the disposition of, or response to, ingested cylindrospermopsin. As discussed in Section 4.1, data from the Palm Island outbreak of a hepatoenteritis-like illness (Blyth, 1980; Griffiths and Saker, 2003) suggest a possible increased sensitivity of children to cylindrospermopsin.

- A 10-fold UF is used to account for deficiencies in the database. There is no information on the longer-term toxicity of cylindrospermopsin in humans. Other database deficiencies include a lack of particular kinds of animal studies on purified cylindrospermopsin, including a chronic study, subchronic or chronic studies in a second species and reproductive and developmental toxicity studies.

The NOAEL/LOAEL approach and an UF of 1000 would also yield an RfD of 0.00003 mg/kg-day due to the similarity of the NOAEL and BMDL for increased kidney weight (30 and 33.1 μg/kg-day, respectively).

5.2.5. Chronic Duration

5.2.5.1. Choice of Principal Study and Critical Effect - with Rationale and Justification

Derivation of a chronic oral RfD for cylindrospermopsin is precluded by insufficient data. No information is available on the chronic toxicity of cylindrospermopsin by any route of exposure. The 11-week study (Humpage and Falconer, 2003) used to derive the subchronic RfD was considered for use in the derivation of a chronic RfD; however, this approach was rejected due to the lack of information on the potential progression of cylindrospermopsin-induced adverse effects with increased exposure duration. The use of the POD from the 11-week subchronic study for the derivation of a chronic RfD would require the application of a subchronic-to-chronic UF to account for the uncertainties involved in extrapolating across exposure durations. The application of a full subchronic-to-chronic UF of 10, along with UFs of 10 in three other areas of uncertainty (interspecies UF, intraspecies UF, database UF), would result in a total composite uncertainty factor of 10,000. A composite uncertainty factor of this

magnitude suggests that the database is insufficient to support the derivation of an RfD for chronic exposure; therefore, no chronic oral RfD is derived.

5.2.6. Route-to-Route Extrapolation

Derivation of acute, short-term, and chronic RfD values for cylindrospermopsin by route-to-route extrapolation could not be considered due to a lack of inhalation data.

5.3. INHALATION REFERENCE CONCENTRATION (RfC)

No information is available on the toxicity of inhaled cylindrospermopsin.

5.4. CANCER ASSESSMENT

No dose-response or other information is available regarding the carcinogenicity of pure cylindrospermopsin.

6. MAJOR CONCLUSIONS IN THE CHARACTERIZATION OF HAZARD AND DOSE RESPONSE

6.1. HUMAN HAZARD POTENTIAL

Cylindrospermopsin is a naturally occurring chemical produced by *Cylindrospermopsis* (particularly *C. raciborskii*) and at least four other genera of freshwater cyanobacteria. Toxicokinetic studies of cylindrospermopsin have not been performed using natural routes of exposure, but oral toxicity studies show that it is absorbed from the gastrointestinal tract, and i.p. toxicokinetic studies indicate that it is mainly distributed to the liver and excreted in the urine as unmetabolized compound. Main targets of cylindrospermopsin toxicity include the liver and kidneys, and possible modes of action include inhibition of protein synthesis, bioactivation to a reactive intermediate and covalent binding of parent compound or a metabolite to DNA and/or RNA.

The main information on the toxicity of cylindrospermopsin in humans is from qualitative reports of a hepatoenteritis-like illness that is attributed to the acute or short-term consumption of drinking water containing *C. raciborskii*. The database on oral toxicity of purified cylindrospermopsin in animals is limited by a small number of studies and insufficient reporting. No studies have been performed assessing the acute oral toxicity of pure cylindrospermopsin. Information on short-term oral toxicity is available from inadequately reported 14- and 21-day studies in mice and rats. Data on the subchronic oral toxicity of pure cylindrospermopsin are available from a comprehensive 11-week study that identified NOAELs and LOAELs for kidney and liver effects in mice. No chronic toxicity, reproductive toxicity, developmental toxicity or carcinogenicity studies of cylindrospermopsin have been conducted. Testing following inhalation has not been performed.

6.2. DOSE RESPONSE

Kidney effects data in the 11-week toxicity study (Humpage and Falconer, 2003) provide a suitable basis for deriving a subchronic oral RfD. The most sensitive effect in this study was increased relative kidney weight; decreased urinary protein and minor histopathological damage to the liver occurred at the next highest dose. As discussed in Section 4.5.2, increased kidney weight and decreased urinary protein are consistent with suppressed protein synthesis, a known mode of action of cylindrospermopsin, and represent part of the progression of effects leading to toxicity. Based on a BMDL of 33.1 μg/kg-day for increased relative kidney weight in mice, a subchronic RfD of $3x10^{-5}$ mg/kg-day was derived by dividing the BMDL by a UF of 1000. The UF comprises component factors of 10 for interspecies extrapolation, 10 for interindividual variability and 10 for database deficiencies. Acute, short-term and chronic oral RfDs could not be derived due to inadequate data. Inhalation RfC derivation is precluded by the lack of data for this route of exposure. There is inadequate information to evaluate the carcinogenicity of cylindrospermopsin.

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APPENDIX A

BENCHMARK DOSE MODELING RESULTS FOR CYLINDROSPERMOPSIN

Part I.

Humpage and Falconer 2003

male mice treated with purified cylindrospermopsin

rel kidney wt

```
 ========================================================================
        Polynomial Model. Revision: 2.2  Date: 9/12/2002
        Input Data File: C:\BMDS\DATA\CYLINDRO.(d)
        Gnuplot Plotting File:  C:\BMDS\DATA\CYLINDRO.plt
                                          Thu May 12 22:04:04 2005
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 BMDS MODEL RUN
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   The form of the response function is:

   Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


   Dependent variable = MEAN
   Independent variable = dose
   rho is set to 0
   Signs of the polynomial coefficients are not restricted
   A constant variance model is fit

   Total number of dose groups = 5
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                          alpha =     0.018741
                            rho =            0   Specified
                         beta_0 =        1.551
                         beta_1 =   0.00123333



                               Parameter Estimates

                                                95.0% Wald Confidence Interval
    Variable   Estimate    Std. Err.     Lower Conf. Limit   Upper Conf. Limit
    alpha      0.0215477   0.00459399    0.0125437           0.0305518
    beta_0     1.54205     0.0311925     1.48091             1.60318
    beta_1     0.00138389  0.000287871   0.000819671         0.00194811


           Asymptotic Correlation Matrix of Parameter Estimates

                   alpha       beta_0       beta_1
     alpha             1     2.1e-009     1.2e-010
    beta_0     2.1e-009            1         -0.7
    beta_1     1.2e-010         -0.7            1
     Table of Data and Estimated Values of Interest
```

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	1.48	0.1	1.54	0.147	-1.34
30	10	1.57	0.14	1.58	0.147	-0.292
60	9	1.66	0.16	1.63	0.147	0.714
120	9	1.82	0.12	1.71	0.147	2.29
240	6	1.78	0.17	1.87	0.147	-1.57

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	68.148702	6	-124.297404
A2	69.554943	10	-119.109885
fitted	62.424690	2	-120.849381
R	52.631671	2	-101.263343

Test 1: Does response and/or variances differ among dose levels
(A2 vs. R)
Test 2: Are Variances Homogeneous (A1 vs A2)
Test 3: Does the Model for the Mean Fit (A1 vs. fitted)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	33.8465	8	<.0001
Test 2	2.81248	4	0.5897
Test 3	11.448	3	0.009534

The p-value for Test 1 is less than .05. There appears to be a
difference between response and/or variances among the dose levels.
It seems appropriate to model the data

The p-value for Test 2 is greater than .05. A homogeneous variance
model appears to be appropriate here

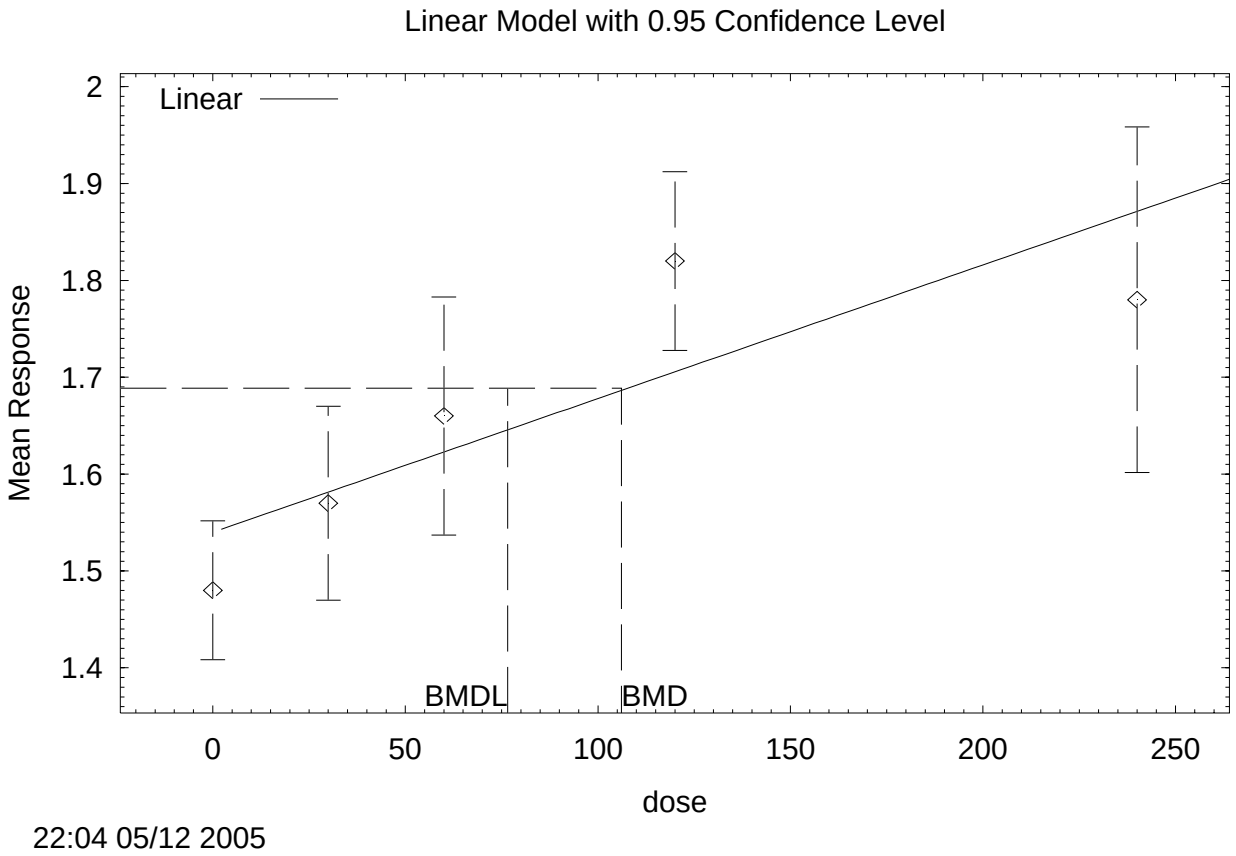
```
The p-value for Test 3 is less than .05.  You may want
to try a
different model
 Benchmark Dose Computation
Specified effect =             1

Risk Type        =     Estimated standard deviations from the control mean


Confidence level =          0.95

             BMD =       106.072


            BMDL =       76.5569
```



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 ========================================================================
        Polynomial Model. Revision: 2.2  Date: 9/12/2002
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        Gnuplot Plotting File:  C:\BMDS\DATA\CYLINDRO.plt
                                          Thu May 12 22:27:06 2005
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 BMDS MODEL RUN
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   The form of the response function is:

   Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


   Dependent variable = MEAN
   Independent variable = dose
   rho is set to 0
   The polynomial coefficients are restricted to be positive
   A constant variance model is fit

   Total number of dose groups = 5
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                          alpha =     0.018741
                            rho =            0   Specified
                         beta_0 =      1.46523
                         beta_1 =            0
                         beta_2 =            0



                                Parameter Estimates

                                                    95.0% Wald Confidence Interval
   Variable Estimate    Std. Err.     Lower Conf. Limit   Upper Conf. Limit
   alpha    0.0215477   0.00459399    0.0125437           0.0305518
   beta_0   1.54205     0.0311925     1.48091             1.60318
   beta_1   0.00138389  0.000287871   0.000819671         0.00194811
   beta_2   0           NA

NA - Indicates that this parameter has hit a bound
     implied by some inequality constraint and thus
     has no standard error.

           Asymptotic Correlation Matrix of Parameter Estimates

                    alpha       beta_0       beta_1
     alpha              1     1.4e-010     7.2e-011
    beta_0     1.4e-010            1         -0.7
    beta_1     7.2e-011         -0.7            1
```

The following parameter(s) have been estimated at a boundary
point or have been specified. Correlations are not computed:

beta_2

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	1.48	0.1	1.54	0.147	-1.34
30	10	1.57	0.14	1.58	0.147	-0.292
60	9	1.66	0.16	1.63	0.147	0.714
120	9	1.82	0.12	1.71	0.147	2.29
240	6	1.78	0.17	1.87	0.147	-1.57

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	68.148702	6	-124.297404
A2	69.554943	10	-119.109885
fitted	62.424690	2	-120.849381
R	52.631671	2	-101.263343

Test 1: Does response and/or variances differ among dose levels
(A2 vs. R)
Test 2: Are Variances Homogeneous (A1 vs A2)
Test 3: Does the Model for the Mean Fit (A1 vs. fitted)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	33.8465	8	<.0001
Test 2	2.81248	4	0.5897
Test 3	11.448	2	0.003267

The p-value for Test 1 is less than .05. There appears
to be a
difference between response and/or variances among the
dose levels.
It seems appropriate to model the data

The p-value for Test 2 is greater than .05. A
homogeneous variance
model appears to be appropriate here

The p-value for Test 3 is less than .05. You may want
to try a
different model

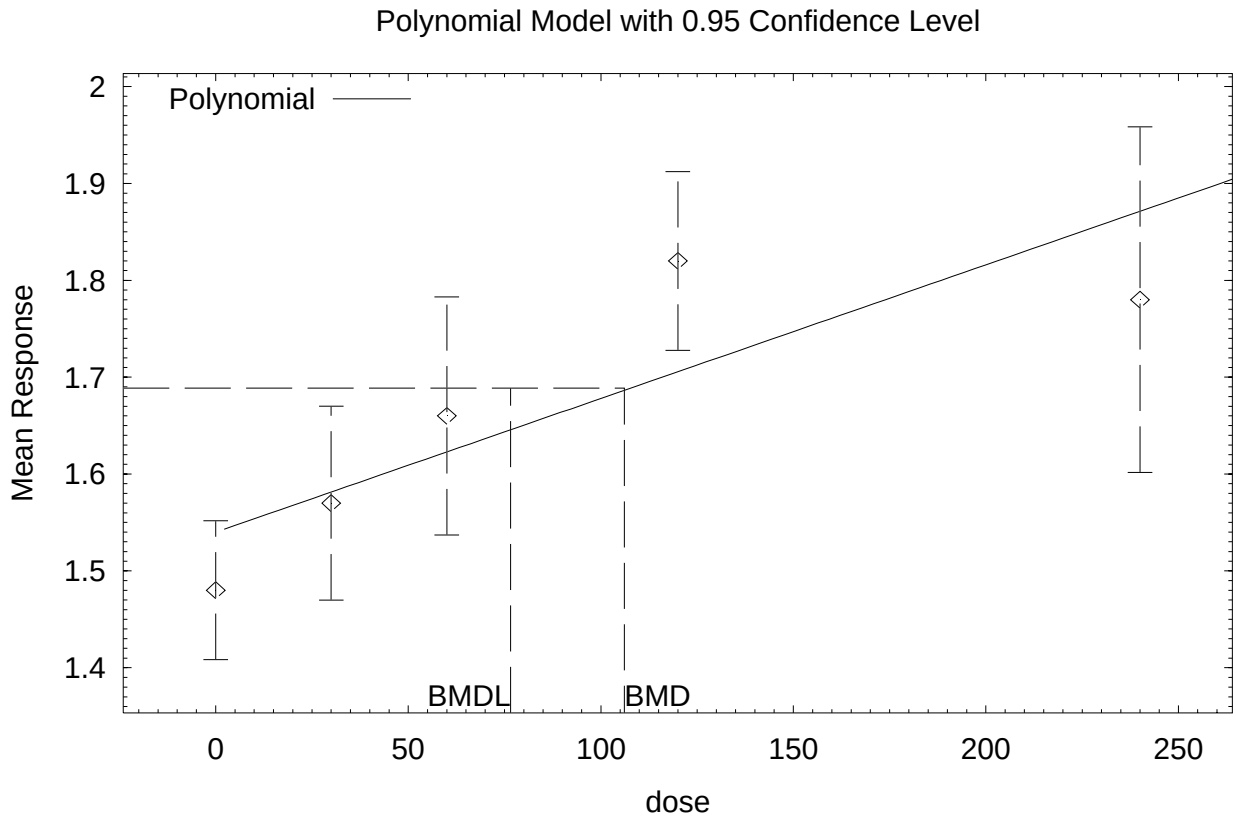
 Benchmark Dose Computation
Specified effect =   1

Risk Type   =   Estimated standard deviations from the control mean

Confidence level =   0.95

             BMD =   106.072

            BMDL =   76.5569

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                                          Thu May 12 22:28:38 2005
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BMDS MODEL RUN
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  The form of the response function is:

  Y[dose] = control + slope * dose^power


  Dependent variable = MEAN
  Independent variable = dose
  rho is set to 0
  The power is restricted to be greater than or equal to 1
  A constant variance model is fit

  Total number of dose groups = 5
  Total number of records with missing values = 0
  Maximum number of iterations = 250
  Relative Function Convergence has been set to: 1e-008
  Parameter Convergence has been set to: 1e-008
```

Default Initial Parameter Values

alpha =	0.018741	
rho =	0	Specified
control =	1.48	
slope =	0.0132605	
power =	0.612843	

Asymptotic Correlation Matrix of Parameter Estimates

	alpha	rho	control	slope	power
alpha	1	-1	NA	NA	NA
rho	-1	1	NA	NA	NA
control	NA	NA	NA	NA	NA
slope	NA	NA	NA	NA	NA
power	NA	NA	NA	NA	NA

NA - This parameter's variance has been estimated at zero.

Parameter Estimates

Variable	Estimate	Std. Err.
alpha	0.0215477	0.0399798
rho	0	3.81654
control	1.54205	0.0380886
slope	0.00138389	0.00299534
power	1	0.337065

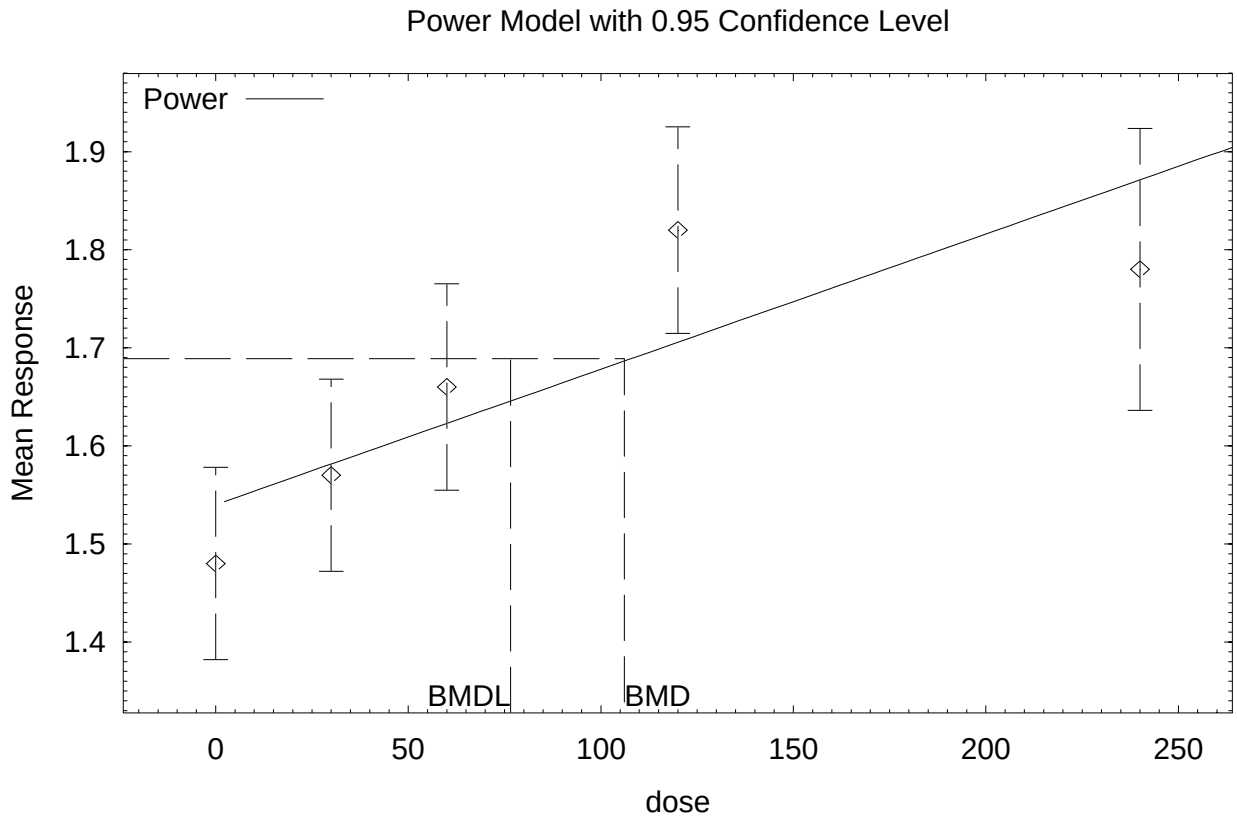
Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	1.48	0.1	1.54	0.147	-0.423
30	10	1.57	0.14	1.58	0.147	-0.0924
60	9	1.66	0.16	1.63	0.147	0.238
120	9	1.82	0.12	1.71	0.147	0.762
240	6	1.78	0.17	1.87	0.147	-0.642

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

```
 Model A2:        Yij = Mu(i) + e(ij)
          Var{e(ij)} = Sigma(i)^2

 Model  R:         Yi = Mu + e(i)
           Var{e(i)} = Sigma^2


                     Likelihoods of Interest

            Model      Log(likelihood)   DF        AIC
              A1           68.148702       6    -124.297404
              A2           69.554943      10    -119.109885
           fitted          62.424690       4    -116.849381
              R            52.631671       2    -101.263343

 Test 1:  Does response and/or variances differ among dose levels
          (A2 vs. R)
 Test 2:  Are Variances Homogeneous (A1 vs A2)
 Test 3:  Does the Model for the Mean Fit (A1 vs. fitted)

                    Tests of Interest

   Test    -2*log(Likelihood Ratio)     df        p-value

   Test 1              33.8465          8         <.00001
   Test 2              2.81248          4          0.5897
   Test 3               11.448          2        0.003267

The p-value for Test 1 is less than .05.  There appears to be a
difference between response and/or variances among the dose levels.
It seems appropriate to model the data

The p-value for Test 2 is greater than .05.  A homogeneous variance
model appears to be appropriate here


The p-value for Test 3 is less than .05.  You may want to try a
different model


 Benchmark Dose Computation
Specified effect =            1

Risk Type        =     Estimated standard deviations from the control mean

Confidence level =          0.95

             BMD =       106.072


            BMDL =       76.5569
```



22:28 05/12 2005

```
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       Gnuplot Plotting File:  C:\BMDS\DATA\CYLINDRO.plt
                                        Thu May 12 22:29:31 2005
=================================================================

BMDS MODEL RUN
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  The form of the response function is:

  Y[dose] = intercept + v*dose^n/(k^n + dose^n)


  Dependent variable = MEAN
  Independent variable = dose
  rho is set to 0
  Power parameter restricted to be greater than 1
  A constant variance model is fit

  Total number of dose groups = 5
  Total number of records with missing values = 0
  Maximum number of iterations = 250
  Relative Function Convergence has been set to: 1e-008
  Parameter Convergence has been set to: 1e-008
```

Default Initial Parameter Values

alpha =	0.0183633	
rho =	0	Specified
intercept =	1.48	
v =	0.34	
n =	0.959904	
k =	63.3333	

Asymptotic Correlation Matrix of Parameter Estimates

	alpha	rho	intercept	v	n	k
alpha	1	0	0	0	0	0
rho	0	1	0	0	0	0
intercept	0	0	1	0	0	0
v	0	0	0	1	0	0
n	0	0	0	0	1	0
k	0	0	0	0	0	1

Parameter Estimates

Variable	Estimate	Std. Err.
alpha	0.0172084	1
rho	0	1
intercept	1.4859	1
v	0.33478	1
n	2.46401	1
k	51.624	1

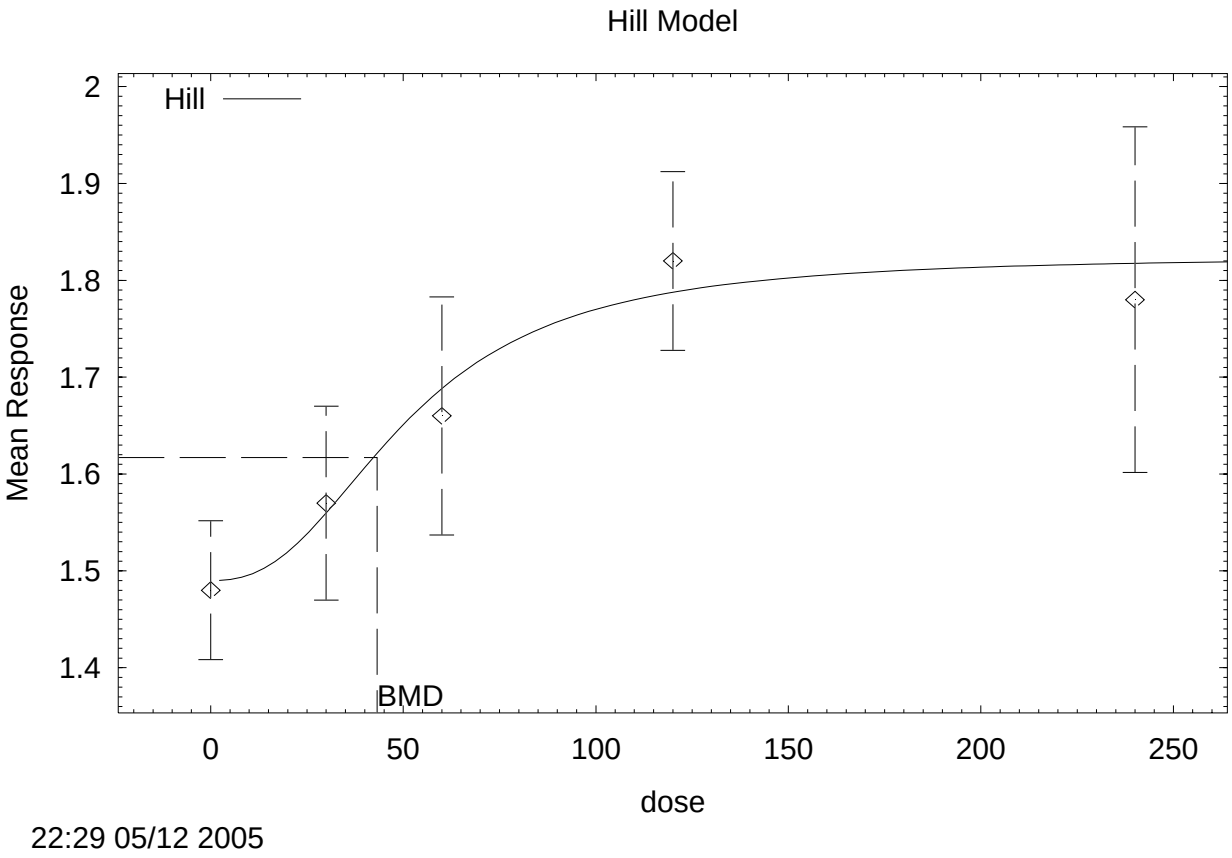
Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	1.48	0.1	1.49	0.131	-0.0449
30	10	1.57	0.14	1.56	0.131	0.11
60	9	1.66	0.16	1.68	0.131	-0.183
120	9	1.82	0.12	1.78	0.131	0.279
240	6	1.78	0.17	1.81	0.131	-0.253

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

```
 Model A2:        Yij = Mu(i) + e(ij)
          Var{e(ij)} = Sigma(i)^2

 Model  R:         Yi = Mu + e(i)
           Var{e(i)} = Sigma^2


                     Likelihoods of Interest

            Model      Log(likelihood)   DF        AIC
              A1          68.148702       6    -124.297404
              A2          69.554943      10    -119.109885
           fitted         67.371885       5    -124.743770
              R           52.631671       2    -101.263343

 Test 1:  Does response and/or variances differ among dose levels
          (A2 vs. R)
 Test 2:  Are Variances Homogeneous (A1 vs A2)
 Test 3:  Does the Model for the Mean Fit (A1 vs. fitted)

                    Tests of Interest

   Test    -2*log(Likelihood Ratio)  Test df     p-value

   Test 1              33.8465          8          <.0001
   Test 2              2.81248          4          0.5897
   Test 3              1.55363          1          0.2126

The p-value for Test 1 is less than .05.  There appears to be a
difference between response and/or variances among the dose levels.
It seems appropriate to model the data

The p-value for Test 2 is greater than .05.  A homogeneous variance
model appears to be appropriate here


The p-value for Test 3 is greater than .05.  The model chosen appears
to adequately describe the data



 Benchmark Dose Computation
Specified effect =            1

Risk Type        =     Estimated standard deviations from the control mean

Confidence level =          0.95

             BMD =       43.1891

Warning:  optimum may not have been found.  Bad completion code in
Optimization routine.

BMDL computation failed.
```


Hill Model
Hill
Mean Response
2
1.9
1.8
1.7
1.6
1.5
1.4
BMD
0
50
100
150
200
250
dose
22:29 05/12 2005

Part II.

Humpage and Falconer 2003

male mice treated with purified cylindrospermopsin

rel kidney wt

drop high dose group

```
 ========================================================================
        Polynomial Model. Revision: 2.2  Date: 9/12/2002
        Input Data File: C:\BMDS\DATA\CYLINDRO.(d)
        Gnuplot Plotting File:  C:\BMDS\DATA\CYLINDRO.plt
                                          Thu May 12 22:31:50 2005
 ========================================================================

 BMDS MODEL RUN
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   The form of the response function is:

   Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


   Dependent variable = MEAN
   Independent variable = dose
   rho is set to 0
   The polynomial coefficients are restricted to be positive
   A constant variance model is fit

   Total number of dose groups = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                          alpha =    0.0172471
                            rho =            0   Specified
                         beta_0 =        1.484
                         beta_1 =   0.00282857



                               Parameter Estimates

                                                95.0% Wald Confidence Interval
   Variable   Estimate     Std. Err.     Lower Conf. Limit   Upper Conf. Limit
   alpha      0.0154483    0.00354407       0.008502            0.0223945
   beta_0     1.4838       0.0306522        1.42373             1.54388
   beta_1     0.00283099   0.000456935      0.00193541          0.00372656


           Asymptotic Correlation Matrix of Parameter Estimates

                   alpha       beta_0       beta_1
     alpha             1    -1.7e-010     1.4e-010
    beta_0    -1.7e-010            1        -0.75
    beta_1     1.4e-010        -0.75            1
```

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	1.48	0.1	1.48	0.124	-0.0968
30	10	1.57	0.14	1.57	0.124	0.0323
60	9	1.66	0.16	1.65	0.124	0.153
120	9	1.82	0.12	1.82	0.124	-0.085

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	60.255446	5	-110.510893
A2	61.376237	8	-106.752474
fitted	60.234922	2	-116.469843
R	46.462327	2	-88.924654

Test 1: Does response and/or variances differ among dose levels
(A2 vs. R)
Test 2: Are Variances Homogeneous (A1 vs A2)
Test 3: Does the Model for the Mean Fit (A1 vs. fitted)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	29.8278	6	<.0001
Test 2	2.24158	3	0.5238
Test 3	0.0410496	2	0.9797

The p-value for Test 1 is less than .05. There appears to be a
difference between response and/or variances among the dose levels.
It seems appropriate to model the data

The p-value for Test 2 is greater than .05. A
homogeneous variance
model appears to be appropriate here

The p-value for Test 3 is greater than .05. The model
chosen appears
to adequately describe the data

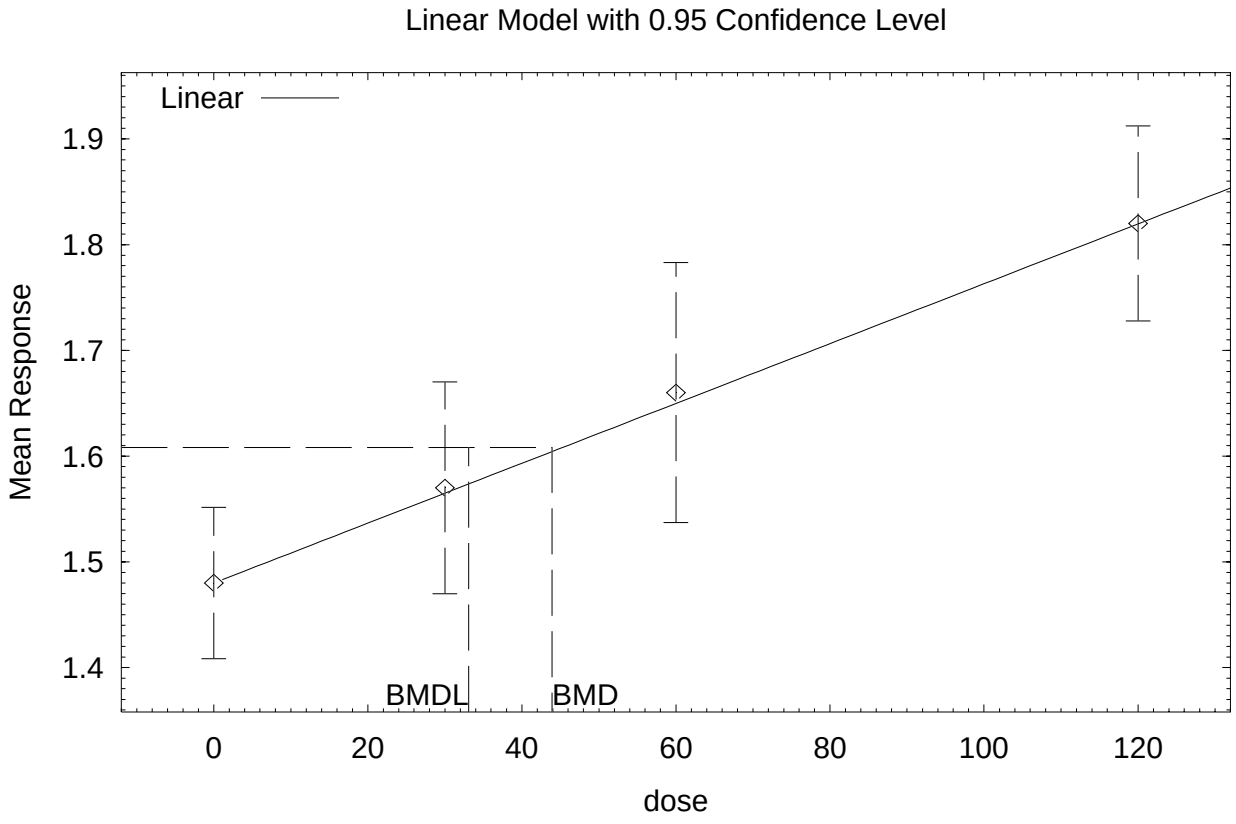
 Benchmark Dose Computation
Specified effect =   1

Risk Type   =   Estimated standard deviations from the control mean

Confidence level =   0.95

             BMD =   43.9038

            BMDL =   33.0684



```
 ========================================================================
        Polynomial Model. Revision: 2.2  Date: 9/12/2002
        Input Data File: C:\BMDS\DATA\CYLINDRO.(d)
        Gnuplot Plotting File:  C:\BMDS\DATA\CYLINDRO.plt
                                          Thu May 12 22:33:17 2005
 ========================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the response function is:

   Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


   Dependent variable = MEAN
   Independent variable = dose
   rho is set to 0
   The polynomial coefficients are restricted to be positive
   A constant variance model is fit

   Total number of dose groups = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                          alpha =    0.0172471
                            rho =            0   Specified
                         beta_0 =      1.47945
                         beta_1 =   0.00314242
                         beta_2 =            0



                                Parameter Estimates

                                          95.0% Wald Confidence Interval
   Variable    Estimate    Std. Err.     Lower Conf. Limit  Upper Conf. Limit
   alpha       0.0154483   0.00354407     0.008502          0.0223945
   beta_0      1.4838      0.0306522      1.42373           1.54388
   beta_1      0.00283099  0.000456935    0.00193541        0.00372656
   beta_2      0           NA

NA - Indicates that this parameter has hit a bound
     implied by some inequality constraint and thus
     has no standard error.

           Asymptotic Correlation Matrix of Parameter Estimates

                   alpha       beta_0       beta_1
    alpha              1     4.9e-011     9.5e-011
   beta_0     4.9e-011            1        -0.75
   beta_1     9.5e-011        -0.75            1
```

The following parameter(s) have been estimated at a boundary
point or have been specified. Correlations are not computed:

beta_2

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	1.48	0.1	1.48	0.124	-0.0968
30	10	1.57	0.14	1.57	0.124	0.0323
60	9	1.66	0.16	1.65	0.124	0.153
120	9	1.82	0.12	1.82	0.124	-0.085

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	60.255446	5	-110.510893
A2	61.376237	8	-106.752474
fitted	60.234922	2	-116.469843
R	46.462327	2	-88.924654

Test 1: Does response and/or variances differ among dose levels
(A2 vs. R)
Test 2: Are Variances Homogeneous (A1 vs A2)
Test 3: Does the Model for the Mean Fit (A1 vs. fitted)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	29.8278	6	<.0001
Test 2	2.24158	3	0.5238
Test 3	0.0410496	1	0.8394

The p-value for Test 1 is less than .05. There appears to be a
difference between response and/or variances among the dose levels.
It seems appropriate to model the data

The p-value for Test 2 is greater than .05. A homogeneous variance
model appears to be appropriate here

The p-value for Test 3 is greater than .05. The model chosen appears
to adequately describe the data

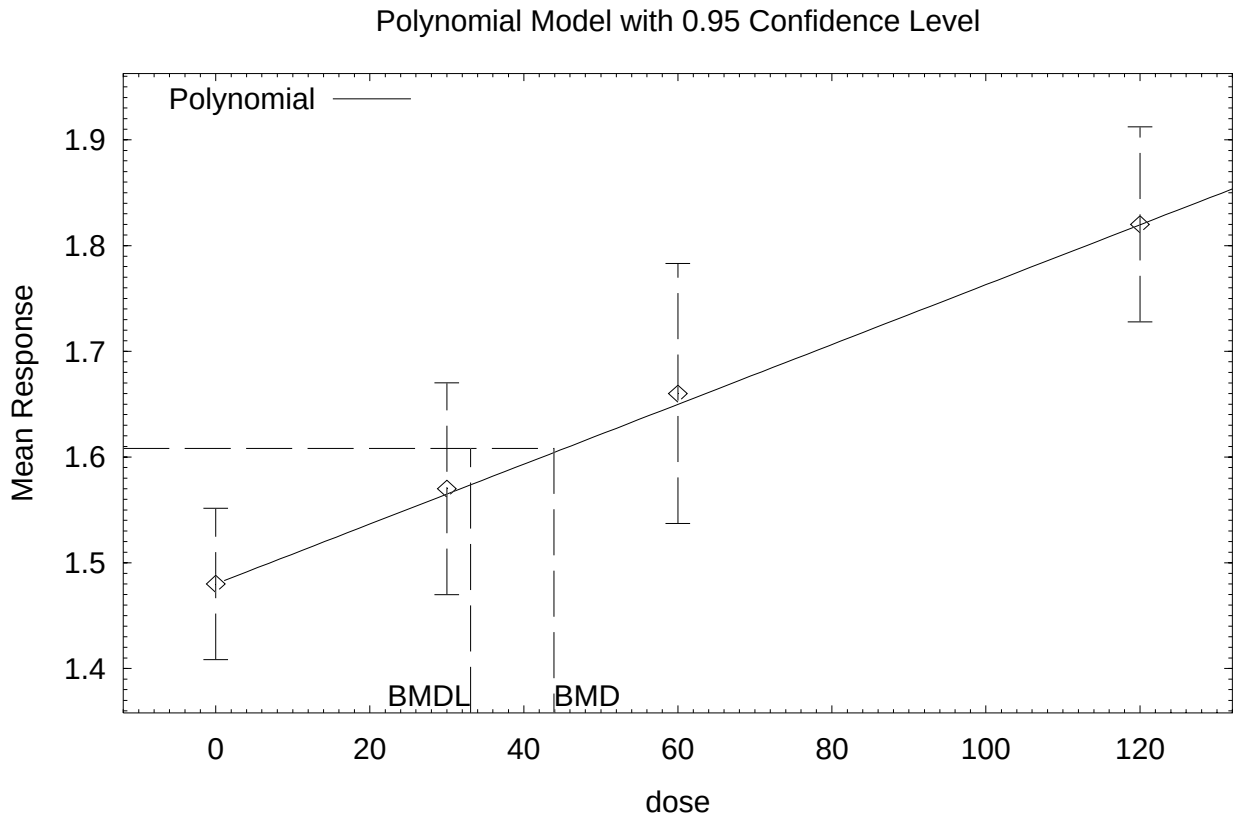
Benchmark Dose Computation
Specified effect = 1

Risk Type = Estimated standard deviations from the control mean

Confidence level = 0.95

BMD = 43.9038

BMDL = 33.0684



22:33 05/12 2005

```
=================================================================
       Power Model. $Revision: 2.1 $ $Date: 2000/10/11 20:57:36 $
       Input Data File: C:\BMDS\DATA\CYLINDRO.(d)
       Gnuplot Plotting File:  C:\BMDS\DATA\CYLINDRO.plt
                                        Thu May 12 22:34:08 2005
=================================================================

BMDS MODEL RUN
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  The form of the response function is:

  Y[dose] = control + slope * dose^power


  Dependent variable = MEAN
  Independent variable = dose
  rho is set to 0
  The power is restricted to be greater than or equal to 1
  A constant variance model is fit

  Total number of dose groups = 4
  Total number of records with missing values = 0
  Maximum number of iterations = 250
  Relative Function Convergence has been set to: 1e-008
  Parameter Convergence has been set to: 1e-008
```

Default Initial Parameter Values

alpha =	0.0172471	
rho =	0	Specified
control =	1.48	
slope =	0.00345163	
power =	0.958769	

Asymptotic Correlation Matrix of Parameter Estimates

	alpha	rho	control	slope	power
alpha	1	-0.99	0.25	-0.29	0.29
rho	-0.99	1	-0.25	0.29	-0.28
control	0.25	-0.25	1	-0.71	0.66
slope	-0.29	0.29	-0.71	1	-1
power	0.29	-0.28	0.66	-1	1

Parameter Estimates

Variable	Estimate	Std. Err.
alpha	0.0154483	0.0279175
rho	0	3.65432
control	1.4838	0.0410106
slope	0.00283099	0.00517499
power	1	0.372848

Table of Data and Estimated Values of Interest

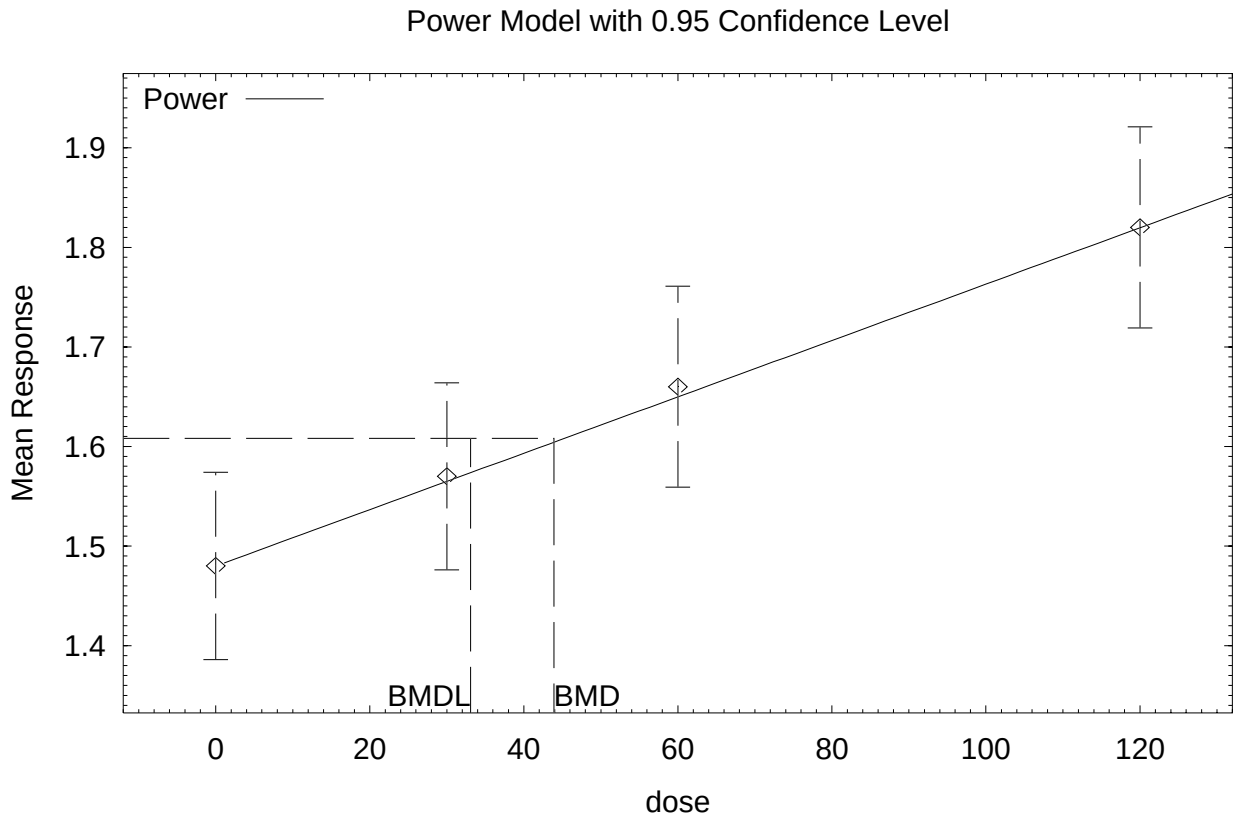
Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	1.48	0.1	1.48	0.124	-0.0306
30	10	1.57	0.14	1.57	0.124	0.0102
60	9	1.66	0.16	1.65	0.124	0.051
120	9	1.82	0.12	1.82	0.124	-0.0283

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

```
 Model  R:         Yi = Mu + e(i)
           Var{e(i)} = Sigma^2


                     Likelihoods of Interest

            Model      Log(likelihood)   DF        AIC
              A1          60.255446       5    -110.510893
              A2          61.376237       8    -106.752474
           fitted         60.234922       4    -112.469843
              R           46.462327       2     -88.924654

 Test 1:  Does response and/or variances differ among dose levels
          (A2 vs. R)
 Test 2:  Are Variances Homogeneous (A1 vs A2)
 Test 3:  Does the Model for the Mean Fit (A1 vs. fitted)

                    Tests of Interest

   Test    -2*log(Likelihood Ratio)     df        p-value

   Test 1              29.8278          6         <.00001
   Test 2              2.24158          3          0.5238
   Test 3            0.0410496          1          0.8394

The p-value for Test 1 is less than .05.  There appears to be a
difference between response and/or variances among the dose levels.
It seems appropriate to model the data

The p-value for Test 2 is greater than .05.  A homogeneous variance
model appears to be appropriate here


The p-value for Test 3 is greater than .05.  The model chosen appears
to adequately describe the data


 Benchmark Dose Computation
Specified effect =             1

Risk Type        =     Estimated standard deviations from the control mean

Confidence level =          0.95

             BMD =       43.9038


            BMDL =       33.0684
```



22:34 05/12 2005

```
==========================================================================
       Hill Model. $Revision: 2.1 $ $Date: 2000/10/11 21:21:23 $
       Input Data File: C:\BMDS\DATA\CYLINDRO.(d)
       Gnuplot Plotting File:  C:\BMDS\DATA\CYLINDRO.plt
                                          Thu May 12 22:34:43 2005
==========================================================================

BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

  The form of the response function is:

  Y[dose] = intercept + v*dose^n/(k^n + dose^n)


  Dependent variable = MEAN
  Independent variable = dose
  rho is set to 0
  Power parameter restricted to be greater than 1
  A constant variance model is fit

  Total number of dose groups = 4
  Total number of records with missing values = 0
  Maximum number of iterations = 250
  Relative Function Convergence has been set to: 1e-008
  Parameter Convergence has been set to: 1e-008
```

Default Initial Parameter Values

alpha =	0.0163951	
rho =	0	Specified
intercept =	1.48	
v =	0.34	
n =	0.68364	
k =	63.3333	

Asymptotic Correlation Matrix of Parameter Estimates

	alpha	rho	intercept	v	n	k
alpha	1	0	0	0	0	0
rho	0	1	0	0	0	0
intercept	0	0	1	0	0	0
v	0	0	0	1	0	0
n	0	0	0	0	1	0
k	0	0	0	0	0	1

Parameter Estimates

Variable	Estimate	Std. Err.
alpha	0.0154316	1
rho	0	1
intercept	1.48	1
v	1.62004	1
n	1.08746	1
k	406.089	1

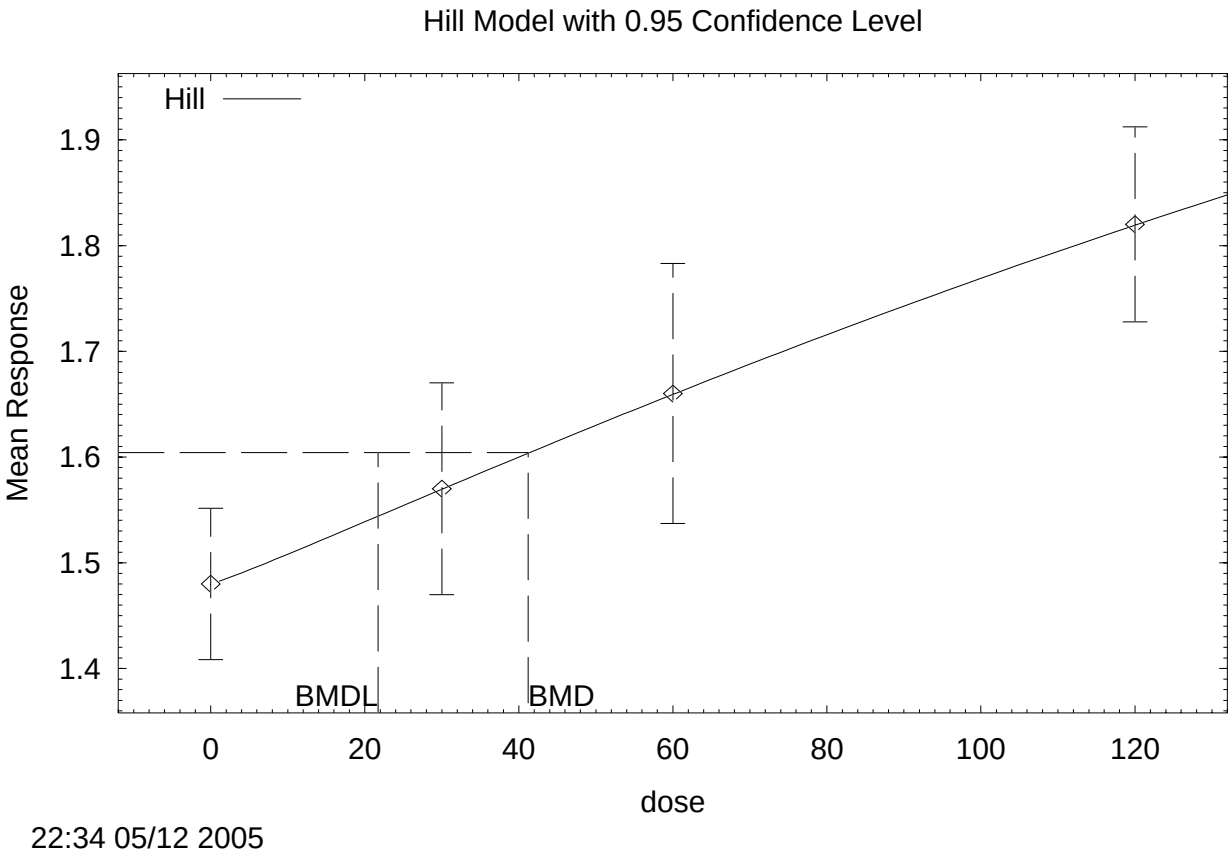
Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	1.48	0.1	1.48	0.124	-2.33e-007
30	10	1.57	0.14	1.57	0.124	-4.42e-007
60	9	1.66	0.16	1.66	0.124	8.2e-007
120	9	1.82	0.12	1.82	0.124	-1.25e-008

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

```
 Model A2:        Yij = Mu(i) + e(ij)
          Var{e(ij)} = Sigma(i)^2

 Model  R:         Yi = Mu + e(i)
           Var{e(i)} = Sigma^2


 Warning: Likelihood for fitted model larger than the Likelihood for model
A1.

                      Likelihoods of Interest

            Model      Log(likelihood)   DF        AIC
              A1          60.255446       5    -110.510893
              A2          61.376237       8    -106.752474
           fitted         60.255447       5    -110.510893
              R           46.462327       2     -88.924654

 Test 1:  Does response and/or variances differ among dose levels
          (A2 vs. R)
 Test 2:  Are Variances Homogeneous (A1 vs A2)
 Test 3:  Does the Model for the Mean Fit (A1 vs. fitted)

                    Tests of Interest

   Test    -2*log(Likelihood Ratio)  Test df     p-value

   Test 1              29.8278          6          <.0001
   Test 2              2.24158          3          0.5238
   Test 3        -4.58279e-007          0              NA

The p-value for Test 1 is less than .05.  There appears to be a
difference between response and/or variances among the dose levels.
It seems appropriate to model the data

The p-value for Test 2 is greater than .05.  A homogeneous variance
model appears to be appropriate here


NA - Degrees of freedom for Test 3 are less than or equal to 0.  The Chi-
Square
     test for fit is not valid


 Benchmark Dose Computation
Specified effect =            1

Risk Type        =     Estimated standard deviations from the control mean

Confidence level =          0.95

             BMD =       41.1966

            BMDL =        21.722
```


Hill Model with 0.95 Confidence Level
Hill
Mean Response
1.9
1.8
1.7
1.6
1.5
1.4
BMDL
BMD
0
20
40
60
80
100
120
dose
22:34 05/12 2005

Part III.

Humpage and Falconer 2003

male mice treated with purified cylindrospermopsin

urinary protein levels

```
 ========================================================================
        Polynomial Model. Revision: 2.2  Date: 9/12/2002
        Input Data File: C:\BMDS\DATA\CYLINDRO.(d)
        Gnuplot Plotting File:  C:\BMDS\DATA\CYLINDRO.plt
                                          Thu May 12 23:15:09 2005
 ========================================================================

 BMDS MODEL RUN
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   The form of the response function is:

   Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


   Dependent variable = MEAN
   Independent variable = dose
   rho is set to 0
   Signs of the polynomial coefficients are not restricted
   A constant variance model is fit

   Total number of dose groups = 5
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                          alpha =    0.0467949
                            rho =            0   Specified
                         beta_0 =      4.00125
                         beta_1 =   -0.0114583



                                Parameter Estimates

                                                95.0% Wald Confidence Interval
   Variable   Estimate    Std. Err.     Lower Conf. Limit    Upper Conf. Limit
   alpha      0.109717    0.0233918          0.0638701            0.155564
   beta_0     4.03293     0.070386           3.89498              4.17088
   beta_1     -0.0120086  0.000649584        -0.0132818           -0.0107354


           Asymptotic Correlation Matrix of Parameter Estimates

                   alpha       beta_0       beta_1
     alpha             1     2.5e-009     9.1e-010
    beta_0     2.5e-009            1         -0.7
    beta_1     9.1e-010         -0.7            1
```

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	4.25	0.3	4.03	0.331	2.07
30	10	3.7	0.25	3.67	0.331	0.261
60	9	3.25	0.05	3.31	0.331	-0.565
120	9	2.15	0.2	2.59	0.331	-4
240	6	1.5	0.15	1.15	0.331	2.58

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	48.017412	6	-84.034824
A2	59.392540	10	-98.785081
fitted	26.616696	2	-49.233392
R	-21.651321	2	47.302642

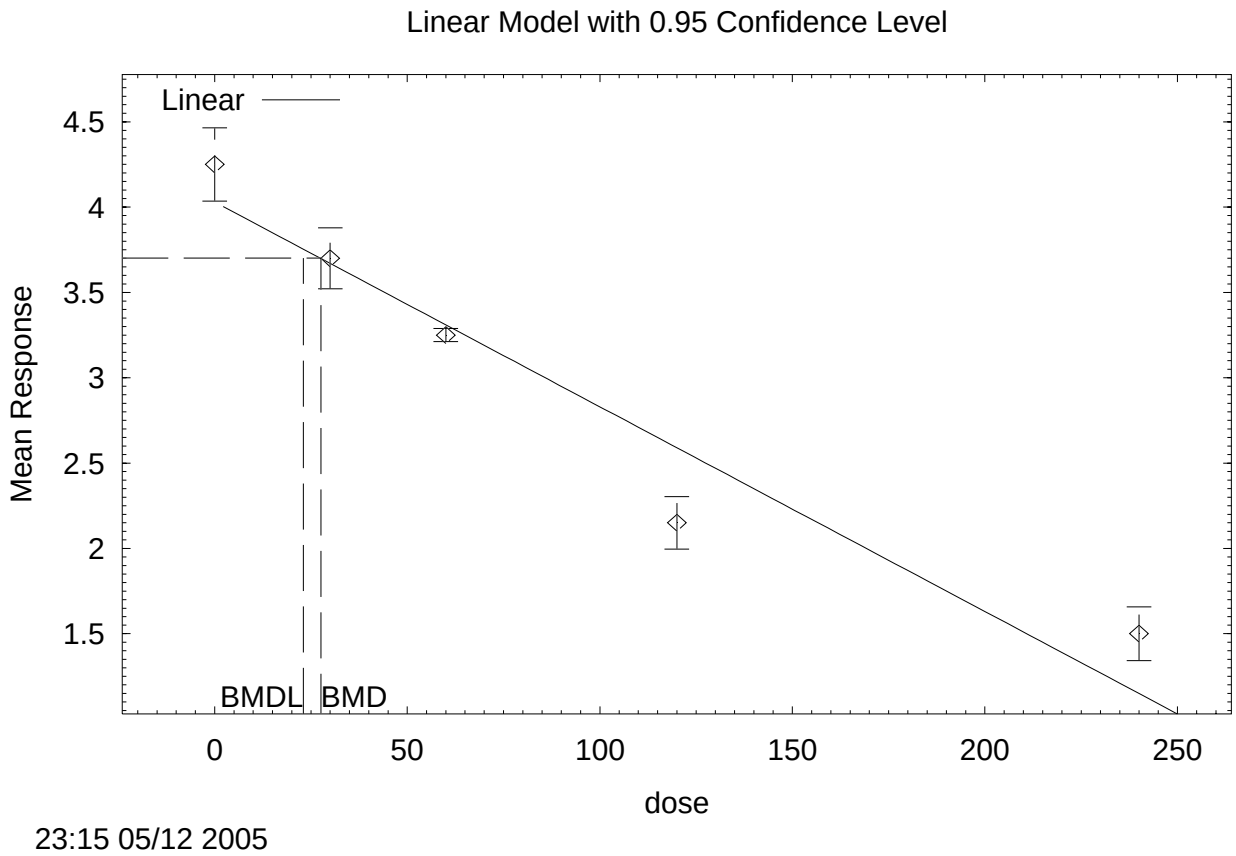
Test 1: Does response and/or variances differ among dose levels
(A2 vs. R)
Test 2: Are Variances Homogeneous (A1 vs A2)
Test 3: Does the Model for the Mean Fit (A1 vs. fitted)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	162.088	8	<.0001
Test 2	22.7503	4	0.000142
Test 3	42.8014	3	<.0001

The p-value for Test 1 is less than .05. There appears to be a
difference between response and/or variances among the dose levels.
It seems appropriate to model the data

```
The p-value for Test 2 is less than .05.  Consider
running a
non-homogeneous variance model

The p-value for Test 3 is less than .05.  You may want
to try a
different model


 Benchmark Dose Computation
Specified effect =            1

Risk Type        =     Estimated standard deviations from the control mean


Confidence level =          0.95

             BMD =       27.5832


            BMDL =       22.9758
```



```
 ========================================================================
       Polynomial Model. Revision: 2.2  Date: 9/12/2002
       Input Data File: C:\BMDS\DATA\CYLINDRO.(d)
       Gnuplot Plotting File:  C:\BMDS\DATA\CYLINDRO.plt
                                        Thu May 12 23:16:16 2005
 ========================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the response function is:

   Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


   Dependent variable = MEAN
   Independent variable = dose
   Signs of the polynomial coefficients are not restricted
   The variance is to be modeled as Var(i) = alpha*mean(i)^rho

   Total number of dose groups = 5
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                          alpha =    0.0467949
                            rho =            0
                         beta_0 =      4.00125
                         beta_1 =   -0.0114583



                               Parameter Estimates

                                                95.0% Wald Confidence Interval
   Variable    Estimate      Std. Err.     Lower Conf. Limit   Upper Conf. Limit
   alpha       0.0612302     0.0136561       0.0344646         0.0879957
   rho         -0.214753     0.0359694       -0.285252         -0.144255
   beta_0      4.26301       0.0535233       4.15811           4.36791
   beta_1      -0.0177625    0.00084855      -0.0194257        -0.0160994


           Asymptotic Correlation Matrix of Parameter Estimates

                  alpha          rho       beta_0       beta_1
     alpha            1         0.12         0.11        -0.18
       rho         0.12            1        -0.18         0.29
    beta_0         0.11        -0.18            1        -0.75
    beta_1        -0.18         0.29        -0.75            1
```

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	4.25	0.3	4.26	0.212	-0.194
30	10	3.7	0.25	3.73	0.215	-0.444
60	9	3.25	0.05	3.2	0.218	0.724
120	9	2.15	0.2	2.13	0.228	0.243
240	6	1.5	0.15	4.95e-008	1.51	2.44

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model A3: Yij = Mu(i) + e(ij)
Var{e(ij)} = alpha*(Mu(i))^rho

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	48.017412	6	-84.034824
A2	59.392540	10	-98.785081
A3	49.799962	7	-85.599924
fitted	33.618701	4	-59.237401
R	-21.651321	2	47.302642

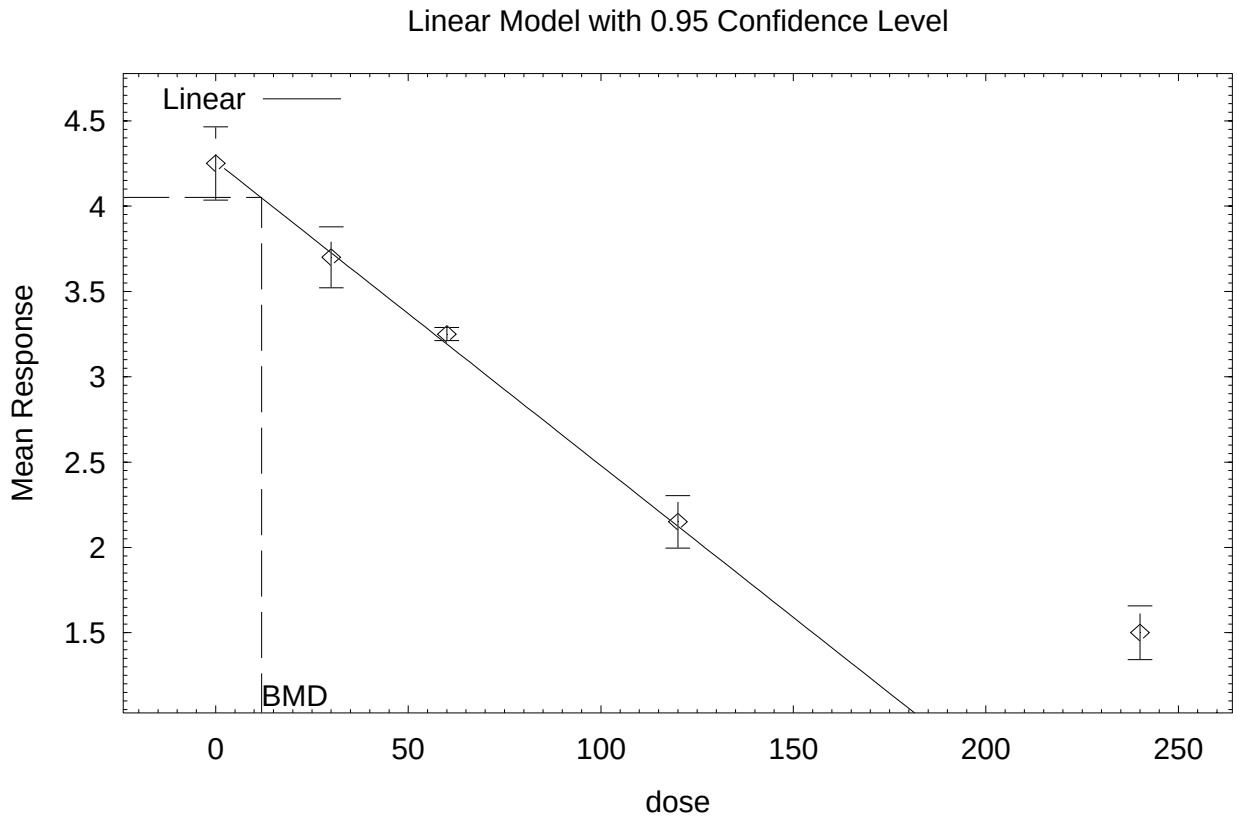
Explanation of Tests

Test 1: Does response and/or variances differ among Dose levels?
(A2 vs. R)
Test 2: Are Variances Homogeneous? (A1 vs A2)
Test 3: Are variances adequately modeled? (A2 vs. A3)
Test 4: Does the Model for the Mean Fit? (A3 vs. fitted)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	162.088	8	<.0001
Test 2	22.7503	4	0.000142
Test 3	19.1852	3	0.0002503
Test 4	32.3625	3	<.0001

```
The p-value for Test 1 is less than .05.  There appears
to be a
difference between response and/or variances among the
dose levels
It seems appropriate to model the data

The p-value for Test 2 is less than .05.  A
non-homogeneous variance
model appears to be appropriate

The p-value for Test 3 is less than .05.  You may want
to consider a
different variance model

The p-value for Test 4 is less than .05.  You may want
to try a different
model


 Benchmark Dose Computation
Specified effect =            1

Risk Type        =     Estimated standard deviations from the control mean


Confidence level =         0.95

             BMD =      11.9223


BMDL computation failed.
```



23:16 05/12 2005

```
=====================================================================
       Polynomial Model. Revision: 2.2  Date: 9/12/2002
       Input Data File: C:\BMDS\DATA\CYLINDRO.(d)
       Gnuplot Plotting File:  C:\BMDS\DATA\CYLINDRO.plt
                                        Thu May 12 23:17:30 2005
=====================================================================

BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

  The form of the response function is:

  Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


  Dependent variable = MEAN
  Independent variable = dose
  rho is set to 0
  Signs of the polynomial coefficients are not restricted
  A constant variance model is fit

  Total number of dose groups = 5
  Total number of records with missing values = 0
  Maximum number of iterations = 250
  Relative Function Convergence has been set to: 1e-008
  Parameter Convergence has been set to: 1e-008
```

Default Initial Parameter Values

alpha =	0.0467949	
rho =	0	Specified
beta_0 =	4.23439	
beta_1 =	-0.0148331	
beta_2 =	-5.51541e-005	
beta_3 =	2.89474e-007	

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
alpha	0.0424211	0.0090442	0.0246948	0.0601474
beta_0	4.23509	0.0633648	4.1109	4.35929
beta_1	-0.0149779	0.00335279	-0.0215492	-0.00840654
beta_2	-5.33342e-005	4.06738e-005	-0.000133053	2.63851e-005
beta_3	2.84322e-007	1.17673e-007	5.36875e-008	5.14956e-007

Asymptotic Correlation Matrix of Parameter Estimates

	alpha	beta_0	beta_1	beta_2	beta_3
alpha	1	-1.8e-008	-4.3e-009	1.3e-009	1.5e-009
beta_0	-1.8e-008	1	-0.71	0.55	-0.48
beta_1	-4.3e-009	-0.71	1	-0.96	0.91
beta_2	1.3e-009	0.55	-0.96	1	-0.99
beta_3	1.5e-009	-0.48	0.91	-0.99	1

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	4.25	0.3	4.24	0.206	0.229
30	10	3.7	0.25	3.75	0.206	-0.698
60	9	3.25	0.05	3.21	0.206	0.643
120	9	2.15	0.2	2.16	0.206	-0.161
240	6	1.5	0.15	1.5	0.206	0.0141

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2
Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	48.017412	6	-84.034824
A2	59.392540	10	-98.785081
fitted	47.522415	4	-87.044831
R	-21.651321	2	47.302642

Test 1: Does response and/or variances differ among dose levels
(A2 vs. R)
Test 2: Are Variances Homogeneous (A1 vs A2)
Test 3: Does the Model for the Mean Fit (A1 vs. fitted)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	162.088	8	<.0001
Test 2	22.7503	4	0.000142
Test 3	0.989994	1	0.3197

The p-value for Test 1 is less than .05. There appears to be a
difference between response and/or variances among the dose levels.
It seems appropriate to model the data

The p-value for Test 2 is less than .05. Consider running a
non-homogeneous variance model

The p-value for Test 3 is greater than .05. The model chosen appears
to adequately describe the data

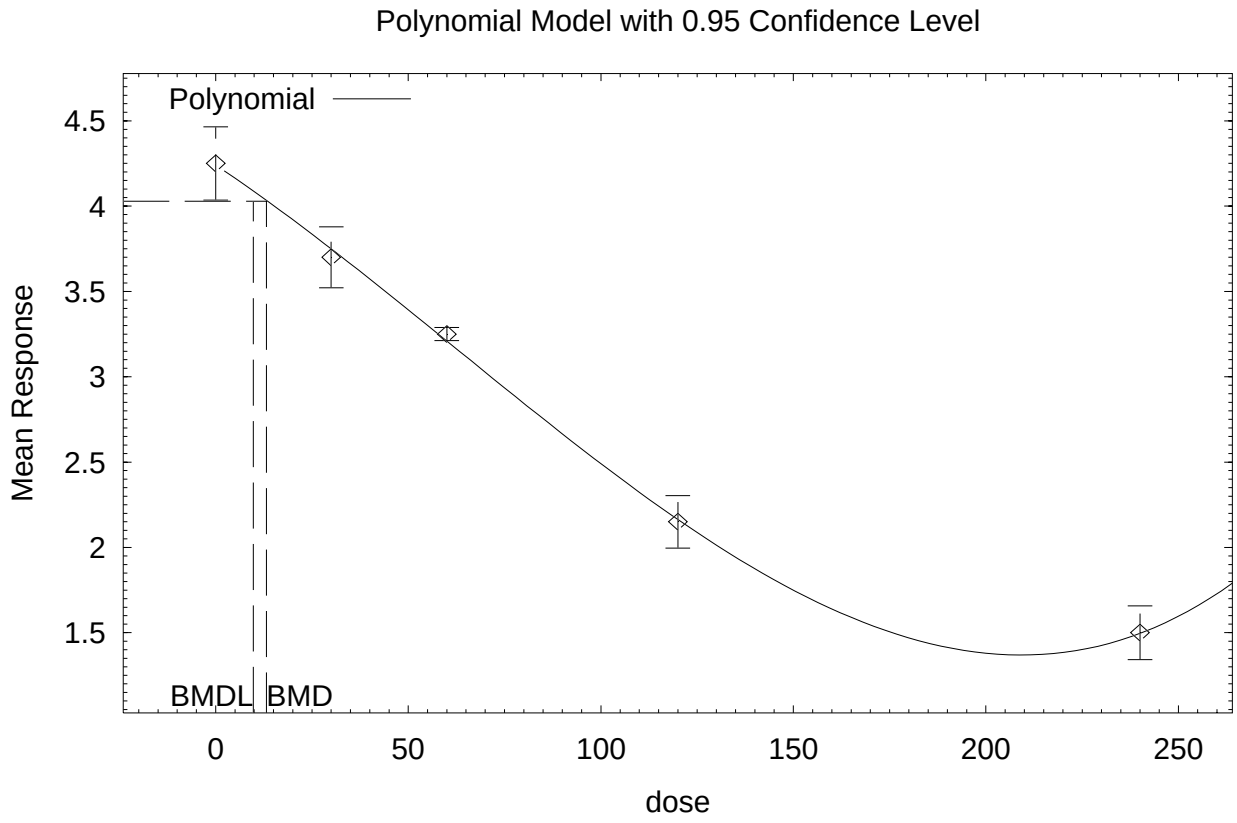
Benchmark Dose Computation
Specified effect = 1

Risk Type = Estimated standard deviations from the control mean

Confidence level = 0.95

BMD = 13.1764

BMDL = 9.7619



23:17 05/12 2005

```
 ========================================================================
        Polynomial Model. Revision: 2.2  Date: 9/12/2002
        Input Data File: C:\BMDS\DATA\CYLINDRO.(d)
        Gnuplot Plotting File:  C:\BMDS\DATA\CYLINDRO.plt
                                          Thu May 12 23:18:39 2005
 ========================================================================

 BMDS MODEL RUN
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   The form of the response function is:

   Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


   Dependent variable = MEAN
   Independent variable = dose
   rho is set to 0
   The polynomial coefficients are restricted to be negative
   A constant variance model is fit

   Total number of dose groups = 5
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008
```

Default Initial Parameter Values

alpha =	0.0467949	
rho =	0	Specified
beta_0 =	4.23439	
beta_1 =	-0.0148331	
beta_2 =	-5.51541e-005	
beta_3 =	0	

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
alpha	0.109717	0.0233918	0.0638701	0.155564
beta_0	4.03293	0.070386	3.89498	4.17088
beta_1	-0.0120086	0.000649584	-0.0132818	-0.0107354
beta_2	0	NA		
beta_3	0	NA		

NA - Indicates that this parameter has hit a bound
implied by some inequality constraint and thus
has no standard error.

Asymptotic Correlation Matrix of Parameter Estimates

	alpha	beta_0	beta_1
alpha	1	-1.2e-007	8.2e-008
beta_0	-1.2e-007	1	-0.7
beta_1	8.2e-008	-0.7	1

The following parameter(s) have been estimated at a
boundary
point or have been specified. Correlations are not
computed:

beta_2 beta_3

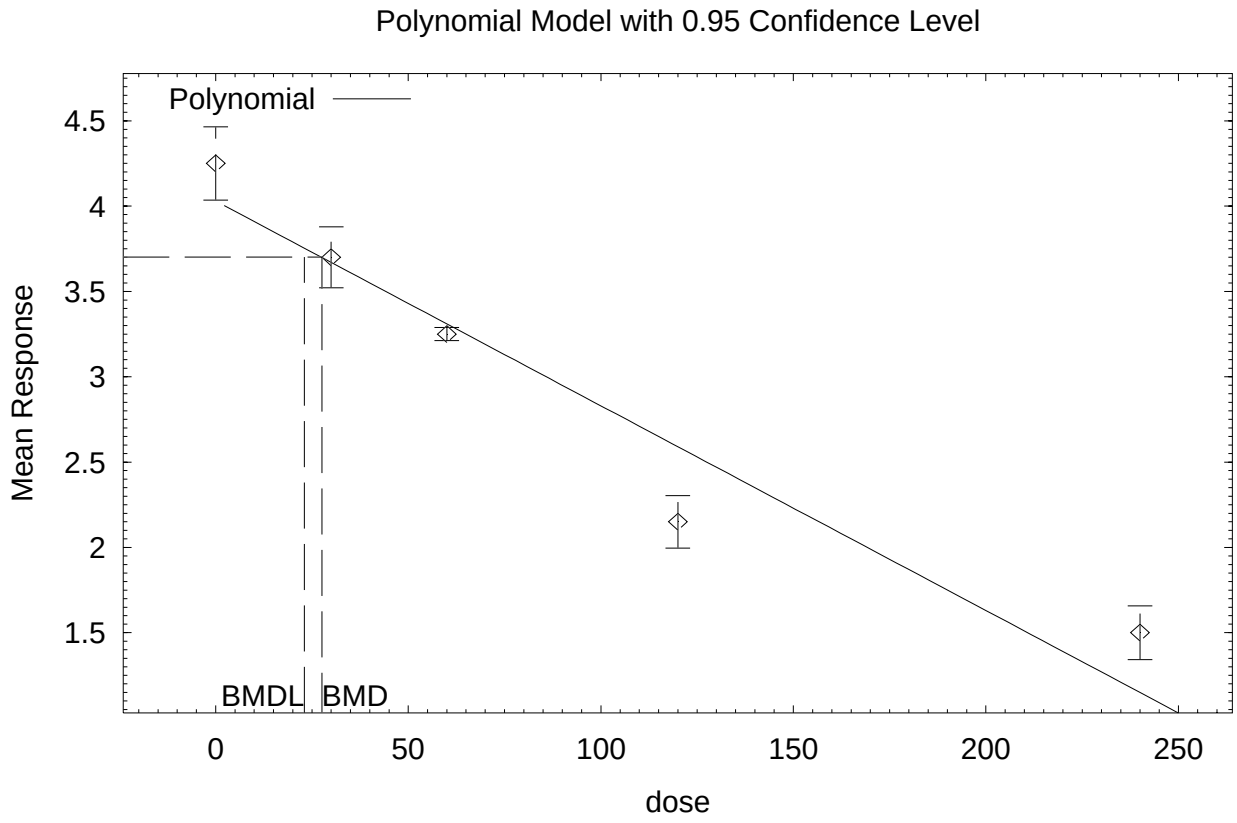
Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	4.25	0.3	4.03	0.331	2.07
30	10	3.7	0.25	3.67	0.331	0.261
60	9	3.25	0.05	3.31	0.331	-0.565
120	9	2.15	0.2	2.59	0.331	-4
240	6	1.5	0.15	1.15	0.331	2.58

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

```
 Model A2:        Yij = Mu(i) + e(ij)
          Var{e(ij)} = Sigma(i)^2

 Model  R:         Yi = Mu + e(i)
           Var{e(i)} = Sigma^2


                     Likelihoods of Interest

            Model      Log(likelihood)   DF        AIC
             A1           48.017412       6     -84.034824
             A2           59.392540      10     -98.785081
           fitted         26.616696       2     -49.233392
              R           -21.651321       2      47.302642

 Test 1:  Does response and/or variances differ among dose
levels
          (A2 vs. R)
 Test 2:  Are Variances Homogeneous (A1 vs A2)
 Test 3:  Does the Model for the Mean Fit (A1 vs. fitted)

                    Tests of Interest

   Test    -2*log(Likelihood Ratio)  Test df     p-value

   Test 1              162.088          8          <.0001
   Test 2              22.7503          4         0.000142
   Test 3              42.8014          1          <.0001

The p-value for Test 1 is less than .05.  There appears
to be a
difference between response and/or variances among the
dose levels.
It seems appropriate to model the data

The p-value for Test 2 is less than .05.  Consider
running a
non-homogeneous variance model

The p-value for Test 3 is less than .05.  You may want
to try a
different model


 Benchmark Dose Computation
Specified effect =            1

Risk Type        =     Estimated standard deviations from the control mean


Confidence level =          0.95

             BMD =       27.5832


            BMDL =       22.9758
```



23:18 05/12 2005

```
=================================================================
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       Input Data File: C:\BMDS\DATA\CYLINDRO.(d)
       Gnuplot Plotting File:  C:\BMDS\DATA\CYLINDRO.plt
                                        Thu May 12 23:19:29 2005
=================================================================

BMDS MODEL RUN
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  The form of the response function is:

  Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


  Dependent variable = MEAN
  Independent variable = dose
  rho is set to 0
  Signs of the polynomial coefficients are not restricted
  A constant variance model is fit

  Total number of dose groups = 5
  Total number of records with missing values = 0
  Maximum number of iterations = 250
  Relative Function Convergence has been set to: 1e-008
  Parameter Convergence has been set to: 1e-008
```

Default Initial Parameter Values

alpha =	0.0467949	
rho =	0	Specified
beta_0 =	4.31231	
beta_1 =	-0.0224959	
beta_2 =	4.45961e-005	

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
alpha	0.0480497	0.0102442	0.0279713	0.068128
beta_0	4.30818	0.0592565	4.19204	4.42432
beta_1	-0.0223839	0.00144605	-0.0252181	-0.0195497
beta_2	4.40376e-005	5.86023e-006	3.25518e-005	5.55235e-005

Asymptotic Correlation Matrix of Parameter Estimates

	alpha	beta_0	beta_1	beta_2
alpha	1	3.3e-009	-6.2e-010	-7.7e-009
beta_0	3.3e-009	1	-0.75	0.62
beta_1	-6.2e-010	-0.75	1	-0.95
beta_2	-7.7e-009	0.62	-0.95	1

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	4.25	0.3	4.31	0.219	-0.839
30	10	3.7	0.25	3.68	0.219	0.342
60	9	3.25	0.05	3.12	0.219	1.73
120	9	2.15	0.2	2.26	0.219	-1.45
240	6	1.5	0.15	1.47	0.219	0.306

Model Descriptions for likelihoods calculated

Model A1: $Y_{ij} = Mu(i) + e(ij)$
$Var\{e(ij)\} = Sigma^2$

Model A2: $Y_{ij} = Mu(i) + e(ij)$
$Var\{e(ij)\} = Sigma(i)^2$

Model R: $Y_i = Mu + e(i)$
$Var\{e(i)\} = Sigma^2$

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	48.017412	6	-84.034824
A2	59.392540	10	-98.785081
fitted	44.781449	3	-83.562899
R	-21.651321	2	47.302642

Test 1: Does response and/or variances differ among dose levels
(A2 vs. R)
Test 2: Are Variances Homogeneous (A1 vs A2)
Test 3: Does the Model for the Mean Fit (A1 vs. fitted)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	162.088	8	<.0001
Test 2	22.7503	4	0.000142
Test 3	6.47193	2	0.03932

The p-value for Test 1 is less than .05. There appears to be a
difference between response and/or variances among the dose levels.
It seems appropriate to model the data

The p-value for Test 2 is less than .05. Consider running a
non-homogeneous variance model

The p-value for Test 3 is less than .05. You may want to try a
different model

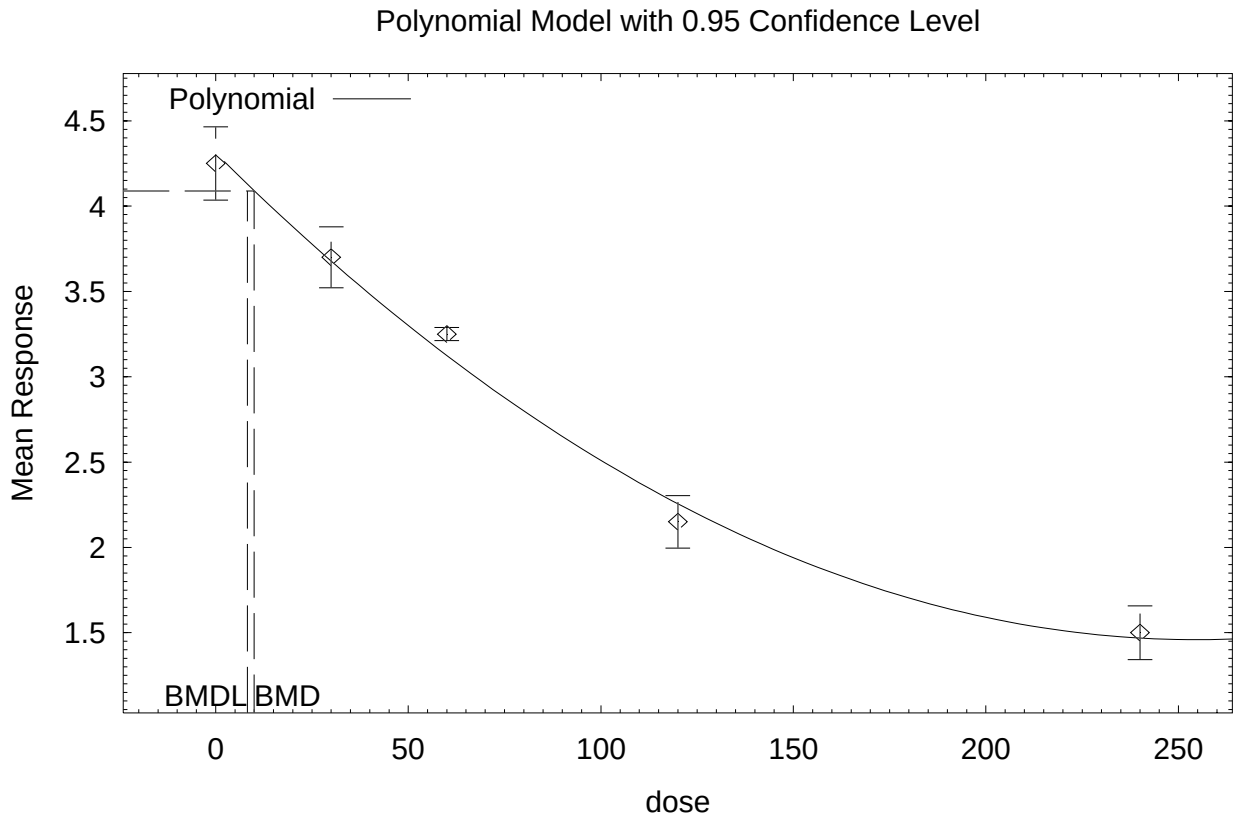
Benchmark Dose Computation
Specified effect = 1

Risk Type = Estimated standard deviations from the control mean

Confidence level = 0.95

BMD = 9.98916

BMDL = 8.24167



23:19 05/12 2005

```
 ======================================================================
        Polynomial Model. Revision: 2.2  Date: 9/12/2002
        Input Data File: C:\BMDS\DATA\CYLINDRO.(d)
        Gnuplot Plotting File:  C:\BMDS\DATA\CYLINDRO.plt
                                          Thu May 12 23:20:33 2005
 ======================================================================

 BMDS MODEL RUN
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   The form of the response function is:

   Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


   Dependent variable = MEAN
   Independent variable = dose
   rho is set to 0
   The polynomial coefficients are restricted to be negative
   A constant variance model is fit

   Total number of dose groups = 5
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008
```

Default Initial Parameter Values

alpha =	0.0467949	
rho =	0	Specified
beta_0 =	4.31231	
beta_1 =	-0.0224959	
beta_2 =	0	

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
alpha	0.109717	0.0233918	0.0638701	0.155564
beta_0	4.03293	0.070386	3.89498	4.17088
beta_1	-0.0120086	0.000649584	-0.0132818	-0.0107354
beta_2	0	NA		

NA - Indicates that this parameter has hit a bound
implied by some inequality constraint and thus
has no standard error.

Asymptotic Correlation Matrix of Parameter Estimates

	alpha	beta_0	beta_1
alpha	1	1.4e-008	2.1e-009
beta_0	1.4e-008	1	-0.7
beta_1	2.1e-009	-0.7	1

The following parameter(s) have been estimated at a
boundary
point or have been specified. Correlations are not
computed:

beta_2

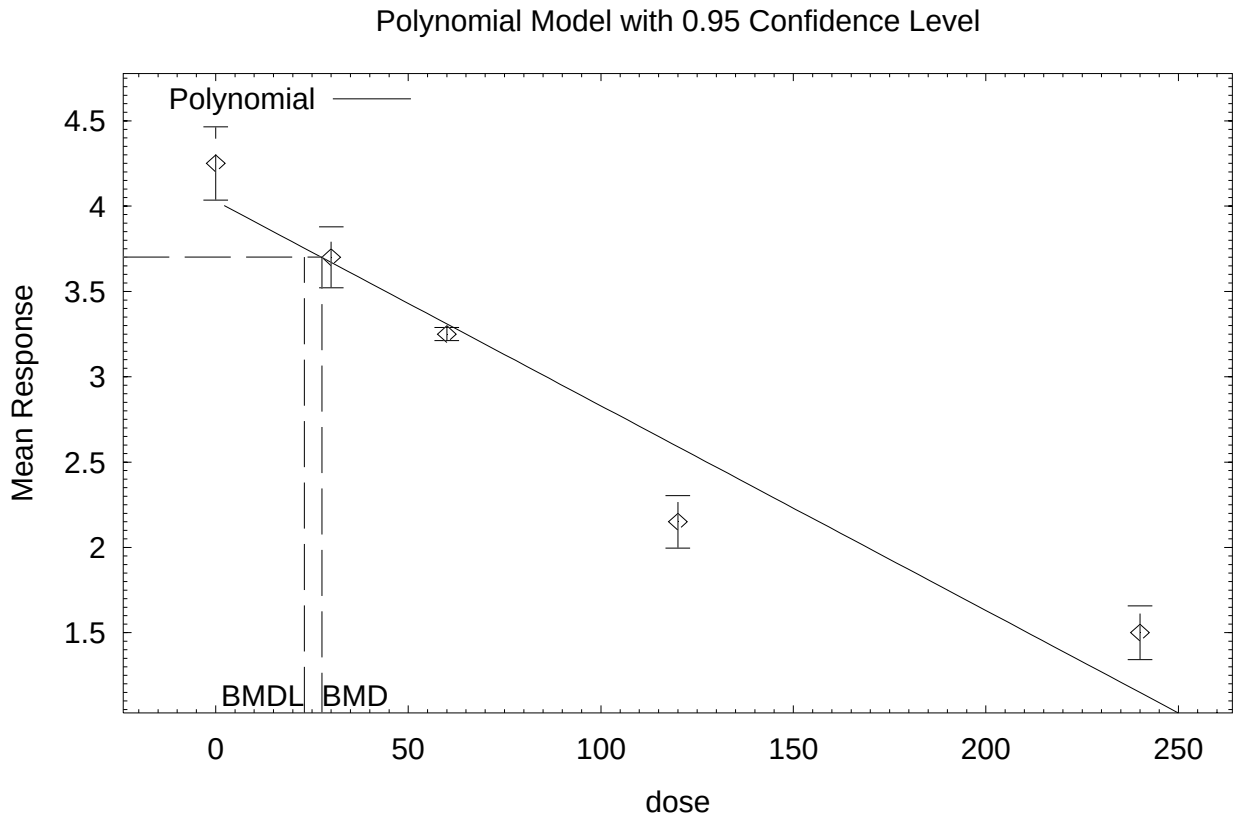
Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	4.25	0.3	4.03	0.331	2.07
30	10	3.7	0.25	3.67	0.331	0.261
60	9	3.25	0.05	3.31	0.331	-0.565
120	9	2.15	0.2	2.59	0.331	-4
240	6	1.5	0.15	1.15	0.331	2.58

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

```
 Model A2:        Yij = Mu(i) + e(ij)
          Var{e(ij)} = Sigma(i)^2

 Model  R:         Yi = Mu + e(i)
           Var{e(i)} = Sigma^2


                     Likelihoods of Interest

            Model      Log(likelihood)   DF        AIC
              A1           48.017412       6     -84.034824
              A2           59.392540      10     -98.785081
           fitted          26.616696       2     -49.233392
              R           -21.651321       2      47.302642

 Test 1:  Does response and/or variances differ among dose
levels
          (A2 vs. R)
 Test 2:  Are Variances Homogeneous (A1 vs A2)
 Test 3:  Does the Model for the Mean Fit (A1 vs. fitted)

                    Tests of Interest

   Test    -2*log(Likelihood Ratio)  Test df     p-value

   Test 1              162.088          8          <.0001
   Test 2              22.7503          4         0.000142
   Test 3              42.8014          2          <.0001
The p-value for Test 1 is less than .05.  There appears
to be a
difference between response and/or variances among the
dose levels.
It seems appropriate to model the data

The p-value for Test 2 is less than .05.  Consider
running a
non-homogeneous variance model

The p-value for Test 3 is less than .05.  You may want
to try a
different model


 Benchmark Dose Computation
Specified effect =             1

Risk Type        =     Estimated standard deviations from the control mean


Confidence level =          0.95

             BMD =       27.5832


            BMDL =       22.9758
```



23:20 05/12 2005

```
=====================================================================
       Power Model. $Revision: 2.1 $ $Date: 2000/10/11 20:57:36 $
       Input Data File: C:\BMDS\DATA\CYLINDRO.(d)
       Gnuplot Plotting File:  C:\BMDS\DATA\CYLINDRO.plt
                                          Thu May 12 23:21:09 2005
=====================================================================

BMDS MODEL RUN
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  The form of the response function is:

  Y[dose] = control + slope * dose^power


  Dependent variable = MEAN
  Independent variable = dose
  rho is set to 0
  The power is restricted to be greater than or equal to 1
  A constant variance model is fit

  Total number of dose groups = 5
  Total number of records with missing values = 0
  Maximum number of iterations = 250
  Relative Function Convergence has been set to: 1e-008
  Parameter Convergence has been set to: 1e-008
```

Default Initial Parameter Values

alpha =	0.0467949	
rho =	0	Specified
control =	4.25	
slope =	-340.972	
power =	-0.879496	

Asymptotic Correlation Matrix of Parameter Estimates

	alpha	rho	control	slope	power
alpha	NA	NA	NA	NA	NA
rho	NA	NA	NA	NA	NA
control	NA	NA	NA	NA	NA
slope	NA	NA	NA	NA	NA
power	NA	NA	NA	NA	NA

NA - This parameter's variance has been estimated at zero.

Parameter Estimates

Variable	Estimate	Std. Err.
alpha	0.109717	0.114352
rho	0	0.935809
control	4.03293	0.129954
slope	-0.0120086	0.0133934
power	1	0.202697

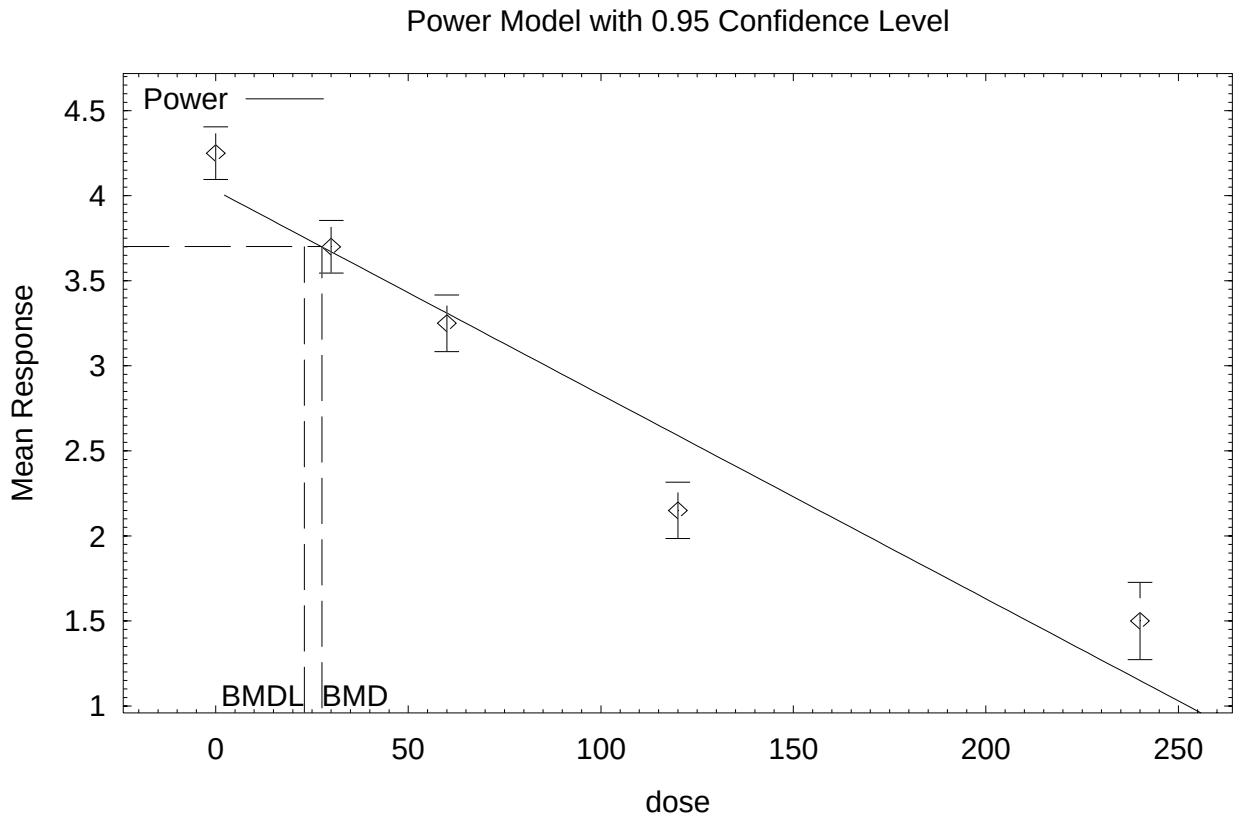
Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	4.25	0.3	4.03	0.331	0.655
30	10	3.7	0.25	3.67	0.331	0.0825
60	9	3.25	0.05	3.31	0.331	-0.188
120	9	2.15	0.2	2.59	0.331	-1.33
240	6	1.5	0.15	1.15	0.331	1.05

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

```
 Model A2:        Yij = Mu(i) + e(ij)
          Var{e(ij)} = Sigma(i)^2

 Model  R:         Yi = Mu + e(i)
           Var{e(i)} = Sigma^2


                     Likelihoods of Interest

            Model      Log(likelihood)   DF        AIC
             A1           48.017412       6     -84.034824
             A2           59.392540      10     -98.785081
           fitted         26.616696       4     -45.233392
              R           -21.651321       2      47.302642

 Test 1:  Does response and/or variances differ among dose levels
          (A2 vs. R)
 Test 2:  Are Variances Homogeneous (A1 vs A2)
 Test 3:  Does the Model for the Mean Fit (A1 vs. fitted)

                    Tests of Interest

   Test    -2*log(Likelihood Ratio)     df        p-value

   Test 1             162.088          8         <.00001
   Test 2             22.7503          4        0.000142
   Test 3             42.8014          2         <.00001

The p-value for Test 1 is less than .05.  There appears to be a
difference between response and/or variances among the dose levels.
It seems appropriate to model the data

The p-value for Test 2 is less than .05.  Consider running a
non-homogeneous variance model

The p-value for Test 3 is less than .05.  You may want to try a
different model


 Benchmark Dose Computation
Specified effect =            1

Risk Type        =     Estimated standard deviations from the control mean

Confidence level =          0.95

             BMD =       27.5832


            BMDL =       22.9758
```



23:21 05/12 2005

```
=================================================================
       Power Model. $Revision: 2.1 $ $Date: 2000/10/11 20:57:36 $
       Input Data File: C:\BMDS\DATA\CYLINDRO.(d)
       Gnuplot Plotting File:  C:\BMDS\DATA\CYLINDRO.plt
                                        Thu May 12 23:21:44 2005
=================================================================

BMDS MODEL RUN
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  The form of the response function is:

  Y[dose] = control + slope * dose^power


  Dependent variable = MEAN
  Independent variable = dose
  rho is set to 0
 The power is not restricted
  A constant variance model is fit

  Total number of dose groups = 5
  Total number of records with missing values = 0
  Maximum number of iterations = 250
  Relative Function Convergence has been set to: 1e-008
  Parameter Convergence has been set to: 1e-008
```

Default Initial Parameter Values

alpha =	0.0467949	
rho =	0	Specified
control =	4.25	
slope =	-0.0336166	
power =	0.803617	

Asymptotic Correlation Matrix of Parameter Estimates

	alpha	rho	control	slope	power
alpha	1	-0.97	-0.22	0.57	0.6
rho	-0.97	1	0.22	-0.58	-0.62
control	-0.22	0.22	1	-0.67	-0.6
slope	0.57	-0.58	-0.67	1	0.99
power	0.6	-0.62	-0.6	0.99	1

Parameter Estimates

Variable	Estimate	Std. Err.
alpha	0.0696661	0.0661961
rho	0	0.846718
control	4.30267	0.0817494
slope	-0.0726613	0.0255026
power	0.676841	0.0658079

Table of Data and Estimated Values of Interest

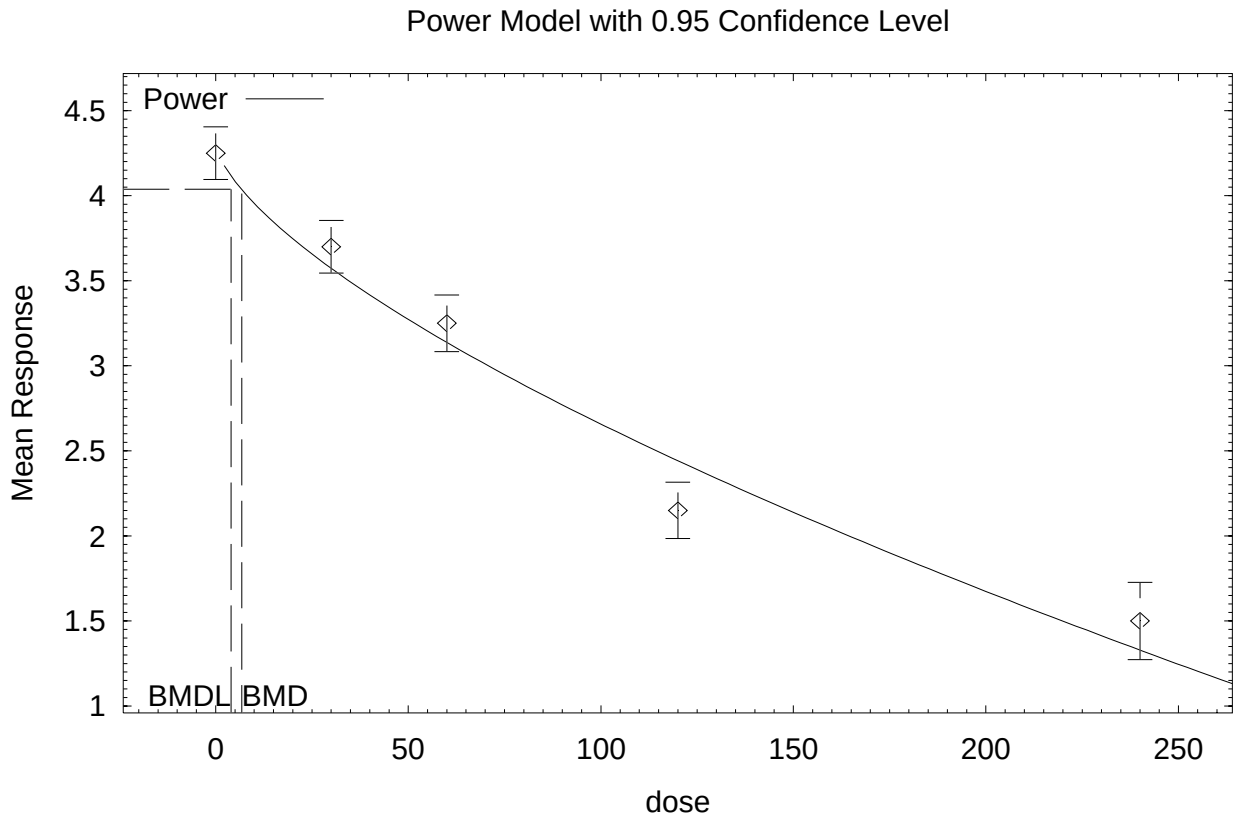
Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	4.25	0.3	4.3	0.264	-0.2
30	10	3.7	0.25	3.58	0.264	0.468
60	9	3.25	0.05	3.14	0.264	0.41
120	9	2.15	0.2	2.45	0.264	-1.12
240	6	1.5	0.15	1.34	0.264	0.623

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

```
 Model  R:         Yi = Mu + e(i)
            Var{e(i)} = Sigma^2


                      Likelihoods of Interest

             Model      Log(likelihood)   DF        AIC
              A1           48.017412       6     -84.034824
              A2           59.392540      10     -98.785081
            fitted         36.608912       4     -65.217824
               R          -21.651321       2      47.302642

 Test 1:  Does response and/or variances differ among dose levels
          (A2 vs. R)
 Test 2:  Are Variances Homogeneous (A1 vs A2)
 Test 3:  Does the Model for the Mean Fit (A1 vs. fitted)

                    Tests of Interest

   Test    -2*log(Likelihood Ratio)     df        p-value

   Test 1              162.088          8         <.00001
   Test 2              22.7503          4        0.000142
   Test 3               22.817          2       1.11e-005

The p-value for Test 1 is less than .05.  There appears to be a
difference between response and/or variances among the dose levels.
It seems appropriate to model the data

The p-value for Test 2 is less than .05.  Consider running a
non-homogeneous variance model

The p-value for Test 3 is less than .05.  You may want to try a
different model


 Benchmark Dose Computation
Specified effect =             1

Risk Type        =     Estimated standard deviations from the control mean

Confidence level =          0.95

             BMD =       6.72481


            BMDL =       3.97902
```



23:21 05/12 2005

```
 ======================================================================
        Hill Model. $Revision: 2.1 $ $Date: 2000/10/11 21:21:23 $
        Input Data File: C:\BMDS\DATA\CYLINDRO.(d)
        Gnuplot Plotting File:  C:\BMDS\DATA\CYLINDRO.plt
                                            Thu May 12 23:22:53 2005
 ======================================================================

 BMDS MODEL RUN
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   The form of the response function is:

   Y[dose] = intercept + v*dose^n/(k^n + dose^n)


   Dependent variable = MEAN
   Independent variable = dose
   rho is set to 0
   Power parameter restricted to be greater than 1
   A constant variance model is fit

   Total number of dose groups = 5
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008
```

Default Initial Parameter Values

alpha =	0.0263306	
rho =	0	Specified
intercept =	4.25	
v =	-2.75	
n =	2.08208	
k =	80.4545	

Asymptotic Correlation Matrix of Parameter Estimates

	alpha	rho	intercept	v	n	k
alpha	1	0	0	0	0	0
rho	0	1	0	0	0	0
intercept	0	0	1	0	0	0
v	0	0	0	1	0	0
n	0	0	0	0	1	0
k	0	0	0	0	0	1

Parameter Estimates

Variable	Estimate	Std. Err.
alpha	0.0466116	1
rho	0	1
intercept	4.22518	1
v	-3.35427	1
n	1.63976	1
k	94.2147	1

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	4.25	0.3	4.23	0.216	0.115
30	10	3.7	0.25	3.78	0.216	-0.369
60	9	3.25	0.05	3.14	0.216	0.502
120	9	2.15	0.2	2.22	0.216	-0.323
240	6	1.5	0.15	1.47	0.216	0.156

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	48.017412	6	-84.034824
A2	59.392540	10	-98.785081
fitted	45.449933	5	-80.899866
R	-21.651321	2	47.302642

Test 1: Does response and/or variances differ among dose levels (A2 vs. R)
Test 2: Are Variances Homogeneous (A1 vs A2)
Test 3: Does the Model for the Mean Fit (A1 vs. fitted)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	162.088	8	<.0001
Test 2	22.7503	4	0.000142
Test 3	5.13496	1	0.02345

The p-value for Test 1 is less than .05. There appears to be a difference between response and/or variances among the dose levels. It seems appropriate to model the data

The p-value for Test 2 is less than .05. Consider running a non-homogeneous variance model

The p-value for Test 3 is less than .05. You may want to try a different model

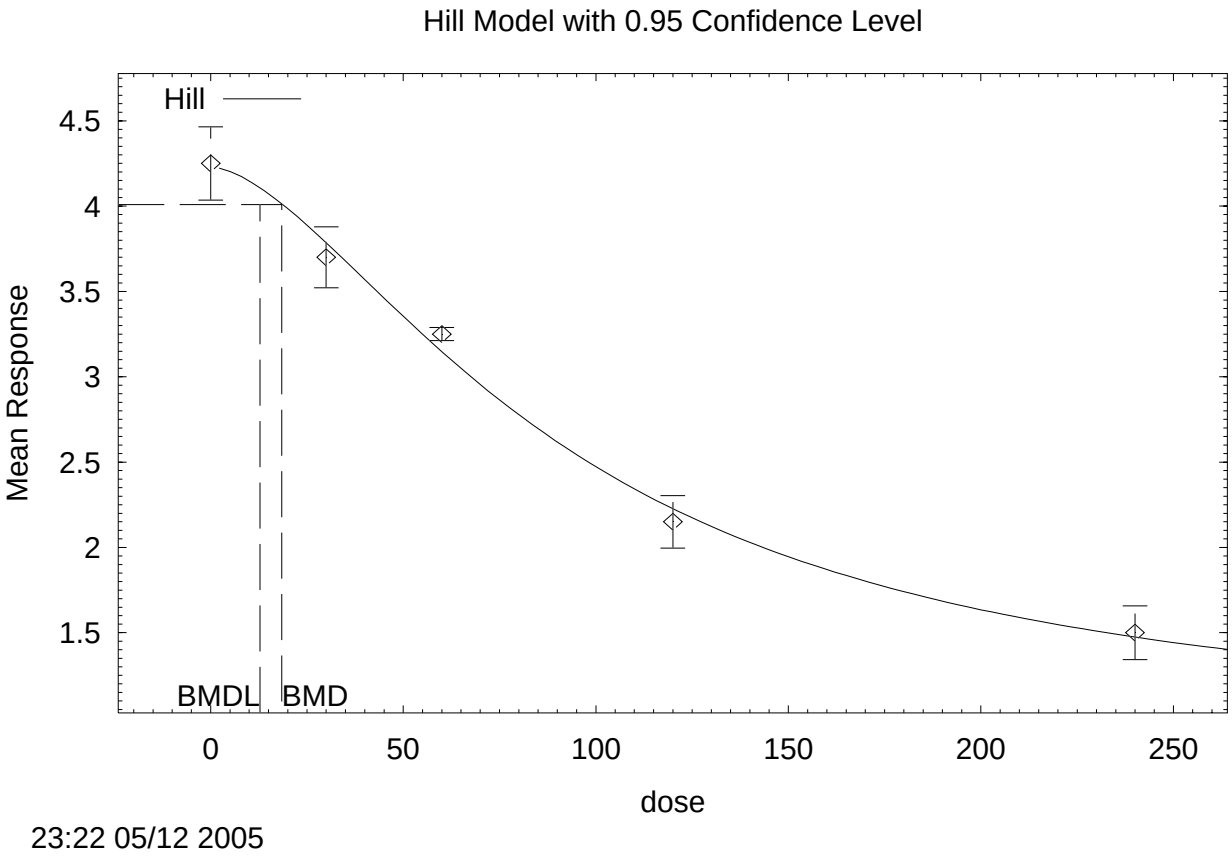
Benchmark Dose Computation
Specified effect = 1

Risk Type = Estimated standard deviations from the control mean

Confidence level = 0.95

BMD = 18.4161

BMDL = 12.8257


Hill Model with 0.95 Confidence Level
Hill
Mean Response
4.5
4
3.5
3
2.5
2
1.5
BMDL
BMD
0
50
100
150
200
250
dose
23:22 05/12 2005

Part IV.

Humpage and Falconer 2003

male mice treated with purified cylindrospermopsin

urinary protein levels

drop high dose

```
 ========================================================================
        Polynomial Model. Revision: 2.2  Date: 9/12/2002
        Input Data File: C:\BMDS\DATA\CYLINDRO.(d)
        Gnuplot Plotting File:  C:\BMDS\DATA\CYLINDRO.plt
                                          Thu May 12 23:24:32 2005
 ========================================================================

 BMDS MODEL RUN
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   The form of the response function is:

   Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


   Dependent variable = MEAN
   Independent variable = dose
   rho is set to 0
   Signs of the polynomial coefficients are not restricted
   A constant variance model is fit

   Total number of dose groups = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                          alpha =    0.0503676
                            rho =            0   Specified
                         beta_0 =         4.25
                         beta_1 =    -0.017381



                               Parameter Estimates

                                                95.0% Wald Confidence Interval
   Variable    Estimate     Std. Err.     Lower Conf. Limit  Upper Conf. Limit
   alpha       0.0457633    0.0104988        0.025186           0.0663406
   beta_0      4.24885      0.052757         4.14545            4.35225
   beta_1      -0.017373    0.000786454      -0.0189144         -0.0158316


           Asymptotic Correlation Matrix of Parameter Estimates

                   alpha       beta_0       beta_1
     alpha             1     2.2e-009       1e-009
    beta_0     2.2e-009            1        -0.75
    beta_1       1e-009        -0.75            1
```

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	4.25	0.3	4.25	0.214	0.017
30	10	3.7	0.25	3.73	0.214	-0.409
60	9	3.25	0.05	3.21	0.214	0.611
120	9	2.15	0.2	2.16	0.214	-0.198

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	39.893006	5	-69.786011
A2	50.462856	8	-84.925712
fitted	39.601194	2	-75.202387
R	-10.831438	2	25.662876

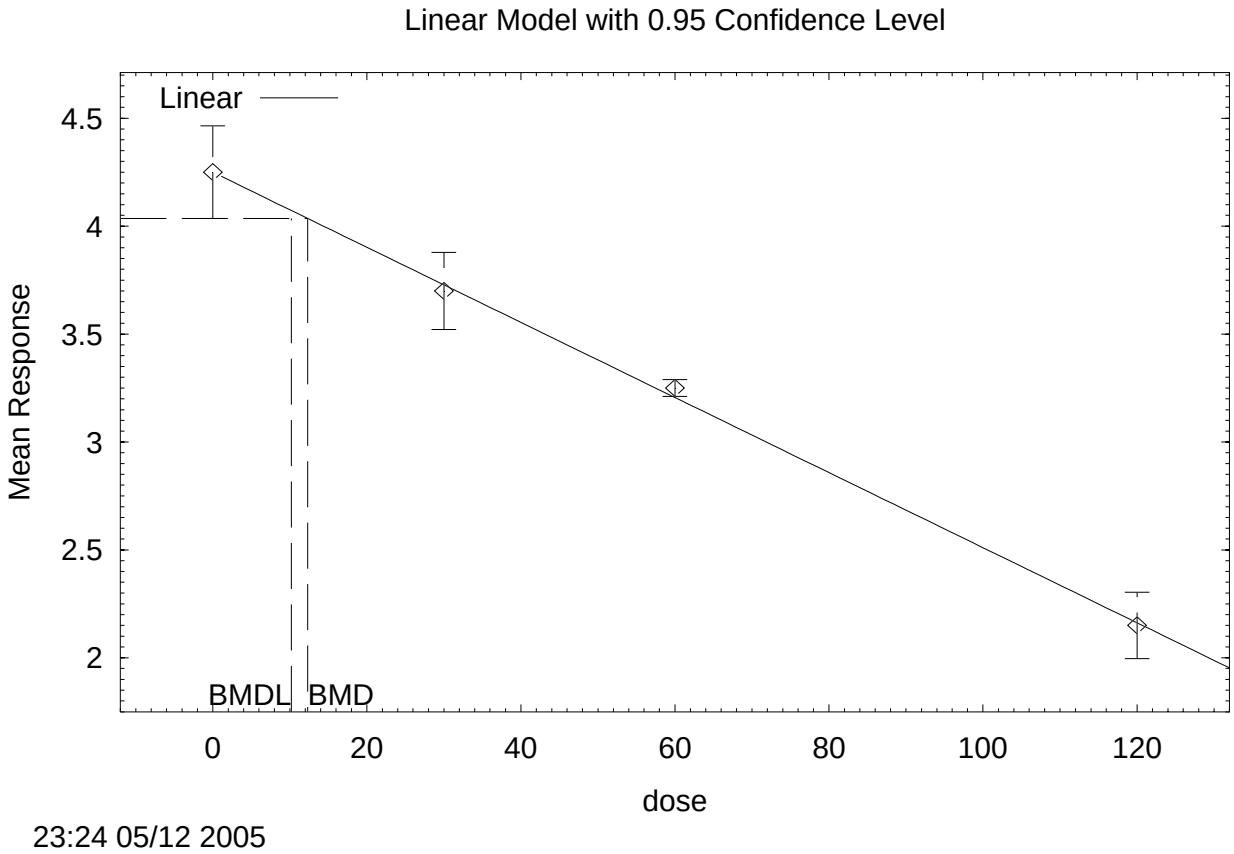
Test 1: Does response and/or variances differ among dose levels
(A2 vs. R)
Test 2: Are Variances Homogeneous (A1 vs A2)
Test 3: Does the Model for the Mean Fit (A1 vs. fitted)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	122.589	6	<.0001
Test 2	21.1397	3	<.0001
Test 3	0.583624	2	0.7469

The p-value for Test 1 is less than .05. There appears to be a
difference between response and/or variances among the dose levels.
It seems appropriate to model the data

```
The p-value for Test 2 is less than .05.  Consider
running a non-homogeneous variance model

The p-value for Test 3 is greater than .05.  The model
chosen appears to adequately describe the data


 Benchmark Dose Computation
Specified effect =            1

Risk Type        =     Estimated standard deviations from the control mean


Confidence level =         0.95

             BMD =      12.3135


            BMDL =      10.1957
```



```
 ========================================================================
        Polynomial Model. Revision: 2.2  Date: 9/12/2002
        Input Data File: C:\BMDS\DATA\CYLINDRO.(d)
        Gnuplot Plotting File:  C:\BMDS\DATA\CYLINDRO.plt
                                          Thu May 12 23:26:08 2005
 ========================================================================

 BMDS MODEL RUN
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   The form of the response function is:

   Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


   Dependent variable = MEAN
   Independent variable = dose
   rho is set to 0
   Signs of the polynomial coefficients are not restricted
   A constant variance model is fit

   Total number of dose groups = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                          alpha =    0.0503676
                            rho =            0   Specified
                         beta_0 =      4.23636
                         beta_1 =   -0.0164394
                         beta_2 = -7.57576e-006



                                Parameter Estimates

                                          95.0% Wald Confidence Interval
   Variable   Estimate        Std. Err.   Lower Conf. Limit  Upper Conf. Limit
   alpha      0.0456423       0.0104711      0.0251194         0.0661652
   beta_0     4.23685         0.0648402      4.10977           4.36394
   beta_1     -0.016519       0.00280362     -0.022014         -0.011024
   beta_2     -6.92416e-006   2.18203e-005   -4.96911e-005     3.58428e-005


           Asymptotic Correlation Matrix of Parameter Estimates

                   alpha       beta_0       beta_1       beta_2
     alpha             1     1.2e-010    -1.4e-013     1.1e-010
    beta_0      1.2e-010            1        -0.73         0.58
    beta_1     -1.4e-013        -0.73            1        -0.96
    beta_2      1.1e-010         0.58        -0.96            1
```

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	4.25	0.3	4.24	0.214	0.195
30	10	3.7	0.25	3.74	0.214	-0.519
60	9	3.25	0.05	3.22	0.214	0.41
120	9	2.15	0.2	2.15	0.214	-0.0684

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	39.893006	5	-69.786011
A2	50.462856	8	-84.925712
fitted	39.651475	3	-73.302950
R	-10.831438	2	25.662876

Test 1: Does response and/or variances differ among dose levels
(A2 vs. R)
Test 2: Are Variances Homogeneous (A1 vs A2)
Test 3: Does the Model for the Mean Fit (A1 vs. fitted)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	122.589	6	<.0001
Test 2	21.1397	3	<.0001
Test 3	0.483061	1	0.487

The p-value for Test 1 is less than .05. There appears to be adifference between response and/or variances among the dose levels.It seems appropriate to model the data

The p-value for Test 2 is less than .05. Consider running a non-homogeneous variance model
The p-value for Test 3 is greater than .05. The model chosen appears to adequately describe the data

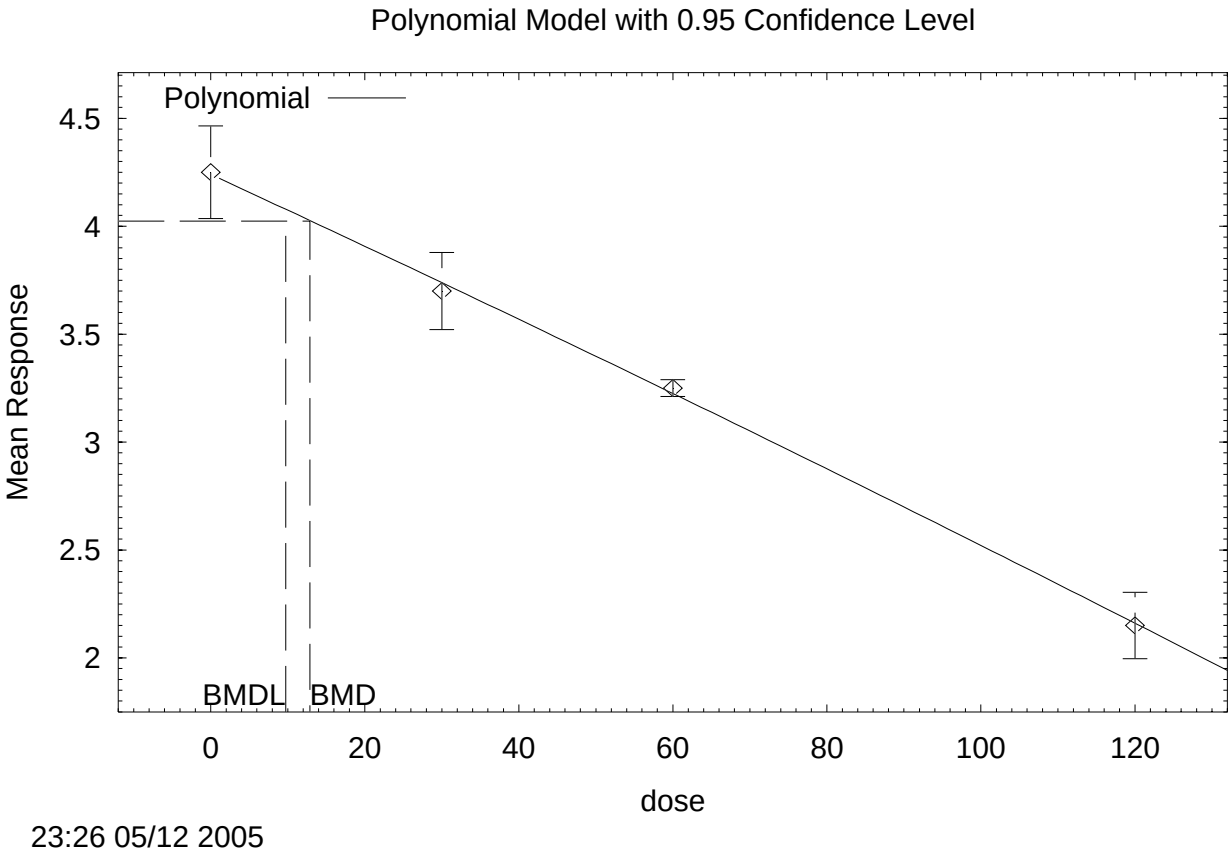
```
 Benchmark Dose Computation
Specified effect =            1

Risk Type        =     Estimated standard deviations from the control mean


Confidence level =          0.95

             BMD =       12.8637


            BMDL =       9.74645
```



```
==========================================================================
       Polynomial Model. Revision: 2.2  Date: 9/12/2002
       Input Data File: C:\BMDS\DATA\CYLINDRO.(d)
       Gnuplot Plotting File:  C:\BMDS\DATA\CYLINDRO.plt
                                        Thu May 12 23:27:07 2005
==========================================================================

BMDS MODEL RUN
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  The form of the response function is:

  Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


  Dependent variable = MEAN
  Independent variable = dose
  rho is set to 0
  The polynomial coefficients are restricted to be negative
  A constant variance model is fit

  Total number of dose groups = 4
  Total number of records with missing values = 0
  Maximum number of iterations = 250
  Relative Function Convergence has been set to: 1e-008
  Parameter Convergence has been set to: 1e-008



                Default Initial Parameter Values
                        alpha =    0.0503676
                          rho =            0   Specified
                       beta_0 =      4.23636
                       beta_1 =   -0.0164394
                       beta_2 = -7.57576e-006



                              Parameter Estimates

                                        95.0% Wald Confidence Interval
  Variable   Estimate       Std. Err.    Lower Conf. Limit  Upper Conf. Limit
  alpha      0.0456423      0.0104711      0.0251194          0.0661652
  beta_0     4.23685        0.0648402      4.10977            4.36394
  beta_1     -0.016519      0.00280362     -0.022014          -0.011024
  beta_2     -6.92416e-006  2.18203e-005   -4.96911e-005      3.58428e-005


          Asymptotic Correlation Matrix of Parameter Estimates

                 alpha       beta_0       beta_1       beta_2
    alpha            1     1.2e-010    -1.4e-013     1.1e-010
   beta_0     1.2e-010            1        -0.73         0.58
   beta_1    -1.4e-013        -0.73            1        -0.96
   beta_2     1.1e-010         0.58        -0.96            1
```

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	4.25	0.3	4.24	0.214	0.195
30	10	3.7	0.25	3.74	0.214	-0.519
60	9	3.25	0.05	3.22	0.214	0.41
120	9	2.15	0.2	2.15	0.214	-0.0684

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	39.893006	5	-69.786011
A2	50.462856	8	-84.925712
fitted	39.651475	3	-73.302950
R	-10.831438	2	25.662876

Test 1: Does response and/or variances differ among dose levels
(A2 vs. R)
Test 2: Are Variances Homogeneous (A1 vs A2)
Test 3: Does the Model for the Mean Fit (A1 vs. fitted)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	122.589	6	<.0001
Test 2	21.1397	3	<.0001
Test 3	0.483061	1	0.487

The p-value for Test 1 is less than .05. There appears to be a difference between response and/or variances among the dose levels.
It seems appropriate to model the data

The p-value for Test 2 is less than .05. Consider running a non-homogeneous variance model
The p-value for Test 3 is greater than .05. The model chosen appears to adequately describe the data

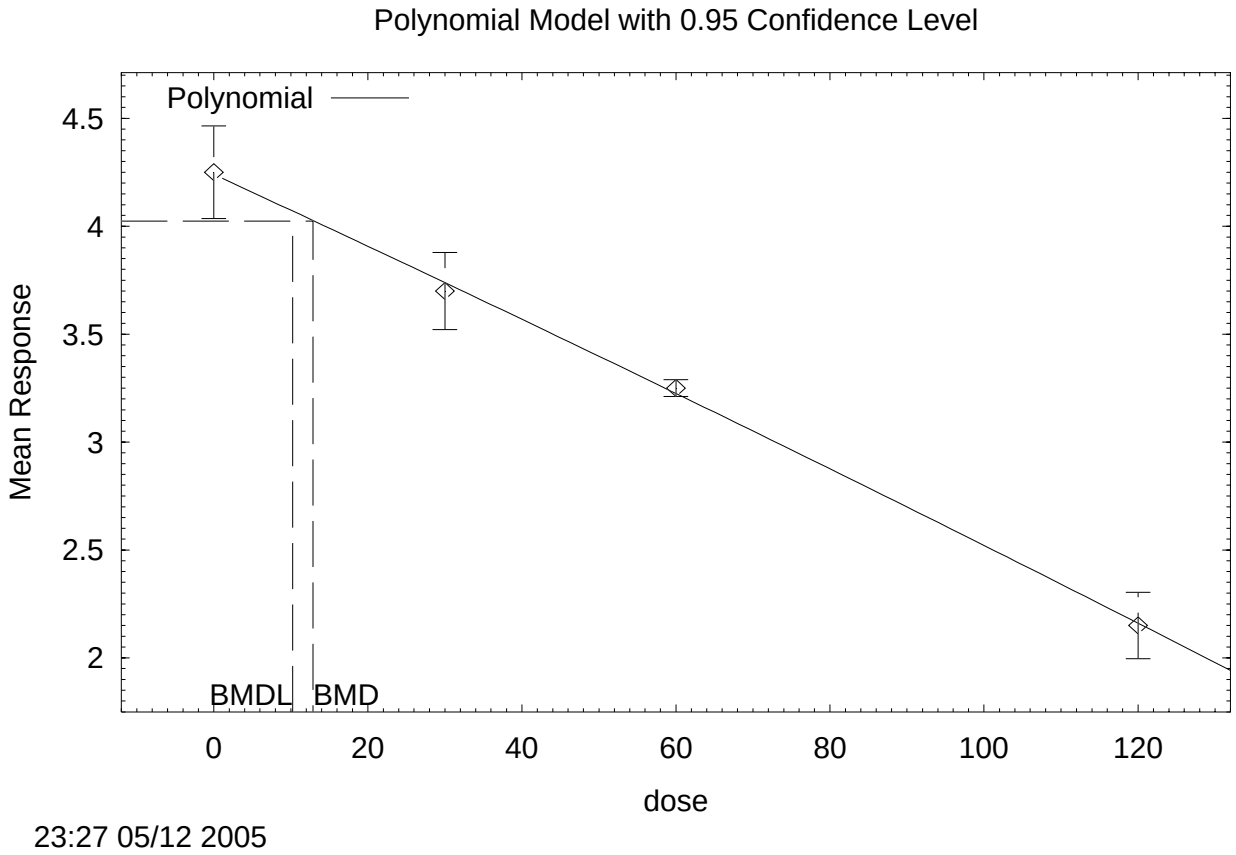
```
 Benchmark Dose Computation
Specified effect =            1

Risk Type        =     Estimated standard deviations from the control mean


Confidence level =          0.95

             BMD =       12.8637


            BMDL =       10.2295
```



```
 ======================================================================
        Power Model. $Revision: 2.1 $ $Date: 2000/10/11 20:57:36 $
        Input Data File: C:\BMDS\DATA\CYLINDRO.(d)
        Gnuplot Plotting File:  C:\BMDS\DATA\CYLINDRO.plt
                                          Thu May 12 23:27:43 2005
 ======================================================================

 BMDS MODEL RUN
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   The form of the response function is:

   Y[dose] = control + slope * dose^power


   Dependent variable = MEAN
   Independent variable = dose
   rho is set to 0
   The power is restricted to be greater than or equal to 1
   A constant variance model is fit

   Total number of dose groups = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                          alpha =    0.0503676
                            rho =            0   Specified
                        control =         4.25
                          slope =     -22.4349
                          power =    -0.494765


           Asymptotic Correlation Matrix of Parameter Estimates

                   alpha          rho      control        slope        power

     alpha             1        -0.98       -0.041      -0.0042       -0.014

       rho         -0.98            1        0.042       0.0043        0.014

   control        -0.041        0.042            1        -0.67        -0.62

     slope       -0.0042       0.0043        -0.67            1            1

     power        -0.014        0.014        -0.62            1            1
```

Parameter Estimates

Variable	Estimate	Std. Err.
alpha	0.0457244	0.0552045
rho	0	0.931145
control	4.24139	0.0671298
slope	-0.0159238	0.00783392
power	1.01795	0.100882

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	4.25	0.3	4.24	0.214	0.0403
30	10	3.7	0.25	3.73	0.214	-0.157
60	9	3.25	0.05	3.21	0.214	0.172
120	9	2.15	0.2	2.16	0.214	-0.0426

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	39.893006	5	-69.786011
A2	50.462856	8	-84.925712
fitted	39.617353	4	-71.234706
R	-10.831438	2	25.662876

Test 1: Does response and/or variances differ among dose levels (A2 vs. R)
Test 2: Are Variances Homogeneous (A1 vs A2)
Test 3: Does the Model for the Mean Fit (A1 vs. fitted)

Tests of Interest

Test	-2*log(Likelihood Ratio)	df	p-value
Test 1	122.589	6	<.00001
Test 2	21.1397	3	9.847e-005
Test 3	0.551305	1	0.4578

The p-value for Test 1 is less than .05. There appears to be a difference between response and/or variances among the dose levels. It seems appropriate to model the data

The p-value for Test 2 is less than .05. Consider running a non-homogeneous variance model

The p-value for Test 3 is greater than .05. The model chosen appears to adequately describe the data

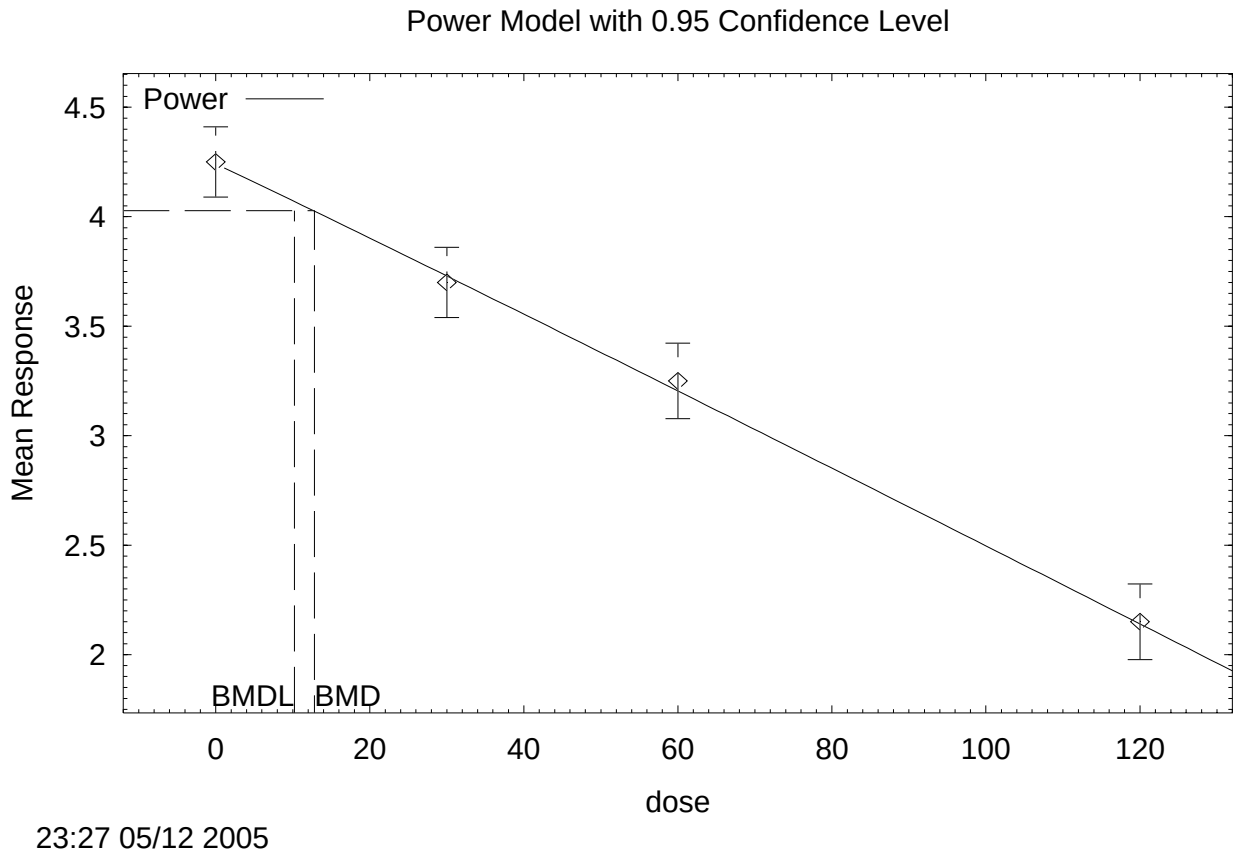
Benchmark Dose Computation
Specified effect = 1

Risk Type = Estimated standard deviations from the control mean

Confidence level = 0.95

BMD = 12.8275

BMDL = 10.2065



```
 ========================================================================
        Hill Model. $Revision: 2.1 $ $Date: 2000/10/11 21:21:23 $
        Input Data File: C:\BMDS\DATA\CYLINDRO.(d)
        Gnuplot Plotting File:  C:\BMDS\DATA\CYLINDRO.plt
                                          Thu May 12 23:28:26 2005
 ========================================================================

 BMDS MODEL RUN
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   The form of the response function is:

   Y[dose] = intercept + v*dose^n/(k^n + dose^n)


   Dependent variable = MEAN
   Independent variable = dose
   rho is set to 0
   Power parameter restricted to be greater than 1
   A constant variance model is fit

   Total number of dose groups = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008


                  Default Initial Parameter Values
                          alpha =    0.0273861
                            rho =            0   Specified
                      intercept =         4.25
                              v =         -2.1
                              n =       1.7744
                              k =      62.7273


           Asymptotic Correlation Matrix of Parameter Estimates

                  alpha     rho       intercept      v       n          k

   alpha          1         0           0            0       0          0

   rho            0         1           0            0       0          0

   intercept      0         0           1            0       0          0

   v              0         0           0            1       0          0

   n              0         0           0            0       1          0

   k              0         0           0            0       0          1
```

Parameter Estimates

Variable	Estimate	Std. Err.
alpha	0.0457348	1
rho	0	1
intercept	4.24125	1
v	-461.591	1
n	1.02098	1
k	23703.4	1

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	4.25	0.3	4.24	0.214	0.0409
30	10	3.7	0.25	3.73	0.214	-0.159
60	9	3.25	0.05	3.21	0.214	0.174
120	9	2.15	0.2	2.16	0.214	-0.0428

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Degrees of freedom for Test A1 vs fitted <= 0

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	39.893006	5	-69.786011
A2	50.462856	8	-84.925712
fitted	39.613009	5	-69.226018
R	-10.831438	2	25.662876

Test 1: Does response and/or variances differ among dose levels (A2 vs. R)
Test 2: Are Variances Homogeneous (A1 vs A2)
Test 3: Does the Model for the Mean Fit (A1 vs. fitted)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value

Test 1	122.589	6	<.0001
Test 2	21.1397	3	<.0001
Test 3	0.559993	0	NA

The p-value for Test 1 is less than .05. There appears to be a difference between response and/or variances among the dose levels. It seems appropriate to model the data

The p-value for Test 2 is less than .05. Consider running a non-homogeneous variance model

NA - Degrees of freedom for Test 3 are less than or equal to 0. The Chi-Square test for fit is not valid

Benchmark Dose Computation
Specified effect = 1

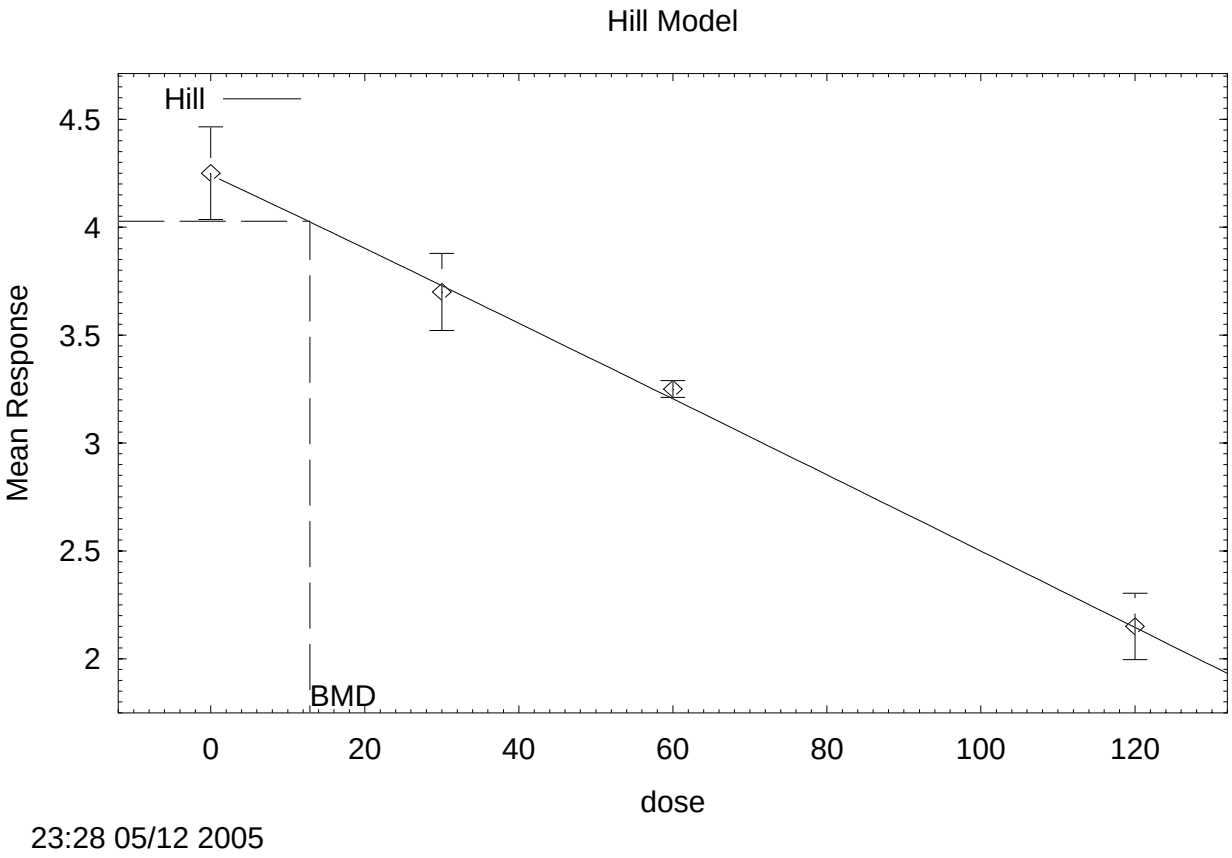
Risk Type = Estimated standard deviations from the control mean

Confidence level = 0.95

BMD = 12.8644

Warning: optimum may not have been found. Bad completion code in Optimization routine.

BMDL computation failed.


Hill Model
Hill
BMD
Mean Response
4.5
4
3.5
3
2.5
2
0
20
40
60
80
100
120
dose
23:28 05/12 2005