Redbook 2000

July 2000; Revised July 2007

Guidance for Industry and Other Stakeholders Toxicological Principles for the Safety Assessment of Food Ingredients *Redbook 2000*

Additional copies are available from:
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http://www.cfsan.fda.gov/guidance.html

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Food Safety and Applied Nutrition
July 2000; Updated July 2007

Note to reader: Individual chapters/sections of this internet version of "Redbook" are available as linked documents via the Table of Contents. The date of revision for each chapter and /or chapter section is provided in the Table of Contents and the individual documents. These revisions supersede previous versions of Redbook

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[1]This guidance has been prepared by the Office of Food Additive Safety in the Center for Food Safety and Applied Nutrition at the U.S. Food and Drug Administration.

[2]*Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food* (also known as Redbook I), U.S. Food and Drug Administration, Bureau of Foods (now CFSAN), 1982. May be purchased from: National Technical Information Services (NTIS), 5285 Port Royal Road, Springfield, VA 22161, Telephone (703) 605-6000, NTIS Order Number PB83-170696.

[3]*Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food* 1993 Draft "Redbook II" Table of Contents[33]

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Redbook 2000: I Introduction

July 2007

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter I. Introduction

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This guidance represents the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

"Toxicological Principles for the Safety Assessment of Food Ingredients [2]" ("Redbook 2000") is the new name for Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food" that was originally published in 1982 ("Redbook I") and a draft revision was published in 1993 ("Redbook II" [3]). Major changes in this revised guidance are presented later in this chapter. This document provides guidance to industry and other stakeholders (e.g. academia, other regulatory groups) regarding toxicological information submitted to the Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety (OFAS) regarding food ingredients. It is a guidance document that is intended to assist petitioners and notifiers in:

- determining the need for toxicity studies
- designing, conducting, and reporting the results of toxicity studies
- conducting statistical analyses of data
- the review of histological data
- the submission of this information to the FDA as part of the safety assessment of food ingredients.

The term "food ingredients", as used in this guidance, includes food additives and color additives used in food, and those substances which are classified as food contact substances (formerly known as indirect food additives), and those substances which are classified as generally recognized as safe (GRAS). The toxicity studies included in this guidance document can also be used in the safety assessment of constituent residues.

Petitioners and notifiers are encouraged to become familiar with the information in this guidance document as well as other toxicology related guidance information available via the following internet links when considering the submission of a petition or notification:

Food and Color Additive (Petition) Program [4]

Food Contact Substances Program [5]

GRAS Notification Program [6]

Additionally, sponsors are encouraged to discuss with the appropriate regulatory divisions in the OFAS the extent and types of toxicity testing they are considering and the type of petition or notification they intend to submit. FDA consistently has taken the position that various types of scientifically valid information may be used to support a determination that the proposed use of an ingredient is safe. Sponsors should consult with the FDA to discuss the use of alternative information to support a determination of safety for the food ingredient prior to the submission of a petition or notification.

BACKGROUND

One of the responsibilities of the FDA and its CFSAN is to ensure the safety of food ingredients added to the food supply in the United States. The "safety" of these ingredients is provided for in Sections 70.3[7] and 170.3[8] of Title 21 of the Code of Federal Regulations (CFR) as, a reasonable certainty that a substance is not harmful under the intended conditions of use. Safety is generally determined by considering the potential cumulative effect of the substance in consumers and the probable consumption of the substance in the diet. The potential cumulative effects are determined by the outcome of toxicity studies and knowledge of compounds and their structures.

FDA's Bureau of Foods (former name of CFSAN) published its guidance, "Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food" (referred to as Redbook I) in 1982 to provide guidance for sponsors regarding the submission of food additive petitions. A revised draft of this document was issued in 1993 (see Notice of Availability, published in the Federal Register (FR) on March 29, 1993, 58 FR 16536) and is referred to as "Redbook II". The FDA has continued to review comments related to this draft which were received from the regulated and scientific communities and other stakeholders. Additionally, the FDA has considered publications and information regarding recent advances and increased knowledge in toxicology, science and the food industry, and other authoritative guidance for toxicity testing in preparing this guidance document.

Food additives are generally defined in Section 201[9](s) of the Federal Food, Drug, and Cosmetic Act (the Act) and in 21 CFR 170.3(e)(1)[10] as substances whose intended use results or may reasonably be expected to result, directly or indirectly, in its becoming a component of food or otherwise affecting the characteristics of food. Food additives have historically, therefore, been referred to as direct or indirect food additives. It is generally understood that direct food additives are compounds that are directly added to the food to achieve a technical effect (i.e., emulsification, sweetening) and indirect food additives include substances used in the production, manufacturing, packing, processing, preparing, treating, packaging, transporting, or holding of food (i.e., can coatings, paper and paperboard, sanitizers, and adhesives). In 1997, the Food and Drug Administration Modernization Act[11] (FDAMA) amended the Act creating a more efficient notification process for food contact substances. Color additives are generally defined in Section 201[12](t) of the Act as a dye, pigment or other substance which when added or applied to a food, drug, or cosmetic is capable of imparting a color thereto. A GRAS substance is one whose safety has been evaluated by qualified experts, and the determination of safety based on evidence and through scientific procedures to be safe under the conditions of its intended use. A description of the eligibility for classification as GRAS can be found in 21 CFR 170.30[13]. Additionally, in 1997 FDA issued a proposed rule (62 FR 18937[14], April 17, 1997) to establish a voluntary notification procedure for notifying FDA of a determination that a particular use of a substance is GRAS.

MAJOR CHANGES IN THE REVISED GUIDANCE

This section summarizes major changes between "Redbook 2000" and "Redbook II". In general, these changes were derived from three major sources: 1) increased scientific knowledge and technological advances since 1993; 2) comments received on the draft 1993 "Redbook II"; and 3) a desire to achieve consistency and harmonization with guidance published by other agencies, countries, and international organizations, when such action does not compromise FDA's ability to ensure the safety of food ingredients.

- The title of this guidance document was changed from "Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food" ("Redbook II") to "Toxicological Principles for the Safety Assessment of Food Ingredients" ("Redbook 2000") to better describe the breadth of circumstances where this guidance is applicable. "Redbook 2000" is the year that the first chapters of the revised Redbook became available on the internet. Rather than change the name of this guidance document when another chapter is added or as revisions are made, the decision was made to retain the name "Redbook 2000".
- "Redbook 2000" is designed as an electronic document available on the internet. It will not be published as a printed and bound document as were previous versions.
- The overall format of this guidance document has changed to facilitate availability on the

internet. Each chapter is a "stand alone" document and can be accessed independently. Because of this, general information related to toxicity testing has been repeated in the separate chapters so that information related to each type of study is complete.

- Chapter III. Concern Levels and Recommended Toxicity Tests in "Redbook II" has been reduced, revised, and renamed "Recommended Toxicity Studies" in "Redbook 2000". Readers are directed to petition and notification web sites for more specific guidance for recommended toxicity studies.
- Chapter IV. C. 1. Short-Term Tests for Genetic Toxicity has been expanded with more complete descriptions and explanations of four separate types of genetic tests.
- "Neurotoxicity Studies" guidance is now located in Chapter IV. Guidelines for Toxicity Studies. "Neurotoxicity Studies" was previously located in "Redbook II", Chapter V. Additional Recommended Studies.
- Chapter IV. C. 7 Combined Chronic Toxicity/Carcinogenicity Studies with Rodents in "Redbook II" is now available in "Redbook 2000" in the following two chapters:
    - IV.C.5a. Chronic Toxicity Studies with Rodents
    - IV.C.7. Combined Chronic Toxicity/Carcinogenicity Studies with Rodents
- Chapter VII. Emerging Issues has not been included in "Redbook 2000". Information related to the emerging issues presented in the "Redbook II" has been considered and incorporated in relevant chapters of "Redbook 2000". The reader is encouraged to become familiar with additional information available via the OFAS web page[15] and to contact OFAS[16] with specific questions.

FLEXIBILITY IN GUIDANCE FOR TOXICITY TESTING

FDA's guidance for toxicity studies for food ingredients continue to emphasize that there is no substitute for sound scientific judgement. This guidance presents recommendations--not hard and fast rules. If an investigator believes that he/she can provide the Agency with useful toxicological information by modifying a recommended study protocol, and is able to support the modification with sound scientific arguments, then the investigator should propose the modified protocol to the appropriate program division within OFAS. As always, petitioners and notifiers should consult with the FDA prior to and during the design of study protocols for toxicity studies and/or before commencement of studies.

FDA's guidance documents, including this guidance, do not establish legally enforceable responsibilities. Instead, guidances describe the Agency's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word should in Agency guidances means that something is suggested or recommended, but not required.

For the benefit of the reader and because each of the chapters and chapter sections of Redbook 2000 are stand alone documents and can be accessed independently on the internet, FDA is repeating the disclaimer in the box at the beginning of each chapter and chapter section of Redbook.

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Chapter II

Agency Review of Toxicology Information in Petitions for Direct Food Additives and Color Additives Used in Food

A. Introduction

The food additive petition review process came into existence in 1958 when Congress enacted the Food Additives Amendment [1] to the Federal Food, Drug, and Cosmetic Act (the Act). [2] This Amendment provides a pre-market safety evaluation process for new substances added to food, "food additives." A similar statute, the Color Additive Amendments of 1960, [3,4] created analogous requirements for color additives used in foods, drugs, cosmetics, or medical devices. "Color additive" used in food is defined in section 201(t) of the Act; "food additive" is defined in section 201(s) of the Act.

Since 1958, before a food additive may be used, an authorizing regulation must be in effect. Approval of a petition for an additive and issuance of an authorizing regulation require that the Agency conclude that the additive is safe for its intended conditions of use. This safety requirement, embodied in section 409(c)(3)(A), is often referred to as the general safety clause for food additives. When the proponent of the proposed use of the additive has shown that the additive is safe for its intended use, the Agency publishes a regulation in the *Federal Register* establishing permitted conditions for the use of the additive.

When a petition for a direct food additive or color additive used in food is submitted to the Agency, or when the petitioner first contacts FDA, a Consumer Safety Officer (CSO) generally is assigned to the petition. One of the CSO's tasks is to coordinate FDA's review of the petition. When appropriate, the CSO can arrange for the petitioner to meet with other individuals in the Agency to discuss specific issues or problems that arise during review of the petition. All communication with the Agency concerning the status or review of the petition should be made through the assigned CSO. General information about the petition review process has been published; [5] specific questions should be addressed to the CSO assigned to the petition.

The Act and the Code of Federal Regulations [6] specify the basic elements that a petition must contain. One of these elements is safety data on the additive, which is usually provided in the form of toxicity studies. Toxicologists, pathologists, and mathematicians evaluate any toxicity studies included in the petition. If appropriate, toxicologists can recommend that carcinogenicity studies be evaluated by special CFSAN committees: the Cancer Assessment Committee (CAC) and the Quantitative Risk Assessment Committee (QRAC); for more information on these committees, see **Chapter II C 5 i and ii**.

Review of toxicity studies and other toxicology information results in an estimate of the acceptable daily intake (ADI) for the direct food additive or color additive used in food. The ADI is typically based on the dose level of the additive in animal studies that was shown to cause no adverse effect, multiplied an appropriate safety factor (often 1/100; see Section 201(s) of the Act [2]). Chronic ingestion of the additive at the ADI is considered consistent with a reasonable certainty of no harm.

FDA urges individuals or corporations preparing to submit petitions for direct food additives or color additives used in food to consult with the Agency early in the planning stages. For example, before the petition is submitted, petitioners can submit toxicity study protocols to FDA for review by Agency scientists. This can help the petitioner perform toxicity studies and prepare data in a form that will expedite the Agency's review of the information in the petition (for more information on expediting review, see **Chapter II B**).

This document delineates the toxicology information deemed appropriate for assessing the safety of direct food additives and color additives used in food. However, guidelines contained in this document are only one possible approach among many to providing the toxicological basis for an assessment of safety. We urge petitioners to discuss alternative approaches and toxicity test protocols with the Agency before toxicity tests are begun.

II B. Expediting Review of Toxicology Information

The Agency recommends that petitioners use the following approaches to minimize requests for additional data and to expedite review of direct food additive and color additive petitions:

- Make sure that petitions are formatted properly and contain complete and adequate information before submitting them for review. Guidelines and recommendations contained in this publication should be consulted before the petition is submitted.

- Initiate interactions between petitioner's representatives and Agency CSOs and scientists before the petition is submitted. Such interactions can involve Agency review of toxicity study protocols and Agency recommendations about the extent of toxicity testing that may be recommended to adequately assess the safety of the food additive or color additive used in food.

- Submit toxicology data in machine-readable form. During review of the safety of a food additive or color additive used in food, it may be necessary for scientific reviewers to re-analyze some of the data in a submission. A large proportion of the work in such a re-analysis is computer entry and verification of data. Therefore, much time would be saved if data are submitted in a machine-readable form (magnetic tape for the IBM mainframe standard or floppy disks for IBM personal computers. Please note that the Agency no longer has the capability to read punched cards). General guidelines for submitting machine-readable data follow, but petitioners are urged to contact the Agency before submitting machine-readable data to discuss modifications to these guidelines.

- Enclosed with the machine-readable data should be:

    i) the name of a contact person;

    ii) a printout of the first 100 to 200 records; and

    iii) the layout of the data. This would include the location of each variable in the record, the type of variable (*e.g.* character, integer), the permissible range of values, and information about how missing data are stored.

- Magnetic tape format needs to be 9-track, with 6250 bpi preferred (although 800 and 1600 bpi are also readable). Data should be recorded in IBM-EBCDIC or ASCII, or should be in IBM-TSO or statistical package datasets; please consult with the Agency statisticians about appropriate datasets. Interior labels should be IBM standard with volume number and dataset names. Unlabeled tapes should be accompanied by the record format, record length, blocking factor, and the name of the program that created the tape.

- Floppy disks should be submitted in duplicate; these should be copy-protected because accidental erasure and destruction of disks can occur. The data should be submitted in a form readable by software programs to which the Agency has access; please consult with Agency statisticians about acceptable software.

II C. Evaluating Toxicology Information

1. Introduction

Toxicity testing requirements for assessing the safety of food and color additives used in food have evolved over the past years as knowledge in the field of toxicology has expanded. While short-term or acute studies were considered adequate even for major food additives several decades ago, today's recommendations generally include comprehensive, long-term toxicity studies. CFSAN toxicologists exercise their best scientific judgement in determining what toxicity studies are needed for the Agency to adequately assess the safety of a direct food additive or color additive used in food. In making these decisions, the toxicologists take into account what is already known about the properties of a compound, its intended conditions of use, and current standards for toxicity testing.

From data submitted by the petitioner in support of the safety of a direct food additive or color additive used in food, Agency toxicologists determine the no-observed-effect level (NOEL), select an appropriate safety factor, and calculate the acceptable daily intake (ADI) for the substance. These steps are briefly summarized below.

2. No-Observed-Effect Level (NOEL)

Non-treatment-related variations in the incidence of toxic endpoints occur and may depend on a number of factors, including the source of the animals, sex, genetic variations, diet, age at death, environmental conditions and the histological criteria used by pathologists.

However, Agency scientists determine the most sensitive treatment-related toxic endpoint (adverse effect) from the data submitted in support of the petition. This endpoint is the adverse or toxic effect that occurs in test animals at the lowest exposure to the test substance. The highest exposure that does not produce this adverse effect is called the no-observed-effect level (NOEL) or the no-observed-adverse-effect level (NOAEL).

3. Safety Factors

Use of safety factors is based on the observation that toxic substances usually have thresholds below which toxic effects cannot be detected. The safety factor attempts to account for differences between animals and humans and differences in sensitivity among humans. Use of the safety factor is intended to provide an adequate margin of safety for consumers.

For non-cancer endpoints, the NOEL is divided by a safety factor to obtain an estimate of the maximum acceptable daily intake (ADI) of the additive for humans. The selection of a safety factor is based on the biological significance of the endpoint, uncertainties inherent in extrapolating information about adverse effects from toxicity studies in animals to human populations, and other judgmental factors. The food additive procedural regulations (21 CFR 170.22) state that a safety factor of 100 will be used as a general rule in applying animal test data to man. However, exceptions to a safety factor of 100 are permitted in accordance with the nature and extent of data available and the circumstances of use of the food additive. For example, safety factors may be modified because of potentially sensitive sub-populations such as children, geriatrics, individuals with deficiency states, and lack of developed enzyme metabolic systems.

II C 4. Acceptable Daily Intake (ADI)

The acceptable daily intake (ADI) is generally estimated by dividing the no-observed-effect level (NOEL) of a test substance by the safety factor. The NOEL may be expressed as mg test substance per kg body weight of the test animal or as percent or ppm (parts per million) of the test diet for the animal. The ADI is usually expressed in mg additive per kg body weight of humans. A food additive generally is considered safe for its intended use if the estimated daily intake (EDI) of the additive is less than, or approximates, the ADI. Because the ADI is calculated to protect against the most sensitive adverse effect, it also protects against other adverse effects occurring at higher exposures to the ingredient.

5. Carcinogenic Risk Assessment

FDA has found risk assessment to be useful for estimating the risk from carcinogenic contaminants of food or color additives used in food, for helping the Agency to set priorities, and for determining the urgency of a regulatory action. [7]

Under the general safety clause of the Act, FDA has used risk assessment procedures to determine the upper limit of risk to the consumer from the presence of a carcinogenic contaminant or constituent chemical. For example, FDA approved for permanent listing D&C Green No. 6, which had not been shown to be a carcinogen in appropriate tests, even though it contains the carcinogenic impurity, *para*-toluidine. In this decision, FDA stated its belief that the lifetime upper limit of risk could adequately be estimated from animal data and extrapolated to humans. Although FDA continues to be concerned about carcinogenic contaminants in the food supply, the Agency believes that this approach can be used, where appropriate, without compromising FDA's mandate to protect the public health.

a. CFSAN's Cancer Assessment Committee (CAC)

The Cancer Assessment Committee (CAC) is comprised of CFSAN experts in such fields as pathology, toxicology, mathematics, food chemistry and technology, epidemiology, and nutrition. These experts are charged with ensuring a uniform and consistent scientific approach for dealing with diverse problems of carcinogenicity throughout the broad regulatory purview of CFSAN. The CAC reviews all lifetime feeding studies submitted to the Agency in support of the safety of direct food additives and color additives used in food. The risk assessment process also can be triggered when a newly petitioned or previously regulated food or color additive presents a question of possible carcinogenicity. If the CAC determines that a substance is a carcinogen, and if it is believed that a quantitative risk assessment may have impact on the regulation of the substance, the CAC informs the Quantitative Risk Assessment Committee (QRAC, see **Chapter II C 5 b**) of this decision.

Figure 1 is a flow chart depicting in schematic fashion the groups involved in the risk assessment process at CFSAN. **Figure 2** identifies the steps involved in risk assessment at CFSAN; each of the steps in **Figure 2** is associated with a particular group or set of groups in **Figure 1**.

Figure 1

Flow Chart Depicting the Various Groups Involved in the Assessment of Cancer Risk at the Center for Food Safety and Applied Nutrition (CFSAN) of the Food and Drug Administration

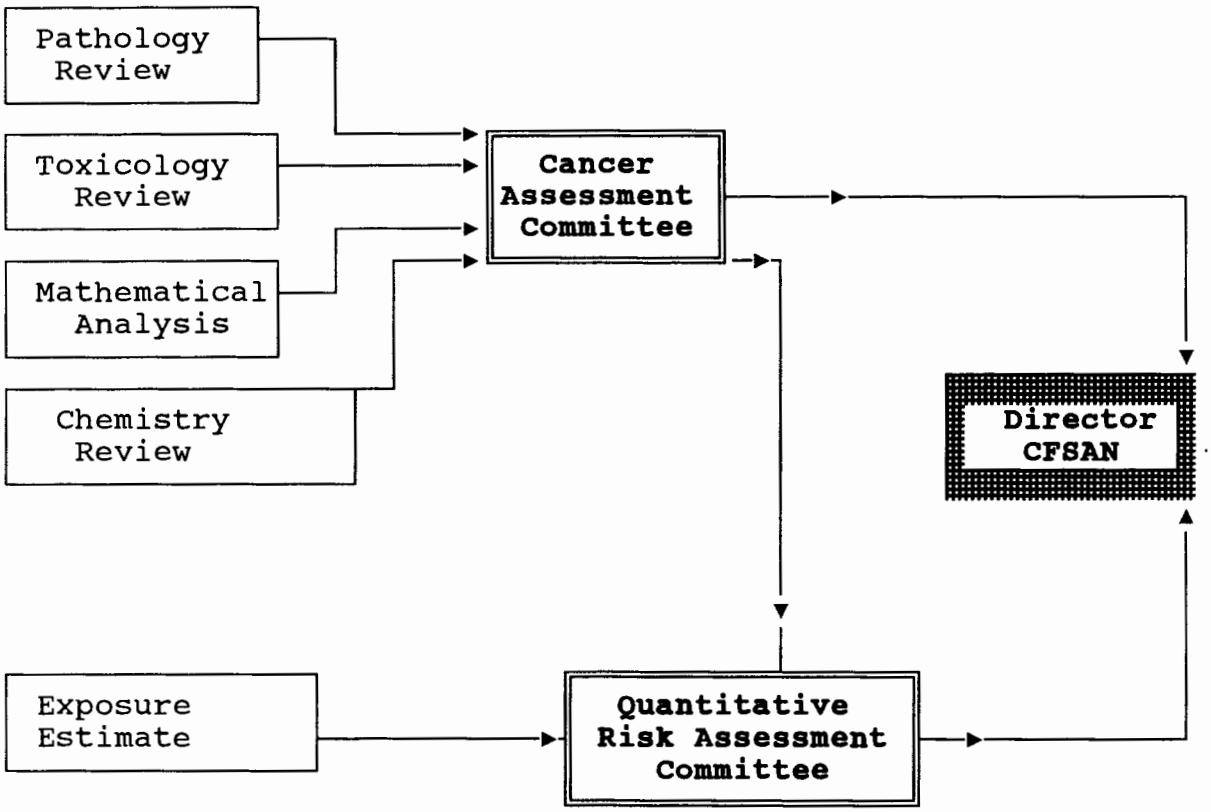

Figure 2

Four Steps in the Risk Assessment of Additives in Food at FDA's Center for Food Safety and Applied Nutrition

1. **Toxicological Evaluation**

- **Input Obtained from Internal Experts**
  - Toxicologists
  - Chemists
  - Other Experts
  - Pathologists
  - Biostatisticians
  - Epidemiologists
- **Input Obtained from External Experts** (where need is indicated)

2. **Cancer Assessment Committee (CAC) Evaluation**

- **CAC Reviews input from Internal and External Experts**
  - Is the Substance a Likely Carcinogen?
    If Yes: CAC recommends the studies, tissue sites, species, and sex suitable for quantitative risk evaluation if risk assessment is allowed under the statute
    If No: No further consideration by CAC or QRAC is needed

3. **Quantitative Risk Assessment Committee (QRAC) Evaluation**

- **QRAC Reviews Data and Exposure Potential**
- **QRAC Chooses Risk Assessment Model and Procedure**
- **QRAC Estimates Magnitude of Potential Human Risk**
  - Calculate the Upper Bound Lifetime Risk

4. **Action Taken by Director of CFSAN, FDA**

- **Makes Risk Management and Policy Recommendations to the Commissioner**

II C 5. Carcinogenic Risk Assessment Continued

As indicated in **Figure 1**, the CAC plays a central role in the risk assessment process at CFSAN. This standing committee, which was established in 1978, is made up of 10 CFSAN individuals with expertise in the various scientific disciplines related to chemical carcinogenesis: pathology, toxicology, mathematics, and food chemistry and epidemiology. The decisions of the CAC with respect to issues of science are authoritative and invariably form the basis for CFSAN's recommendations to the Commissioner.

In addition to reviewing information presented by the disciplines indicated in **Figure 1**, the CAC may request additional information from internal and external experts, such as a review of available epidemiological data or a special review of mutagenicity data. The CAC may choose to postpone a final decision on the carcinogenicity of a compound pending the outcome of ongoing or anticipated animal or analytical experiments. In some cases, the CAC may request that CFSAN pathologists review microscope slides from an animal bioassay. External scientific peer review is sometimes requested by the CAC when a particularly difficult or controversial scientific issue is involved.

In general, FDA and CFSAN follow the National Research Council guidelines for risk assessment, described in *Risk Assessment in the Federal Government: Managing the Process*. [8] FDA and CFSAN also follow the set of principles for risk assessment contained in the 1985 Office of Science and Technology Policy document, "*Chemical Carcinogens; A Review of the Science and its Associated Principles*". [9,10]

There are no universally agreed upon ways of evaluating carcinogenicity data. It is necessary that there be interaction between pathologist, toxicologist and statistician. The role of the pathologist is to decide whether an observed lesion is cancerous or noncancerous. [11] The role of the toxicologist is to determine whether the lesion is related to the treatment. The statistician's role is to analyze the mathematical probability of occurrence of the tumors by chance or as a result of treatment.

Some suggested approaches to the assessment of the evidence of carcinogenicity of a substance are discussed in the following sections.

i) Evaluation of the Adequacy of the Design and Conduct of the Bioassay: The first step in the analysis is a general review of the adequacy of design and conduct of the bioassay to decide whether it is acceptable for evaluation and for deriving conclusions about safety. For example: Was the test chemical properly identified and characterized? Were an adequate number of animals of each sex used per group? Was the test chemical administered for the major part of the life span of the animals? Did sufficient numbers of animals in each group survive long enough for possible late-developing tumors to be manifested? Were there unforeseen events, such as an outbreak of infectious disease, that might invalidate the bioassay? Did the bioassay utilize adequate matched control animals for statistical comparison? Were detailed pathological examinations performed for every tissue?

ii) Evaluation of the Possible Increase in Tumor Incidence: Since it is generally believed that cancers arise independently in various parts of the body, it has become customary to treat each potential target site (*e.g.*, brain, lung, liver, kidney, urinary bladder) separately for evaluation. One general exception is the evaluation of types of tumors that may be multicentric in origin, including leukemia and, possibly, tumors originating in blood vessels or nerves, such as hemangioendotheliomas or neurofibrosarcomas. In general, tumor incidence is defined as the number of tumor-bearing animals having tumors at a specific organ site divided by the total number of animals with that organ examined histopathologically.

II C 5. Carcinogenic Risk Assessment Continued

Judgment of an experienced pathologist is important for proper diagnosing and grouping of lesions for statistical analysis to determine whether or not observed increases in tumor incidence implicate a compound as a carcinogen. The grouping of tumors for statistical evaluation should be based on commonality of histogenic origin. Because it is frequently a matter of arbitrary definition and expert pathologists may disagree about how to designate tumors on the borderline of the continuum between benign and malignant, and because of practical difficulties in categorizing certain tumors as benign or malignant, it is usually necessary to combine the incidence of certain benign tumors with that of malignant tumors occurring in the same tissue and organ for statistical evaluation.

Having recorded the tumors present for each animal, the statistical analysis can be undertaken to evaluate the internal consistency of the data, the reproducibility of the test results, the level of statistical significance, the increase in tumor incidence, the evidence for dose-response relationship or shortened latency period, *etc.* Methods of statistical analysis for carcinogenicity are available. [12,13,14]

iii) Evaluation of the Extent of Evidence for Carcinogenicity: Because the power of carcinogenesis bioassays that use groups of a few dozen animals is relatively weak for determining carcinogenic activity, it is not surprising that evidence of carcinogenicity is sometimes difficult to establish from a single bioassay. This is so for several reasons, including problems of histological diagnosis, sensitivity of the bioassay, and variability of the background tumor incidence. For these reasons, other correlative information may be necessary to add to the weight of evidence of carcinogenicity of a chemical. In general, the extent of the evidence for carcinogenicity can be determined by considering the following information:

- the number of species or strains with an increased tumor incidence;
- the number of positive studies (with different routes of administration and/or doses), if tested in more than one bioassay;
- the degrees of tumor response (incidence, site, type, multiplicity, *etc.*);
- evidence of structure-activity relationship;
- prevalence of dose-response relationship;
- the results of short-term tests for genetic toxicity;
- the presence of preneoplastic lesions; and
- a reduced latency for tumor development or increase in the severity (malignancy of the neoplasia.

Other information, such as whether there was a shortened survival due to the toxicity of the test substance or whether the chemical is tested at or near the MTD, can also add weight to or confound the evidence of carcinogenicity. Information on dose-dependent or nonlinear kinetics from metabolic and pharmacokinetic studies in experimental animals and humans can supplement the assessment of the potential carcinogenic hazard of the additive to humans.

It should be noted that, although general approaches to animal carcinogenesis bioassays are well accepted by the scientific community, opinions about the design, conduct, and interpretation of such test results are not always in agreement and are often the source of scientific debate. This may be due, in large degree, to our lack of knowledge about the mechanisms of cancer induction and progression. Because the Act prohibits the use of

carcinogenic food and color additives, the interpretation of carcinogenicity test results has enormous potential societal and economic impact. Consequently, proper assessment of carcinogenicity data has become an extremely critical function of CFSAN.

b. CFSAN's Quantitative Risk Assessment Committee (QRAC)

The QRAC was formed in 1983. Although quantitative risk assessments were performed under the auspices of the CAC prior to this, the QRAC was formed because of the need for an increasing number of quantitative risk assessments related to food and color additive petitions. Based on its evaluation of all relevant data on a substance, the CAC recommends to the QRAC the bioassays and epidemiological studies most appropriate for low-dose extrapolation. The CAC also recommends to the QRAC the tissue site(s), species, and sex most suitable for quantitative evaluation.

The QRAC then performs a quantitative risk assessment. This portion of the risk assessment process is often controversial, even among experts. Currently, the QRAC uses a linear-at-low-dose approach, similar to that described by Gaylor and Kodell. [15] The QRAC cannot determine the most probable expected human risk for almost any case because of the uncertainties and sources of error inherent in quantitative risk assessment using high-dose animal data. However, the QRAC believes that, in cases where dose-response data are suitable, it can predict a lifetime upper limit of risk with some degree of confidence.

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U.S. Department of Health & Human Services

U.S. Food and Drug Administration

Redbook 2000: III Recommended Toxicity Studies

July 2007

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter III. Recommended Toxicity Studies

Return to *Redbook 2000* table of contents [1]

This guidance represents the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

This chapter provides a general description of how FDA determines which toxicological studies are recommended in order for FDA to evaluate the safety of food ingredients (i.e., direct food additives, color additives used in food, food contact substances, previously referred to as indirect food additives, etc.). A determination that a reasonable certainty of no harm will result from the proposed use(s) of food ingredients is required before manufacturers and distributors can market them in the United States (see sections 70.3 and 170.3 of Title 21 of Code of Federal Regulations). The determination of reasonable certainty of no harm is contingent upon the results of toxicological studies, exposure information, and other types of information submitted in petitions and notifications. Details related to the evaluation of toxicological information is provided in Chapter II of Redbook. Safety data submitted for evaluation of Generally Recognized As Safe (GRAS) substances and bioengineered foods may result from specialized tests which are not represented in Redbook. In certain cases, the classical toxicological studies presented in Redbook may be recommended and useful in the evaluation of these substances. The reader is directed to more specific guidance for each type of submission as follows:

Food and Color Additives Program [2]

- Questions and Answers about the Petition Process [3]
- Guidance for Industry: Summary Table of Recommended Toxicological Testing for Additives Used in Food [4]
- Estimating Exposure to Direct Food Additives [5]
- Concern Levels and Recommended Toxicity Tests for Direct Food Additives and Color Additives Used in Food ("draft" 1993 Redbook II) [6]

Food Contact Substance Notification Program [7]

- Guidance for Industry: Preparation of Food Contact Notifications for Food Contact Substances: Toxicology Recommendations [8]
- Guidance for Industry: Preparation of Food Contact Notifications and Food Additive Petitions for Food Contact Substances: Chemistry Recommendations [9]
- Guidance for Industry: Preparation of Food Contact Notifications: Administrative [10]

GRAS Notification Program and Biotechnology [11]

- Frequently Asked Questions About GRAS [12]
- How to Submit a GRAS Notice [13]
- Biotechnology [14]
- List of Completed Consultations on Bioengineered Foods [15]

Generally speaking, the determination of the types of toxicological studies that should be submitted as part of a petition or notification for a food ingredient is based on information about the compound of interest as well as the exposure to the compound via its proposed use(s). Information about the compound includes the toxicological effects on various biological systems (i.e., nature of effect, target, magnitude of response per unit dose, etc.) which may be derived from toxicological studies which have been conducted with this compound. Toxicological information on a similar compound or knowledge about compounds with a similar chemical structure or substructure may be helpful in instances where toxicological information on the compound of interest is limited.

Detailed guidance for specific toxicity studies are not included in this chapter. Guidance for the conduct of short-term studies for genetic toxicity, short-term toxicity studies with rodents and non-rodents, subchronic toxicity studies with rodents and non-rodents, chronic toxicity studies with rodents and non-rodents, carcinogenicity studies with rodents, combined chronic toxicity/carcinogenicity studies or chronic toxicity studies with rodents, *in-utero* exposure phase for addition to carcinogenicity studies or chronic toxicity studies with rodents, neurotoxicity, and reproduction and developmental toxicity studies, can be found in Chapter IV. C. of Redbook 2000. Guidance to assist the petitioner and notifier in developing strategies for assessing the metabolism and pharmacokinetics and immunotoxicity can be found in Chapter V of the "draft" 1993 Redbook II.

It is important to note that as data from the recommended set of toxicity studies are obtained, the results may be used to refine or adjust the type, sensitivity, and rigor of subsequent studies needed to evaluate the potential toxicological effects of the compound.

Links on this page:

1. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/default.htm
2. http://www.fda.gov/Food/FoodIngredientsPackaging/FoodAdditives/default.htm
3. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/ucm078136.htm
4. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/ucm054658.htm
5. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/ucm074725.htm
6. http://www.fda.gov/downloads/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/UCM078759.pdf
7. http://www.fda.gov/Food/FoodIngredientsPackaging/FoodContactSubstancesFCS/default.htm
8. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/ucm081825.htm
9. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/ucm081818.htm
10. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/ucm081807.htm

11. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/ucm081807.htm

12. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/ucm061846.htm

13. http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/ucm083062.htm

14. http://www.fda.gov/Food/Biotechnology/Announcements/default.htm

15. http://www.fda.gov/Food/Biotechnology/default.htm

Redbook 2000: IV. A Introduction: Guidelines for Toxicity Studies

November 2003

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter IV. A. Introduction: Guidelines for Toxicity Studies

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The Agency published its first set of guidelines for toxicity studies for food additives and color additives used in food in 1982 and the first revision in 1993. The guidelines presented in Chapter IV. of Redbook 2000 reflect current scientific knowledge relevant to the design, conduct, and reporting of toxicity studies used in the safety evaluation of food ingredients.

Information contained within Chapter IV.B. provides recommendations which generally apply to all types of toxicity studies and should be considered when designing toxicity studies. Chapter IV. B. currently includes: "General Guidelines for Designing and Conducting Toxicity Studies" (IV.B.1.), "Guidelines for Reporting the Results of Toxicity Studies" (IV.B. 2.), "Pathology Considerations in Toxicity Studies" (IV.B.3.), and "Statistical Considerations in Toxicity Studies" (IV.B.4.). Relevant information contained in the "General Guidelines for Designing and Conducting Toxicity Studies" (IV.B.1.) has also been incorporated into the specific studies (IV.C.3.-5.) in Redbook 2000, for your convenience.

Information presented within Chapter IV.C. of Redbook 2000 provides recommendations for the following specific types of toxicity studies:

1. IV.C.1. Short-Term Tests for Genetic Toxicity
2. IV.C.1.a. Bacterial Reverse Mutation Test
3. IV.C.1.b. *In vitro* Mammalian Chromosomal Aberration Test
4. IV.C.1.c. *In vitro* Mouse Lymphoma Thymidine Kinase Gene Mutation Assay
5. IV.C.1.d. *In vivo* Mammalian Erythrocyte Micronucleus Test
6. IV.C.3.a. Short-Term Toxicity Studies with Rodents
7. IV.C.3.b. Short-Term Toxicity Studies with Non-Rodents
8. IV.C.4.a. Subchronic Toxicity Studies with Rodents
9. IV.C.4.b. Subchronic Toxicity Studies with Non-Rodents
10. IV.C.5 One-Year Toxicity Studies with Non-Rodents
11. IV.C.9.a. Guidelines for Reproduction Studies

12. IV.C.9.b. Guidelines for Developmental Toxicity Studies
13. IV.C.10. Neurotoxicity Studies

Other specific toxicity studies listed in the Table of Contents of Redbook 2000 will be added in the coming months. These include: acute toxicity studies and/or alternatives (Chapter IV.C.2.), carcinogenicity studies with rodents (Chapter IV.C.6.), combined chronic toxicity/carcinogenicity studies with rodents (Chapter IV.C.7.), and *in utero* exposure phase for addition to carcinogenicity studies (Chapter IV.C. 8.).

Links on this page:

1. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/default.htm

Redbook 2000: IV.B.1 General Guidelines for Designing and Conducting Toxicity Studies

November 2003

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter IV.B.1. General Guidelines for Designing and Conducting Toxicity Studies

Return to *Redbook 2000* table of contents [1]

This guidance represents the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

General Guidelines for Toxicity Studies

Guidelines that are common to several or all toxicity studies are described in this section. Guidelines for specific recommended toxicity studies are found in Chapter IV.C., including: genetic toxicity studies (Chapter IV.C.1.), acute oral toxicity studies (Chapter IV.C.2. in the 1993 draft "Redbook II"), short-term toxicity studies with rodents and non-rodents (Chapter IV.C.3.a. and b., respectively), subchronic toxicity studies with rodents and non-rodents (Chapter IV.C.4.a. and b., respectively), one-year toxicity studies with non-rodents (Chapter IV.C.5.), carcinogenicity studies with rodents (Chapter IV.C.6. in the 1993 draft "Redbook II"), combined chronic toxicity/carcinogenicity studies with rodents (Chapter IV.C.7. in the 1993 draft "Redbook II"), *in utero* exposure phase for addition to rodent toxicity studies (Chapter IV.C.8. in the 1993 draft "Redbook II"), reproduction and developmental toxicity studies (Chapter IV.C.9.a. and b., respectively), and neurotoxicity studies (Chapter IV.C.10.). We encourage sponsors/submitters of petitions/notifications to also become familiar with the Guidance for Reporting Results of Toxicity Studies (Chapter IV.B.2.), Pathology Considerations in Toxicity Studies (Chapter IV.B.3.), and Statistical Considerations in Toxicity Studies (Chapter IV.B.4.) during the development of study design.

Scientifically justified changes to this section of the 1993 draft "Redbook II" have been made following consultation with other authoritative guidelines and publications[1-8].

I. Good Laboratory Practice

Nonclinical laboratory studies must be conducted according to U.S. FDA good laboratory practice (GLP) regulations, issued under Part 58. Title 21. Code of Federal Regulations. This document may be obtained from the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C., 20402, (toll free 866-512-1800).

II. Test Animals

A. Care, Maintenance and Housing:

Recommendations about the care, maintenance, and housing of animals contained in NIH publication 85-23, "Guide for the Care and Use of Laboratory Animals"[9], and the DHEW publication no. 78-23 should be followed unless they conflict with specific recommendations in these guidelines.

B. Selection of Species, Strains and Sex:

These guidelines are for studies with rodents (usually rats) and non-rodents (usually dogs); if other species are used, modifications of these guidelines may be necessary. Both male and female test animals, that are healthy and have not been subjected to previous experimental procedures should be used.

It is important to consider the test animals' general sensitivity and the responsiveness of particular organs and tissues of test animals to toxic chemicals when selecting rodent species, strains, and substrains for toxicity studies. The selection of the use of inbred, out-bred, or hybrid rodent strains for toxicity tests should be based upon the scientific questions to be answered. Additionally, it is important that test animals come from well-characterized and healthy colonies. Because recent information suggests survivability problems exist for some strains of rats, test animals should be selected that are likely to achieve the recommended duration of the study. FDA encourages petitioners and notifiers to consult with Agency scientists before toxicity testing is begun if they have questions about the appropriateness of a particular species, strain, or substrain.

C. Age:

Testing should be performed on young animals, with dosing beginning as soon as possible after weaning and following an acclimation period of at least 5 days.Dosing of rodents should begin no later than 6 to 8 weeks of age. When dogs are used, dosing should begin no later than 4 to 6 months of age.

D. Number and Sex:

Equal numbers of males and females of each species and strain should be used for the study. In general, for subchronic toxicity studies, experimental and control groups should have at least 20 rodents per sex per group or at least 4 dogs per sex per group. Ten rodents/sex/group may be acceptable for subchronic rodent studies when the study is considered to be range-finding in nature or when longer term studies are anticipated. These recommendations will help ensure that the number of animals that survive until the end of the study will be sufficient to permit a meaningful evaluation of toxicological effects.

If interim necropsies are planned, the number of animals per sex per group should be increased by the number scheduled to be sacrificed before completion of the study; for rodents, at least 10 animals per sex per group should be available for interim necropsy.

E. Infected Animals:

Generally, it is not possible to treat animals for infection during the course of a study without risking interaction between the compound used for treatment and the test substance. This interaction may confound or complicate the interpretation of study results.

F. Animal Identification:

Test animals should be characterized by reference to their species, strain (and substrain), sex, age, and weight. Each animal must be assigned a unique identification number (e.g., ear tag, implanted identification chip, tatoo).

G. Caging:

Animals should be housed one per cage or run (single-caged) except during mating and lactation and for acute toxicity studies. This recommendation reflects three points of consideration:

- The amount of feed consumed by each animal in the study cannot be determined when more than one

animal is housed in each cage. This information is necessary in the determination of feed efficiency (relationship of feed consumed to body weight gained).

- Minimizing the possibility of confounding analyses and determining whether decreases in body weight gain are due to decreased palatability or substance mediated toxicity.

- Organs and tissues from moribund and dead animals which are single-caged would not be lost due to cannibalism.

H. Diet:

In general, feed and water should be provided *ad libitum* to animals in toxicity studies, and the diets for these studies should meet the nutritional requirements of the species[10-13] for normal growth and reproduction. Unless special circumstances apply which justify otherwise, care should be taken to ensure that the diets of the compound treated groups of animals are isocaloric (equivalent in caloric density) with and contain the same levels of nutrients (e.g., fiber, micronutrients) as the diets of the control group. Unrecognized or inadequately controlled dietary variables may result in nutritional imbalances or caloric deprivation that could confound interpretation of the toxicity study results (e.g., lifespan, background rates of tumor incidences) and alter the outcome and reproducibility of the studies.

The following issues are important to consider when establishing diets for animals in toxicity studies:

When the test substance has no caloric value and constitutes a substantial amount of the diet (e.g., more than 5%), both caloric and nutrient densities of the high dose diet would be diluted in comparison to the diets of the other groups. As a consequence, some high dose animals may receive higher test article doses than expected because animals fed such diluted diets *ad libitum* may eat more than animals in other dosed groups to compensate for the differences in energy and nutrient content of the high dose diets. Such circumstances make it especially important that feed consumption of these animals be as closely and accurately monitored as possible in order to determine whether changes observed could be due to overt toxicity of the test substance or to a dietary imbalance. To further aid in this assessment, two control groups can be used; one group would be fed the undiluted control diet and a second group would be fed the control diet supplemented with an inert filler (e.g., methylcellulose) at a percentage equal to the highest percentage of the test substance in the diet.

When the vehicle for the test substance is expected to have caloric and/or nutritional values, which are greater than that of the control ration, an adjustment in the caloric and/or nutritional components may be necessary.

When administration of the test substance is expected to have an effect on feed intake because of its unpleasant taste or texture, paired feeding can be used to eliminate the differences in consumption between control and compound treated groups. When a paired feeding study design is to be employed, pairs of litter-mate weanling rats of the same sex and approximate size are selected and fed the control or the experimental diet. Animals should be single-caged so that feed consumption can be determined daily, and the control animal is then fed an amount of food equal to that which the paired experimental animal ate on the preceding day. If the test substance is non-nutritive and composes a significant proportion of the diet, the pair-fed control animals should be fed an amount of its feed such that it consumes a nutritionally equivalent amount of diet as the paired experimental animal. Additionally, the study should include a second group of control animals fed *ad libitum* to ensure that the impact on any observed experimental result is due to differences in energy or nutrient intake.

When the test substance interferes with the absorption of nutrients, leading to nutritional deficiencies or changes in nutrient ratios, this can confound assessment of the toxicological endpoints under consideration. For example, fat soluble vitamins may preferentially partition with a mineral oil or fat substitute which is largely unabsorbed, such that a potential deficiency in these vitamins may result. This potential may be eliminated by additional nutrient fortification of the feed for those groups receiving the test substance. Appropriate levels of nutrient fortification should be determined experimentally.

It may be preferable to use a semi-purified diet prepared with known amounts of well-characterized ingredients for short-term and subchronic toxicity studies because of batch to batch variations in diet composition (e.g., fiber, mineral, vitamins, isoflavones) in some of the commonly used laboratory animal

chows. The use of these semi-purified diets, however, may not be advisable in long-term and reproductive studies due to inadequate historical data related to their influences on animal survival and toxicological endpoints. For example, loss of necessary but unidentified micronutrients in the semipurified diet may interfere with normal reproduction.

Related issues are discussed in the section on Diets for Toxicity Studies in Chapter IV.B.5. in the 1993 draft "Redbook II".

I. Assignment of Control and Compound Treated Animals:

Animals should be assigned to control and compound treated groups in a stratified random manner; this will help minimize bias and assure comparability of pertinent variables across compound treated and control groups (for example: mean body weights and body weight ranges). If other characteristics are used as the basis for randomization then that characterization should be described and justified.

Animals in all groups should be placed on study on the same day; if this is not possible because of the large number of animals in a study, animals may be placed on study over several days. If the latter recommendation is followed, a preselected portion of the control and experimental animals should be placed on the study each day in order to maintain concurrence.

J. Mortality:

Excessive mortality due to poor animal management is unacceptable and may be cause to repeat the study. For example, under normal circumstances, mortality in the control group should not exceed 10% in short and intermediate length (not lifetime) toxicity studies.

K. Autolysis:

Adequate animal husbandry practices should result in considerably less than 10% of animals and tissues or organs lost to a study because of autolysis. Autolysis in excess of this standard may be cause to repeat the study.

L. Necropsy:

Necropsy should be performed soon after an animal is sacrificed or found dead, so that loss of tissues due to autolysis is minimized. When necropsy cannot be performed immediately, the animal should be refrigerated at a temperature that is low enough to prevent autolysis but not so low as to cause cell damage. If histopathological examination is to be conducted, tissue specimens should be taken from the animals and placed in appropriate fixatives when the necropsy is performed.

III. Test Substance

The test substance used in toxicity studies should be the same substance that the petitioner/notifier intends to market. A single lot of test substance should be used throughout the study, when possible. Alternatively, lots that are as similar as possible in purity and composition should be used.

A. Identity:

The identity of the test substance or mixture of substances to be tested should be known. We urge petitioners/notifiers to consult with the Agency in determination of test compound and to provide a Chemical Abstract Service (CAS) Registry Number or Numbers.

B. Composition/Purity:

The composition of the test substance should be known including the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials.

C. Conditions of Storage:

The test sample should be stored under conditions that maintain its stability, quality, and purity until the studies are complete.

D. Expiration Date:

The expiration date of the test material should be known and easily available. Test materials should not be used past their expiration date.

IV. Experimental Design

A. Duration of Testing:

Animals should be exposed to the test substance 7 days per week for the designated length of time of the study.

B. Route of Administration:

The route of administration of the test substance should approximate that of normal human exposure, if possible. For food ingredients (e.g., food and color additives) the oral route of administration is preferred. A justification should be provided when other routes are used. The same method of administration should be used for all test animals throughout the study.

The test substance should be administered in one of the following ways:

- **In the diet**, if human exposure to the test substance is likely to be through consumption of solid foods or a combination of solid and liquid foods. If the test substance is added to the diet, animals should not be able to selectively consume either basal diet or test substance in the diet on the basis of color, smell, or particle size. If the compound is mixed with ground feed and pelleted, nothing in the pelleting process should affect the test substance (for example, heat-labile substances may be destroyed during pellet production by a steam process). When the test substance is administered in the diet, dietary levels should be expressed as mg of the test substance per kg of feed.

- **Dissolved in the drinking water**, if the test substance is likely to be ingested in liquid form (for example, in soft drinks or beer), or if administration in the diet is inappropriate for other reasons. The amount of test substance administered in drinking water should be expressed as mg of test substance per ml of water.

- **By encapsulation or oral intubation (gavage)**, if the two previous methods are unsatisfactory or if human exposure is expected to be through daily ingestion of single, large doses instead of continual ingestion of small doses. If the test substance is administered by gavage, it should be given at approximately the same time each day. The maximum volume of solution that can be given by gavage in one dose depends on the test animal's size; for rodents, the volume ordinarily should not exceed 1 ml/100 g body weight. If the gavage vehicle is oil (see Chapter IV.B.5.b. in the 1993 draft "Redbook II"), then the volume should be no more than 0.4 ml/100 g of body weight, and the use of a low-fat diet should be considered. If the test substance must be given in divided doses, all doses should be administered within a 6 hour period. Doses of test substance administered by gavage should be expressed as mg of test substance per ml of gavage vehicle. Finally, the petitioner/notifier should provide information that can allow the reviewer to conclude that administration of the test compound by encapsulation or gavage is equivalent in all toxicologically important respects to administration in the diet or drinking water. Alternatively, metabolic information on both modes of administration should be provided so that appropriate interpretation of data can be accomplished.

C. Dose groups:

Three to five dose levels of the test substance and concurrent control groups should be used with both males and females. Information obtained from acute (Chapter IV.C.2. in the 1993 draft "Redbook II") and short-term (Chapter IV.C.3.a. and b.) toxicity studies can help determine appropriate doses for subchronic studies.

1. Selection of Treatment Doses:

Dose selection for toxicity studies should be based on information related to the toxicity of the test substance.

A minimum of three dose levels of the test substance and a concurrent control group should be used in toxicity studies. When designing and conducting toxicity studies the following should be considered: 1) the high dose should be sufficiently high to induce toxic responses in test animals; 2) the low dose should not induce toxic responses in test animals; and 3) the intermediate dose should be sufficiently high to elicit minimal toxic effects in test animals (such as alterations in enzyme levels or slight decreases in body weight gains). No dose should cause an incidence of fatalities that prevents meaningful evaluation of the data.

Administration of the test substance to all dose groups should be done concurrently.

2. Controls:

A concurrent control group of test animals is required. The control group in dietary studies should be fed the basal diet. Exceptions to this and other related information, including a discussion regarding pair-feeding, was provided above in section "II. Test Animals, H. Diet".

A carrier or vehicle for the test substance should be given to control animals at a volume equal to the maximum volume of carrier or vehicle given to any dosed group of animals. Sufficient toxicology information should be available on the carrier or vehicle to ensure that its use will not compromise the results of the study. If there is insufficient information about the toxic properties of the vehicle used to administer the test substance, an additional control group that is not exposed to the carrier or vehicle should be included. In all other respects, animals in the control group should be treated the same as animals in dosed groups. (See additional information in "II. Test Animals, H. Diet" above.)

D. Computerized systems

Computerized systems that are used in the generation, measurement, or assessment of data should be developed, validated, operated, and maintained in ways that are compliant with Good Laboratory Practice principles.[14]

V. Observations and Clinical Tests

A. Observations of Test Animals:

Routine cage-side observations should be made on all animals at least once or twice a day throughout the study for general signs of pharmacologic and toxicologic effects, morbidity and mortality. The usual interval between observations should be at least 6 hours. Individual records should be maintained for each animal and the time of onset and the characteristics and progression of any effects should be recorded, preferably using a scoring system.

An expanded set of clinical evaluations, performed inside and outside of the cage, should be carried out in short-term and subchronic toxicity studies in rodents and non-rodents, in one-year non-rodent toxicity studies, and reproductive toxicity studies in rodents to enable detection not only of general pharmacologic and toxicologic effects but also of neurologic disorders, behavioral changes, autonomic dysfunctions, and other signs of nervous system toxicity. Specific information about the systematic clinical tests/observations is contained in Chapter IV.C.10. This expanded set of clinical examinations (Chapter IV.C.10.), conducted inside and outside the cage, should be age appropriate and performed on all animals at least once prior to initiation of treatment, and periodically during treatment. Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g., lacrimation, pilorection, pupil size, unusual respiratory pattern). Additionally, changes in gait, posture and response to handling, as well as the presence of clonic or tonic movements, stereotypes (e.g., excessive grooming, repetitive circling) or bizarre behavior (e.g., self-mutilating, walking backwards) should be recorded. Tumor development, particularly in long-term studies, should be followed: the time of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumor should be recorded. During the course of a study, toxic and pharmacologic signs may suggest the need for additional clinical tests or expanded post-mortem examinations.

B. Body Weight and Feed Intake Data:

Recommendations are described in guidelines for specific toxicity study types (see Chapter IV.C. of the 1993 draft "Redbook II" and/or "Redbook 2000"). Feed spillage should be noted and adjustments made in related calculations and appropriate discussion presented in the study report.

C. Clinical Testing:

Ophthalmological examination, hematology profiles, clinical chemistry tests, and urinalyses should be performed as described in the following sections:

1. **Opthalmological Examination:** This examination should be performed by a qualified individual on all animals before the study begins and on control and high-dose animals at the end of the study. If the results of examinations at termination indicate that changes in the eyes may be associated with administration of the test substance, ophthalmological examinations should be performed on all animals

in the study.

2. **Hematology**: The recommended number of animals and time intervals for hematology assessment are found in individual toxicity study guidelines (see Chapter IV.C.3.-5. of "Redbook 2000" and Chapter IV.C.2. and 6.-8. of the 1993 draft "Redbook II"). Ideally, the same rodents should be sampled at each collection time point. Blood samples should be analyzed individually, and not pooled. If animals are sampled on more than one day during a study, blood should be drawn at approximately the same time each sampling day.

    1. The following determinations are recommended:
        1. hematocrit
        2. hemoglobin concentration
        3. erythrocyte count
        4. total and differential leukocyte counts
        5. mean corpuscular hemoglobin
        6. mean corpuscular volume
        7. mean corpuscular hemoglobin concentration
        8. and a measure of clotting potential (such as clotting time, prothrombin time, thromboplastin time, or platelet count).

    Test compounds may have an effect on the hematopoietic system and therefore appropriate measures should be employed so that evaluations of reticulocyte counts and bone marrow cytology may be performed if warranted. Reticulocyte counts should be obtained for each animal using automated reticulocyte counting capabilities, or from air-dried blood smears. Bone marrow slides should be prepared from each animal for evaluating bone marrow cytology. These slides would only need to be examined microscopically if effects on the hematopoietic system were noted.

3. **Clinical Chemistry**: The recommended number of animals and time intervals for clinical chemistry assessment are found in individual toxicity guidelines (see Chapter IV.C.3.-5. of "Redbook 2000" and Chapter IV.C.2. and 6.-8. of the 1993 draft "Redbook II"). Ideally, the same rodents should be sampled at each collection time point. Blood samples should be drawn at the end of the fasting time and before feeding. Fasting duration should be appropriate for the species and the analytical tests to be performed. Blood samples should be analyzed individually, and not pooled. If animals are sampled on more than one day during a study, blood should he drawn at approximately the same time each sampling day.

    Clinical chemistry tests that are appropriate for all test substances include measurements of electrolyte balance, carbohydrate metabolism, and liver and kidney function. Specific determinations should include:

    1. Hepatocellular evaluation: select at least 3 of the following 5
        1. alanine aminotransferase (SGPT, ALT)
        2. aspartate aminotransferase (SGOT, AST)
        3. sorbitol dehydrogenase
        4. glutamate dehydrogenase
        5. total bile acids
    2. Hepatobiliary evaluation: select at least 3 of the following 5
        1. alkaline phosphatase
        2. bilirubin (total)
        3. gamma-glutamyl transpeptidase (GG transferase)
        4. 5' nucleotidase
        5. total bile acids
    3. Other markers of cell changes or cellular function
        1. albumin
        2. calcium
        3. chloride
        4. cholestrol(total)

5. cholinesterase
6. creatinine
7. globulin (calculated)
8. glucose (in fasted animals)
9. phosphorous
10. potassium
11. protein (total)
12. sodium
13. triglycerides (fasting)
14. urea nitrogen

1. However, when adequate volumes of blood cannot be obtained from test animals, the following determinations should generally be given priority. FDA understands that the specific nature of the test compound may warrant the consideration of alternative tests. Appropriate justification for alternative tests should be presented in study reports.

1. alanine aminotransferase
2. alkaline phosphatase
3. chloride
4. creatinine
5. gamma-glutamyl transpeptidase (GG transferase)
6. glucose (in fasted animals)
7. potassium
8. protein (total)
9. sodium
10. urea nitrogen

Additional clinical chemistry tests may be recommended to extend the search for toxic effects attributable to a test substance. The selection of specific tests will be influenced by observations on the mechanism of action of the test substance. Clinical chemistry determinations that may be recommended to ensure adequate toxicological evaluation of the test substance include analyses of acid/base balance, hormones, lipids, methemoglobin, and proteins.

In spite of standard operating procedures and equipment calibration, it is not unusual to observe considerable variation in the results of clinical chemistry analyses from day to day[15]. Ideally, clinical chemistry analyses for all dose groups should be completed during one day. If this is not possible, analyses should be conducted in such a way as to minimize potential variability.

4. **Urinalyses**: Timed urine volume collection should be conducted during the last week of the study and at other time intervals described in specific individual toxicity study guidelines (Chapter IV.C.3.-5. of "Redbook 2000" and Chapter IV.C.2. and 6.-8. of the 1993 draft "Redbook II"). The volume of urine collected, specific gravity, pH, glucose, and protein should be determined as well as conducting a microscopic evaluation of urine for sediment and presence of blood/blood cells [16].
5. **Neurotoxicity Screening/Testing** : Screening for neurotoxic effects should be routinely carried out in short-term and subchronic toxicity studies with rodents (preferably rats) and non-rodents (preferably dogs), one-year studies in non-rodents, and reproductive toxicity studies in rodents. The neurotoxicity screen should be age appropriate and would typically include: (1) specific histopathological examination of tissue samples representative of major areas of the brain, spinal cord, and peripheral nervous system (see organs and tissues listed below under VI.C. Preparation of Tissues for Microscopic Examination) and (2) a functional battery of quantifiable observations and manipulative tests selected to detect signs of neurological, behavioral, and physiological dysfunctions. This functional battery is also referred to as an expanded set of clinical evaluations and is described more fully in section V.A. Observations of Test Animals in this Chapter and in Chapter IV.C.10. Neurotoxicity Studies.

   Toxicity study reports should include an assessment of the potential for the test substance to adversely affect the structural or functional integrity of the nervous system. This assessment should evaluate data

from the neurotoxicity screen and other toxicity data from the study, as appropriate. Based on this assessment, the petitioner should make an explicit statement about whether or not the test substance presents a potential neurotoxic hazard and if additional neurotoxicity testing is deemed appropriate. FDA recommends that additional neurotoxicity testing not be undertaken without first consulting with the Agency.

6. **Immunotoxicity**: For short-term, subchronic and developmental toxicity studies, results of clinical tests that are included in the list of primary indicators for immune toxicity (see Chapter V.C. of the 1993 draft "Redbook II") should also be evaluated as part of an immunotoxicity screen. Additional immunotoxicity tests are discussed in Chapter V.C. of the 1993 draft "Redbook II", but should not be undertaken without first consulting with the Agency.

VI. Necropsy and Microscopic Examination

A. Gross Necropsy

All test animals should be subjected to complete gross necropsy, including examination of external surfaces, orifices, cranial, thoracic and abdominal cavities, carcass, and all organs. The gross necropsy should be performed by, or under the direct supervision of, a qualified pathologist, preferably the pathologist who will later perform the microscopic examination (see below).

B. Organ Weight

Organs that should be weighed include the adrenals, brain, epididymides, heart, kidneys, liver, spleen, testes, thyroid/parathyroid, thymus, ovaries and uterus. Organs should be carefully dissected and trimmed to remove fat and other contiguous tissue and then be weighed immediately to minimize the effects of drying on organ weight.

C. Preparation of Tissues for Microscopic Examination

Generally, the following tissues should be fixed in 10% buffered formalin (or another generally recognized fixative) and sections prepared and stained with hematoxylin and eosin (or another appropriate stain) in preparation for microscopic examination. Lungs should be inflated with fixative prior to immersion in fixative. Sponsors should refer to specific studies (Chapter IV.C.1.-10.) for details.

1. adrenals
2. aorta
3. bone (femur)
4. bone marrow (sternum)
5. brain (at least 3 different levels)
6. cecum
7. colon
8. corpus and cervix uteri
9. duodenum
10. epididymis
11. esophagus
12. eyes
13. gall bladder (if present)
14. Harderian gland (if present)
15. heart
16. ileum
17. jejunum
18. kidneys
19. liver
20. lung (with main-stem bronchi)
21. lymph nodes (1 related to route of administration and 1 from a distant location)

22. mammary glands
23. nasal turbinates
24. ovaries and fallopian tubes
25. pancreas
26. pituitary
27. prostate
28. rectum
29. salivary gland
30. sciatic nerve
31. seminal vesicle (if present)
32. skeletal muscle
33. skin
34. spinal cord (3 locations: cervical, mid-thoracic, lumbar)
35. spleen
36. stomach
37. testes
38. thymus (or thymic region)
39. thyroid/parathyroid
40. trachea
41. urinary bladder
42. vagina
43. Zymbal's gland (if present)
44. all tissues showing abnormality

D. Microscopic Evaluation

All gross lesions should be examined microscopically. All tissues from the animals in the control and high dose groups should be examined. If treatment related effects are noted in certain tissues, then the next lower dose level tested of those specific tissues should be examined. Successive examination of the next lower dose level continues until no effects are noted. In addition, all tissues from animals which died prematurely or were sacrificed during the study should be examined microscopically to assess any potential toxic effects.

E. Histopathology of Lymphoid Organs

Histopathological evaluation of the lymphoid organs should be performed as described in the section on immunotoxicity testing (see Chapter V.C.) for all animals in short-term and subchronic toxicity studies and developmental toxicity studies.

VII. References

1. EPA Health Effects Test Guidelines OPPTS 870.3050, 28-Day Oral Toxicity in Rodents, July 2000
2. EPA Health Effects Test Guidelines OPPTS 870.3150, 90-Day Oral Toxicity in Non-Rodents, August 1998
3. EPA Health Effects Test Guidelines OPPTS 870.3100, 90-Day Oral Toxicity in Rodents, August 1998
4. EPA Health Effects Test Guidelines OPPTS 870.4100, Chronic Toxicity, August 1998
5. OECD Guideline For The Testing Of Chemicals , Repeated Dose 90-day Oral Toxicity Study in Non-Rodents, 409, September 1998
6. OECD Guideline For The Testing Of Chemicals , Repeated Dose 90-day Oral Toxicity Study in Rodents, 408, September 1998
7. OECD Guideline For The Testing Of Chemicals , Repeated Dose 28-day Oral Toxicity Study in Rodents, 407, July 1995
8. Kurt Weingand et al., 1996. "Harmonization of Animal Clinical Pathology Testing in Toxicity and Safety

Studies", *Fundamental and Applied Toxicology*, 29, pp 198-201.

9. National Research Council Institute of Laboratory Animal Resources. 1996. Guide for the Care and Use of Laboratory Animals. National Academy Press, Washington, DC.
10. Nutrient Requirements of Laboratory Animals, Fourth Revised Edition, Subcommittee on Laboratory Animal Nutrition, Committee on Animal Nutrition, Board on Agriculture, National Research Council, 1995.
11. Nutrient Requirements of Dogs, Revised, Committee on Animal Nutrition, National Research Council, 1985.
12. Nutrient Requirements of Swine: 10th Revised Edition, Subcommittee on Swine Nutrition, Committee on Animal Nutrition, National Research Council, 1998.
13. Nutrient Requirements of Rabbits, Second Revised Edition, Committee on Animal Nutrition, National Research Council, 1977.
14. The Application of the Principles of GLP to Computerised Systems, Organisation for Economic Cooperation and Development (OECD), Environmental Monograph No. 116, Paris, 1995.
15. Gaylor, D.W., Suber, R.L., Wolff, G.L. and Crowell, J.A. (1987) Statistical variation of selected clinical pathological and biochemical measurements in rodents (42555) *Proc. Soc. Exp. Biol. Med.* , 185:361-367
16. Ragan, H.A. and Weller, R.E., "Markers of Renal Function and Injury" in *The Clinical Chemistry of Laboratory Animals*, Second Edition 1999, Taylor & Francis, Philadelphia, PA, pp. 520-533

Links on this page:

1. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/default.htm

Redbook 2000: IV.B.2 Guidelines for Reporting the Results of Toxicity Studies

November 2003

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter IV.B.2. Guidelines for Reporting the Results of Toxicity Studies

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This guidance represents the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

Guidelines for reporting the results of toxicity studies are contained in this section. More complete information regarding Pathology and Statistics can be found in Chapters IV.B.3. [2] and IV.B.4. [3], respectively. The study report should include all information necessary to provide a complete and accurate description and evaluation of the study procedures and results in accordance with 21 CFR 58.185 [4]. The following sections should be included:

I. Study Identification and Information

A. Study title and report number
B. Testing facility by name and address
C. Duration of study
D. Dates of:
    1. Acclimation
    2. Initiation (onset of dosing)
    3. Sacrifice/termination
    4. Study report
E. Identification and signatures (where appropriate) of personnel primarily responsible for:
    1. Conduct of the study (i.e., study director, principal investigator)
    2. Analyses of data
    3. Histopathology
    4. Writing the report
    5. Other information contained in the report

II. Good Laboratory Practice for Nonclinical Laboratory Studies Statement

The Good Laboratory Practice (GLP) regulations were designed to establish minimal standards for conduct and reporting of nonclinical safety testing and are intended to assure the quality and integrity of safety data submitted to the FDA. Each food and color additive petition, Generally Recognized as Safe (GRAS) affirmation petition, and Food Contact Notification (FCN) must include a GLP compliance statement as set forth in 21 CFR 171.1(k), 21 CFR 71.1(g), 21 CFR 170.35(c)(1)(vi), or 21 CFR 170.101(c).

III. Quality Assurance Statement

Each study report must include a quality assurance statement signed by the quality assurance unit in accordance with 21 CFR Section 58.35(b)(7) which states: "Prepare and sign a statement to be included with the final study report which shall specify the dates inspections were made and findings reported to management and to the study director."

IV. Protocol and Amendments

A protocol for each non-clinical study should be written so that it satisfies all GLP requirements set forth in Section 21 of CFR § 58.120[5]. The protocol should include clearly stated objectives and methods for the conduct of the study. A copy of the protocol should be attached to the study report. The study report should include statements describing all changes in or revisions of the protocol, the reasons for those changes, and any influence these changes had on the results of the study.

V. Storage, Retrieval, and Retention of Records

This section of the study report should include information regarding the availability and location of original data, specimens and samples of the test substance, in accordance with 21 CFR 58.190[6] and 195[7].

VI. Summary and Conclusions

This section of the study report should contain a brief description of:

A. Methods
B. Summary and analysis of numerical data
C. Summary and analysis of descriptive data (e.g., observations to assess neurotoxic potential)
D. The conclusions drawn from the analyses, including target organ(s) and no observed effect levels (NOELs)

   The summary should highlight all significant changes in data or observations of the test substance treated groups, in comparison to the control groups, which may be an indication of toxic effects of the test substance. The summary should also include a description of the relationship between dose and the incidence/severity of lesions or abnormalities.

   The summary should include a description of all circumstances that may have influenced the quality or integrity of the data or observations.

VII. Test Substance

A. Identification
   1. Chemical name
   2. Chemical Abstracts Service (CAS) registry number (or code number)
   3. Molecular structure and molecular weight
   4. Qualitative and quantitative determination of its chemical composition
B. Manufacturing information
   1. Lot number
   2. Purity, including names and quantities of known contaminants and impurities and the percentage of unidentifiable materials
   3. Expiration date
   4. Stability

5. Storage instructions

C. Physical properties
   1. State (i.e., powder, liquid)
   2. Color
   3. Solubility, in aqueous and in dosing vehicle
   4. pH (pKa where applicable)
   5. Boiling and melting point

D. Identification of diluents, suspending agents, emulsifiers, excipients, or other materials used in administering the test substance.

E. Sampling of test material in administered form
   1. Times when sampling was performed
   2. Verification of stability of test compound in administered form: method and results
   3. Verification of homogeniety of test compound in administered form: method and results
   4. Verification of concentration of test compound in administered form: method and results

F. Storage conditions: during and after the study

VIII. Test Animals

A. Species and strain (and substrain if applicable) used and, particularly if a strain other than a common laboratory strain is used in the study, rationale for selection of the strain

B. Source or supplier of the animals

C. Description of any pre-test conditioning (such as quarantine procedures)

D. Description of the method used to randomize animals into test and control groups

E. Numbers, age, and condition of animals of each sex in all test substance treated and control groups at the beginning and end of the study

F. Diet
   1. Feed
      a. Diet: lot number, composition, etc.
      b. Availability: i.e., *ad libitum*
   2. Water
      a. Availability: i.e., *ad libitum*

G. Caging conditions
   1. Number of animals per cage
   2. Bedding material
   3. Ambient temperature
   4. Humidity
   5. Lighting conditions

IX. Methods

The methods section of the study report should include, but not be limited to, the following information

A. Deviations from these Redbook 2000 guidelines
   1. Describe all ways in which the test procedure deviates from these Redbook 2000 guidelines
   2. State the rationale for each deviation.

B. Experimental design and procedures (full description)

1. Length of the study (including dates study began and ended)
2. Data on dosage administration should include
   a. All dose levels administered, expressed as mg/kg body weight, per unit of time (e.g. day)
   b. Route of administration
   c. Method, frequency, and time of day of administration
   d. Total volume of dose plus vehicle administered to each animal, if the test substance is administered by gavage
   e. Duration of treatment period
3. Observation of the test animals
   a. Duration of individual observation
   b. Frequency
   c. Method
4. Sampling Conditions of specimens (hematology, clinical chemistry, urinalysis, other)
   a. Duration and time of day
   b. Frequency
   c. Method
5. Statistical analyses: All statistical methods used should be fully described or identified by reference. For a complete discussion of the information that should be contained in this section of the study report, see Chapter IV.B.4 [8].

XI. Results and Discussion

A. Individual animal data and results should be provided in tabular format and in sufficient detail to permit independent evaluation of the results. Describe all computerized systems used in the generation, measurement, or assessment of data including the name and version of the system (software) and the specified indications for use. The following information should be included for each test animal:
   1. Time of first observation of each abnormal sign and its subsequent course. These data should be organized, when appropriate, by litter.
   2. Time of death during the study. The "most probable" cause of death should also be determined and reported for those animals that were not sacrificed at the pre-scheduled time.
   3. Feed and water consumption data (including feed spillage)
   4. Body weights and body weight changes
   5. Feed efficiency data
   6. Hematology, clinical chemistry, urinalysis, and other clinical findings
   7. Results of neurotoxicity and immunotoxicity studies, as appropriate
   8. Gross necropsy findings including:
      a. absolute and relative organ weights
      b. description of gross lesions
      c. incidence and severity of gross lesions
   9. Histopathology findings (see Chapter IV.B.3. [9]) including:
      a. description of microscopic lesions
      b. incidence and severity of microscopic lesions

B. Summarized data from individual animals should be organized by sex and dose group and provided in tabular format. When appropriate, data should also be organized by litter. When numerical means are presented, they should be accompanied by an appropriate measure of variability, such as the standard error. For each summarized parameter, the following information should be included:
   1. The number of animals at the beginning of the study

2. The number of animals evaluated for each parameter
3. The day of the study (e.g., day 60, at termination) when animals were evaluated for each parameter

C. All numerical results should be evaluated by an appropriate statistical method. Refer to Chapter IV.B.4.[10] for detailed guidelines about statistical considerations in toxicity studies.

D. Evaluation of the results should include at a minimum:

1. Discussion about the nature of relationships, if any, between exposure to the test substance and the incidence and severity of all general and specific adverse effects (such as neoplastic and non-neoplastic lesions, organ weight effects, and mortality effects), and identification of target organs.
2. Discussion about the relationship between clinical observations made during the course of a study and post mortem findings.
3. A conclusion statement regarding the dosage level at which no effects attributable to the test substance were observed (NOEL), and a discussion of any complex and/or any controversial issues surrounding that determination.

X. References

This section of the study report should include appropriate literature citations or references, for the following:

A. Test procedures
B. Statistical and other methods used to analyze the data
C. Compilation and evaluation of results
D. The basis upon which conclusions were reached

The above guidance document supersedes the previous version dated October, 2001.

Links on this page:

1. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/default.htm
2. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/ucm078318.htm
3. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/ucm078320.htm
4. http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/showCFR.cfm?CFRPart=58
5. http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/showCFR.cfm?CFRPart=58
6. http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/showCFR.cfm?CFRPart=58
7. http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/showCFR.cfm?CFRPart=58
8. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/ucm078320.htm
9. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/ucm078318.htm
10. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/ucm078320.htm

Redbook 2000: IV.B.3 Pathology Considerations in Toxicity Studies

July 2000

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter IV.B.3. Pathology Considerations in Toxicity Studies

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Pathology data make up an essential part of the toxicology information submitted to FDA in support of the safe use of the use of food ingredients. The interpretation of pathology data and other safety data forms the basis for judgement about the safety of a product.

Specific recommendations concerning necropsy of test animals and microscopic examination of organs and tissues for short-term toxicity tests with rodents and non-rodents, subchronic toxicity tests with rodents and non-rodents, one-year toxicity tests with non-rodents, carcinogenicity studies with rodents, combined chronic toxicity/carcinogenicity studies with rodents, reproduction studies, and developmental toxicity studies can be found in Chapter IV.B.1.e. In general, these guidelines recommend that all animals in the studies be subjected to complete gross necropsy, all gross lesions and all protocol-required tissues and organs (see Chapter IV.B.1.e.iii.) from all control and high dose animals in the study should be examined microscopically; all gross lesions and target organs from all other dosed groups should also be examined microscopically.

This section on pathology considerations in toxicity studies describes the review process for pathology data, identifies common problems reviewers encounter in reviewing such data, and presents general guidelines for reporting pathology data. Although not addressed in this chapter, CFSAN pathologists also review and provide advice to petitioners on protocols for proposed toxicity studies; requests for such review should be directed to the CSO assigned to the petition (see Chapter II.A.).

a. Description of the Process for Review of Pathology Data

Review of pathology data may begin with a request for pathology evaluation from regulatory review scientists or from the CAC. This happens when questions about the interpretation of pathology data arise during the scientific review of the toxicology information submitted in support of the safety of food ingredients . Requests for review are generally limited to specific interpretative questions, directing the reviewing pathologist's attention to findings in a particular organ or tissue. Occasionally, a reviewing pathologist is asked to examine all of the pathology findings in a study.

The pathology portion of the study report usually contains mean and individual organ weight parameters, clinical chemistry results, hematological measurements, summary incidences of observed pathological changes, and gross and microscopic pathology observations for individual animals. An evaluation memorandum from the regulatory review scientist may accompany the material; the memorandum contains summaries of toxicology information, including the results of previous toxicity studies and information from relevant scientific literature.

The reviewing pathologist usually begins his/her review by examining the experimental design and methods. He/she carefully reviews general indices of toxicity in test animals (for example, body weight gain, food consumption, clinical or hematologic findings, and organ weight changes); particular attention is paid to the survival of the animals and the number of animals alive at termination. All this information helps the reviewing pathologist evaluate the relationship of observed pathology changes to treatment.

Although the approach to pathology review may vary, the elements listed below are considered in all reviews. The reviewing pathologist:

- Determines how the percentage of animals with lesions in summary incidence tables has been calculated; for example, was the denominator based on the total number of animals in the study, or was it the number of animals for which a particular tissue or organ was examined microscopically;
- Compares gross and microscopic findings to ensure that all gross observations are accounted for by microscopic findings or by other suitable explanations;
- Examines the diagnostic terminology applied to lesions to determine whether it is contemporary and conventional;
- Checks to see that individual animal data provide adequate information on the location, size, and distribution of reported gross lesions;
- Considers the qualitative characteristics, severity of lesions, and the incidence figures in evaluating treatment-related differences among groups of experimental animals;
- Carefully evaluates control data before interpreting findings;
- Evaluates the discussion of significant pathological findings prepared by the study pathologist; and
- Correlates pathology findings, when appropriate, with other observations about treatment-related effects on test animals during the study.

When the pathology review is completed, a formal written report is submitted to the collaborating regulatory review scientist. The report discusses the pathological findings based on review of submitted material and the relationship of pathological findings to treatment. If questions about the pathology data remain, the report may recommend a request for additional, clarifying material.

A follow-up pathology review requires additional data. The additional information most often requested by the Agency is clarification of the diagnostic criteria used and historical control data for a specific lesion. The Agency may ask to review the original microscope slides; in some cases, the petitioner may be asked to prepare new slides from paraffin blocks or wet tissue for FDA review. The Agency's review of slides from a toxicity study provides an independent characterization of the lesions and enables the incidence of lesions to be verified.

When microscope slides and other materials are requested by the Agency for a follow-up review, the Agency provides instructions for their submission. Usually, microscope slides from an organ or tissue site should be

arranged by treatment group, sex, and in the order of pathology accession numbers. If microscope slides are submitted according to the Agency's directions, the follow-up review will be expedited.

b. Common Problems Encountered during Review of Pathology Data

The timely review of pathology data is sometimes hindered by missing, inaccurate, or incomplete information. These problems are often encountered in submissions to the Agency; a general discussion of problems resulting from information deficiencies is presented below. A more detailed discussion of this subject has been published.(1)

i. Lack of Morphologic Descriptions of Lesions

One of the most common problems causing delay in the review of pathology data is the lack of adequate morphologic descriptions of lesions. It is difficult to assess the significance of reported lesions without information on their diagnostic criteria, distribution, and severity. This is particularly important when the terminology for lesions is controversial.

ii. Inconsistency in Applying Diagnostic Terminology

The use of multiple diagnostic terms without explanation for describing a single type of lesion can present problems for the reviewing pathologist. Further clarification is needed to indicate whether two or more terms are being used interchangeably or the results of the study have been evaluated by more than one pathologist, each using different terms for the same morphologic change. For example, in one study the terms "hepatocellular carcinoma" and "hepatoma, malignant" were used in the same set of diagnoses. In another report, four different terms--"c-cell," "clear cell," "light cell," and "parafollicular cell"--were used to describe rat thyroid lesions. In both instances, reasons for using multiple terms for the same diagnosis were not provided.

Differences in the use of diagnostic terms have been encountered when more than one pathologist has examined slides: for example, a study was submitted in which tissues from about one-third of the animals were evaluated by the study pathologist and the remainder were evaluated by a consulting pathologist. The diagnostic terminology was not consistent between pathologists and no attempt was made to explain the inconsistencies in the study report. Although the data appeared to show treatment-related effects, these were subsequently attributed to the way different categories of lesions were summarized.

iii. Incomplete Descriptions of the Results of Gross Pathology Examinations

Incomplete gross descriptions have made it difficult to correlate gross pathology findings with microscopic diagnoses. When microscopic findings do not correlate with gross descriptions, the reviewer must attempt to determine if important information is missing. The report should describe steps taken to resolve discrepancies between gross findings and microscopic diagnoses (for example, recuts of paraffin blocks or additional samples taken from wet tissues).

iv. Inaccurate Summaries of Data

Inaccurate summary numbers resulting from incorrect counts or calculations have caused difficulty in reviewing pathology data. When pathology data are summarized, all experimental animals should be accounted for and incidence figures should be based on the numbers of animals, organs, and tissues actually examined.

v. Failure to Adequately Discuss the Results of Pathology Examinations

Often, submissions fail to adequately discuss the significance of the results of pathology evaluations. Some reports summarize conclusions but do not explain how the conclusions were deduced from the available pathology data. Some reports base conclusions solely on the results of statistical analyses of data, ignoring broader conclusions that may be discerned from considering all relevant biological information from a study.

c. General Recommendations for Reporting Pathology Data

The pathology section in the report of a toxicity study generally includes an introductory statement and sections on materials and methods, results and discussion, and summary and conclusions.

When pathology data are reported separately from the toxicity study, adequate information about the experimental design and methodology of the toxicity study should be included. This information should include the species and strain of the experimental animals, details about the administration of the test compound, number of experimental and control groups, number of animals in each group, type and frequency of in-life observations including clinical chemistry measurements and hematological examinations, and the scope of gross and microscopic evaluation of tissues. In general, information provided should be sufficient to enable a reviewer to evaluate the quality of the pathology data.

Deviations from the original protocol should be explained. For example, if tissues from low- and mid-dose groups were not scheduled for microscopic examination but were examined, the appropriate protocol amendment or reason for this deviation should be given.

i. Arranging Tabular Data and Morphological Observations

The arrangement of tabular information in an easily comprehensible format is especially important for facilitating review. Table titles and row and column headings should be brief but informative. In the tables showing the individual animal findings, descriptive diagnostic categories should be informative. Redundancy of categories of lesions should be avoided. Morphologic diagnoses should reflect currently accepted criteria. Whenever multiple categories of lesions are grouped under a common "diagnosis," the rationale for grouping should be provided. When multiple diagnoses are not grouped under a common diagnosis, it will be assumed that morphologic differences preclude grouping. Severity grades as well as information on the distribution of a lesion within an organ or tissue should be provided; these observations are particularly important when progression of lesions and effects of different dosages are being studied. In paired organs such as adrenal glands, gonads, and kidneys, certain lesions, when appropriate, should be indicated as unilateral or bilateral. All gross lesions should be accounted for by microscopic findings or a written explanation.

ii. Summary Tables

Summary tables in the results section of a report should clearly indicate the number of animals, organs, and tissues actually examined. Unless the number of tissues examined in animals of each group is indicated, the incidence figures or mean values indicating effects are subject to question. Summary tables should be free of double counting. In determining incidence, the denominators should reflect actual numbers of animals whose tissues were examined, not just the number of animals originally assigned to each group. The figure for the number of tissues examined should clearly show any adjustments that reflect loss, autolysis, or missing tissue: For example, the accurate incidence of lesions involving the adrenal medulla should be based upon how many adrenal sections (for an animal) from both adrenal glands contained sufficient medullary tissue for microscopic examination. In summarizing lesions that are disseminated, *e.g.*, tumors of the lymphoreticular tissue, the incidence figures should reflect the number of animals with these lesions, not just the presence of the disease in individual organs.

iii. Cross-Reference Table

A cross-reference table that lists individual lesions on the vertical axis and individual animal numbers along the horizontal axis should be included, if possible. This is convenient both for reviewing lesions within an animal and for comparing lesions across animals in a group or among different groups.

iv. Animal Disposition Table

The report should generally contain an animal disposition table that provides the pathology accession number, sex, group designation, number of days on the study, and fate of the animals (for example, interim sacrifice, moribund sacrifice, found dead, or terminal sacrifice). This serves as a ready reference for the Agency's scientific reviewers and eliminates the need to develop this information from individual animal data.

v. Pathologist's Narrative

Finally, the report should include a section that specifically discusses the pathology data. This pathology narrative should provide an overview of the pathology findings from the study pathologist's perspective. A discussion that includes qualitative description of lesions and that highlights differences among treated and control groups is an essential part of the interpretation and evaluation of pathology data. The description of morphologic characteristics of lesions is particularly important where terminology may be controversial or misunderstood. Remarks about possible pathogenesis, strengthened by references to the scientific literature, could be an important part of the pathologist's narrative. Significant events, such as a disease outbreak during the study, and the impact of such events on the study outcome should be discussed. Differences in the incidence of key histopathologic findings among groups should be discussed; if observed differences are not regarded as treatment-related, then the basis for this conclusion should be provided.

d. Reference

1. Dua, P.N., and B.A. Jackson (1988). Review of Pathology Data for Regulatory Purposes. *Toxicologic Pathology.* **16:**443-450.

Links on this page:

1. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/default.htm

Redbook 2000: IV.B.4 Statistical Considerations in Toxicity Studies

July 2007

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter IV.B.4. Statistical Considerations in Toxicity Studies

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The regulations governing approval for marketing imply that submissions should contain both statistical analyses of toxicology data presented in the submission and documentation of the analyses. The purpose of this section is to guide the submitter in documenting statistical aspects of toxicity studies contained in food ingredient submissions so that CFSAN reviewers can evaluate these studies efficiently. Additional advice in the form of Standard Operational Procedures (SOPs) prepared by the Division of Mathematics of CFSAN's Office of Toxicological Sciences is available upon request from the CSO assigned to the petition.

To ensure the validity of safety assessments of a food ingredient obtained from well-conducted toxicity studies, statistical expertise should be used routinely in the planning, design, execution, analysis, and interpretation of results. This guideline highlights factors that are of primary importance in assessing the validity of evidence from toxicity studies. These factors are 1) study protocol and design, 2) presentation of collected data (individual animal data), 3) presentation and interpretation of analytical results (including tables of summary data), and 4) other considerations.

FDA emphasizes that communication between statisticians and the scientists conducting a particular toxicity study can help ensure that the statistics used are relevant to the biology of the toxicity test. For example, statistical outliers are not always biological outliers, and a "significant" statistical test ($p < 0.05$) does not always indicate biological significance. FDA encourages petitioners to consult with Agency statisticians during the design and conduct of the study and the interpretation of data from the study, as appropriate.

The following recommendations offer general guidance to the petitioner in organizing and documenting the results of toxicity studies:[(1)]

- Data should be submitted in a form that will enable FDA reviewers to easily verify the results by duplicating the analysis or, if necessary, performing an alternative analysis. The best way to accomplish this is to submit the data in tabular form in the petition and, at the same time, in a machine-readable form (see Chapter II.B. for additional information about submission of machine-readable data).
- Summary tables of the data also should be submitted.
- The submission should be organized and documented so as to enable Agency reviewers to move easily between the data and the summary tables. (For example, if the report of a bioassay involving 50 rats in a dose group includes a summary table indicating that the incidence of a given tumor is 3/40, there should be auxiliary tables showing which three rats had the tumor, which 37 rats were examined but did not have the tumor, and which ten rats were not examined for the tumor.)
- When outliers are removed for statistical reasons, the statistical test upon which the decision to remove them was based should be specified.
- The description of a statistical inference should include a statement about the model used, summary data appropriate for the model, analysis of the data with estimates of treatment effects, and reasonable statistical checks on the adequacy of the model.
- In presenting tables of summary data that reference statistical tests of hypotheses, a statement should identify the null and alternative hypotheses, the statistical test, the sampling distribution of the test statistic under the null hypothesis, the value of the test statistic, the degrees of freedom of the test statistic (when appropriate), the p-value, and whether the test is one or two tailed.
- Statistical analyses should be directly linked to specific questions regarding the safety of the additive (*i.e.*, comparing results for treated groups with results for a control group and evaluating the effects of various animal characteristics (sex, species, age, *etc.*) on the results of an experiment).
- Results of the statistical analyses of all toxicity studies (*e.g.*, p-values, confidence intervals) should be tabulated. Additionally, an effort should be made to explain how these results contribute to resolving questions about the safety of the food ingredient .
- The submission should cross-reference related information (*e.g.*, data tabulations, statistical hypotheses tested, models used, *etc.*) that will facilitate FDA's statistical review of the study.

a. Specific Statistical Issues

i. Study protocol and design

The submitted petition should contain the original protocol and a complete account of protocol modifications made during the course of the study. The protocol is a critical document in the evaluation of a bioassay, shaping both the conduct of the study and the ultimate analyses. It sets forth the objectives of the study and relates these objectives to the statistical hypotheses that are tested. It describes critical features of the study's design and execution, such as the purpose of the study, experimental design (subchronic, short-term, multi-generation), selection of species, selection of parameters to be assessed, planned interim analyses of data, planned interim and final sacrifices, events that would trigger early termination of the study, roles and responsibilities of data monitoring boards or quality assurance boards, and proposed statistical methods. By designating in advance the treatment groups and the variables that will be considered to be primary endpoints for statistical analyses, the protocol appropriately defines and limits the hypotheses that the study is able to test.

A well-designed experimental protocol will normally contain, as a minimum, the following items:

- **Statement of objectives:** In addition to the primary objective(s), secondary objective(s) should be stated explicitly. The precise hypotheses that the study is attempting to prove or disprove also should be stated explicitly.
- **Source of test animals:** A clear statement about the species, strain, sex and source of the test animals in the study and how animals are screened from the study (*i.e.*, will "runts" be eliminated; why?).
- **Experimental design:** This should include information about initial baseline periods (if any), the study configuration (short-term, lifetime, *etc.*), the treatment levels, the control group(s), the number of animals in each group (sample size), and the criteria for terminating the study.
- **Randomization procedures:** A description of the randomization procedure(s) used to assign animals to experimental groups. Generally, a computer-driven procedure using a random number generator is

better than a table of random numbers.

- **Administration:** A statement about the route of administration and frequency of administration of the test compound.
- **Diets:** A complete description of any diets used in the study.
- **Control of confounding factors:** A statement about how the effects of confounding response variables of interest (*i.e.*, caging effects) were minimized. If this is not possible, that fact should be stated along with the reason for the inability to discount these effects and the possible impact on the study.
- **Experimental parameters measured:** A description of the parameters that will be measured and a statement about how frequently they will be measured.
- **Power analysis:** If the number of animals being used is within the guidelines given in this publication for the type of study being planned, a power analysis is not necessary. If fewer numbers of animals are to be used, then a power analysis or a statement about the differences in study parameters between compared groups that the study is able to detect should be submitted.
- **Quality control:** A description of the steps taken to ensure accurate, consistent, and reliable data (*e.g.*, standard operating procedures, instruction manuals, data verification).
- **Data analysis:** A description of planned interim analyses of the data, including monitoring procedures, variables to be analyzed, statistical analyses to be used (including the choice of significance level for each interim analysis), and frequency of analysis.
- **Statistical Methods:** A description of the statistical methods to be applied to the data. Here, specific questions that the statistical analyses will address in support of the study objectives are identified. For example, a description of the methodology that would be used to detect outliers may be important. The major end-points for analysis should be identified. If multiple comparisons are to be made, they should be pre-planned.

ii. Presentation of collected data

Information on every animal in the study should be presented. Data should be organized so that the reviewer can easily find all information about any animal used in the study. For example, data should be organized so that the reviewer can view all study parameters for a single animal and a single parameter for all animals. Individual animal records can be presented or data can be tabulated, depending on the study and the type of data collected. The liberal use of data tables and submission of machine-readable data is strongly encouraged (see contact information [2] for electronic submissions). Steps taken to assure the numerical accuracy of the collected data should be documented in detail sufficient to permit the reviewer to judge their accuracy.

As described previously, the identifying number, age upon entry into the study, dose level, sex, initial body weight, and cage identification should be presented for each animal in the study. There also should be a table showing how animals were randomized into their respective dose groups. Other information should include:

- For each animal, length of time in the study, date of death, type of death (*e.g.*, scheduled sacrifice, moribund sacrifice, animal found dead, *etc.*), and reason for early withdrawal from the study, if this occurred (*e.g.*, escaped from cage).
- Food, water, and test compound consumption at each interval specified in the protocol.
- All measured values for defined parameters and the times at which these measurements were taken. If deviations from standard operating procedures occurred in taking the measurements, the nature of the deviation, the reason for the deviation, and its impact on the study should be discussed.
- For all microscopic lesions: a) type of lesions (neoplastic or non-neoplastic) should be clear from the morphologic diagnosis; b) when appropriate, severity grades (e.g., mild, moderate, marked) for non-neoplastic lesions should be included; c) when appropriate, modifiers such as "metastatic", "invasive" or "systemic" should be used for neoplastic lesions; and d) information indicating when the lesion was first observed (in life or at necropsy) should be included in the individual animal data.

iii. Presentation and interpretation of analytical results

Presentation of results of statistical analysis should include a description of, and rationale for, all statistical methods used. Unless the method is well-known (*e.g.*, analysis of variance), references should be provided. A thorough discussion of the statistical analysis, including reasons for the use of a particular analysis,

assumptions, conduct of the analysis, and validity of the conclusions, will guide FDA in deciding whether re-analysis of the data is needed. For each analysis of a relevant variable that is submitted, the following information should be provided:

- **Specific variables and analysis of variance:** A statement identifying the specific variable; if not obvious, a discussion of its relevance to the objectives of the study should be included.
- **Statistical model:** The statistical model underlying the analysis; references should be provided, if necessary.
- **Hypothesis:** A statement of the hypothesis being tested and of the alternative hypothesis.
- **Power calculation:** A power calculation for tests that failed to reject the null hypothesis, particularly to justify the adequacy of the sample size.
- **Confidence intervals:** The statistical methods used to estimate effects, construct confidence intervals, *etc.*; literature references should be supplied when appropriate.
- **Outliers:** The methods used to detect outlying data points (outliers) and the reasons why particular methods were selected. Identified outliers should be studied in an attempt to determine the reason for their deviation from other data in the set.
- **Assumptions underlying the statistical methods:** It should be shown that, insofar as is statistically reasonable, the data satisfy crucial assumptions, especially when such assumptions are necessary to confirm the validity of an inference. For example, in deciding whether to use parametric or non-parametric methods, tests for normality and for equality of variances should be conducted.
- **Survival analyses:** Such analyses will address the question of whether treated animals died earlier than control animals and will help determine if treated animals lived long enough to enable treatment-related tumors to be detected.
- **Analysis of tumors:** Analysis of tumors (benign and malignant) and other lesions for each group of test animals. Whether the tumor is an incidental finding upon death or a cause of death should dictate the method of analysis used. The major theoretical difference between these analyses is the manner in which the number of animals at risk in each time interval is defined. This needs to be taken into account in performing tests such as the standard Cox Life Table test.
- **Trend test:** A trend test, when appropriate. This includes not only a test for linearity, but a test for lack of fit as well.
- **Plots or graphs of summary data:** Care should be taken to generate plots that will convey the most information: For example, in studies with many animals in each dose group, it may be better to plot the mean and confidence limits or plus or minus one (±1) standard deviation than to attempt to plot individual data.

The following points are also important in the presentation of collected data:

- **Transformation of data:** Unnecessary data transformations should be avoided. If data transformation has been performed, a rationale for the transformation and an interpretation of the estimates of treatment effects based on transformed data should be provided.
- **Parametric and non-parametric analyses of data:** Parametric and non-parametric analyses of the same parameter at different time periods should be avoided. For example, if equality of variances in a parameter measured over time is tested, and some tests turn out significant and others do not, the statistician should arrive at a consensus (*i.e.*, does the preponderance of evidence point to equality of variances or not). We recommend that this be done by converting p-values obtained to standard normal deviates (z-scores) and obtaining the p-value for the average score times the square root of the number of p-values.
- **Litter and caging effects:** Litter and caging effects should be taken into account in determining the statistical model. If this is not possible, that fact should be stated along with the reason for the inability to account for these effects and its possible impact on the study.
- **Repetitive measurements:** For parameters that are measured across time, a repeated measures analysis should be considered.
- **Dependent experimental parameters:** If a given parameter depends biologically on another parameter (*i.e.*, organ weight depends on body weight), then the dependent parameter should be adjusted, as in analysis of co-variance.
- **Time of death:** Time of death should be reported as days from the start of the study. For example, if a study began on January 1, 1997 and the animal died on January 1, 1999, then the animal died on Day

730.

- **Reproduction studies:** In reproduction studies, if a dam continues in the study after all pups have died, the number of pups in her litter should be counted as 0.
- **Statistical comparisons:** When statistical comparisons of data were not pre-planned, a statement on how bias was avoided in choosing the particular analysis should be included.
- **Statistic:** The statistic, the sampling distribution of the test statistic under the null hypothesis, the value of the test statistic, the significance level (*i.e.*, p-value), a statement of whether the test used was one or two tailed, and intermediate summary data should be presented in a format that will enable the reviewer to verify the results of the analysis quickly and easily. In most cases, a copy of the computer output will provide the necessary information. For example, documentation of a two-sample t-test should include the two sample sizes, the mean and variance for each of the samples, the pooled estimate of variance, the value of the t-statistic, the associated degrees of freedom, and the p-value.
- **Computer programs:** When possible, commonly available computer programs should be used; please consult with FDA statisticians about appropriate programs. If it is necessary to use a program written by the petitioner itself, the program should be fully documented, including:
    - the source code;
    - test runs against "known" results; that is, textbook examples, examples worked by hand, or examples run with packaged programs. These test runs should cover every case that could arise in connection with the data in the petition. Test cases should be run both before and after the program is used for the submitted data.

iv. Support from CFSAN Statistical Reviewers

In the case of a complex toxicity test or carcinogenicity bioassay, the petitioner is encouraged to consult with CFSAN before conducting the study or submitting the petition to discuss relevant statistical considerations. Requests for comments by statistical reviewers on protocols for proposed toxicity studies can be sent to the CSO assigned to the petition (see Chapter II.A.).

If unusual concerns arise during the conduct of a study, the petitioner may submit preliminary tabulations of the data and materials pertaining to the statistical analysis to CFSAN for advice and guidance.

b. Statistical Considerations Reference

1. Dubey, Satya (June, 1985) Draft Guidelines for the Format and Content of the Statistical Sections of an Application. (Return to text)

Links on this page:

1. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/default.htm
2. http://www.fda.gov/~dms/opa-help.html

Draft

Chapter IV. Guidelines for Toxicity Tests

IV B General Recommendations for Toxicity Studies

IV B 5. Diets for Toxicity Studies

The effects of diet composition on the responses of experimental animals to xenobiotics have been reviewed. [1-6] Some of the most important effects include:

- Diet composition may influence experimental results through effects on background rates of toxicology parameters, such as tumor incidence. [7,8]

- Unrecognized or inadequately controlled nutritional and other dietary variables may alter the outcome and reproducibility of long-term toxicity studies.[8]

- A number of nutrients and non-nutritive dietary components have been shown to enhance or inhibit carcinogenesis; these include calories or energy, fat, protein, fiber, vitamins C and E, selenium, and lipotropes (methionine, choline, folacin, and vitamin B_{12}. Dietary fibers have been shown to reduce, enhance, or have no effect on the toxicity and carcinogenicity of chemicals. [9,10] Detailed reviews of the interactions of nutrients and carcinogens have been reported. [3,11-15]

a. Types of Diets

i. Natural Ingredient Diets

Natural ingredient diets are the most widely used diets in toxicology research. They are prepared from unrefined plant and animal materials such as wheat, corn, oats, fish meal, soybean meal, or wheat bran and are characterized as open formula or closed formula diets. The percentages of ingredients in open formula diets are known, but the composition of closed formula diets is proprietary information. [16] Natural ingredient diets support growth and reproduction and are economical, commercially available, and satisfactory for studies involving additives that will not affect nutrient balances.

Limitations of natural ingredient diets for toxicity studies include:

- Variations in types and quantities of nutrients and other dietary components are due to several factors; for example, the composition of fibers may vary with their sources, [17] the mineral content of natural ingredient diets can vary significantly among production batches, and specifications for essential dietary elements are not always met. [18]

- Diet composition cannot be altered to study the effects of varying a particular nutrient, which makes natural ingredient diets poor choices for research protocols in which nutrition may influence outcome.

- Nutrient excesses well beyond their requirements, and the presence of other non-nutrients substances in natural ingredient diets support rapid weight gain, pregnancy, and lactation in experimental animals and decrease the effects of many xenobiotics.

- Finally, common contaminants of natural ingredient diets that can alter the response of laboratory animals to experimental treatment include pesticides and mycotoxins. [13,19]

ii. Purified Diets

The use of purified diets has been recommended to avoid some of the limitations associated with the use of natural ingredient diets.[13,20,21] Purified diets usually contain refined proteins, carbohydrates, and fat. Vitamin and mineral mixtures including highly purified vitamins and inorganic salts also are added to purified diets. [16] AIN-76A, the most commonly used purified diet, [14] was formulated to provide a diet of known composition that was intended to meet the known nutrient requirements of rodents; it supports growth, reproduction (generally, one or two generations), and lactation in a manner similar to natural ingredient diets. [16]

Advantages of using purified diets for toxicity studies include:

- Ability to reproduce nutrient concentrations from batch to batch, to maintain the nutrient composition of a diet within a narrow range, and to alter the type and composition of dietary components. [20]

- Use of purified diets usually decreases dietary intake of contaminants such as pesticide residues, heavy metals, enzyme inducers and other agents that may alter the responses of test animals to experimental treatment. [14,20,21]

Disadvantages of using purified diets for toxicity studies include:

- Difficulty in assessing the impact of purified diets on animal survival and toxicology endpoints because adequate historical data regarding the use of such diets is lacking;

- Lack of information about the suitability of purified diets for long-term studies, although some researchers have used purified diets successfully for up to 56 weeks; [22,23]

- Errors that may occur in the preparation of purified diets may be more critical than similar errors in the preparation of natural ingredient diets because, in purified diets, each ingredient may be the sole dietary source of an essential nutrient. [20] In general, practical experiences with purified diets in long-term studies have not been satisfactory. [7,24-27]

b. Issues to Consider when Selecting and Preparing Diets for Animals in Toxicity Studies

The following are important issues to consider when selecting diets for animals in toxicity studies:

- Protein requirements for maintenance and growth of laboratory animals are well characterized, [20] but this is not true for most nutrients. Nutrient needs [28] and metabolism of xenobiotics [29,30] change with age. Hence, the general practice of feeding a single diet throughout the life cycle of experimental animals may be inappropriate--nutritional deficiencies may occur during phases of rapid growth and development in young animals and nutrient excess may occur in older animals.

- Individual ingredients in purified diets may cause problems in long-term studies. For example, purified diets high in ingredients such as casein and sucrose may stick to the hair of rodents and cause excessive grooming. Purified sugars as the sole source of carbohydrates in diets that are low in dietary fiber may cause diarrhea, resulting in problems of digestion and absorption of other nutrients.

- For reasons that are incompletely understood, animals may not reproduce well when fed purified diets. The components in natural ingredient diets that are required to support reproduction have not been defined.

- Toxic chemicals in the diet and induced nutrient deficiencies can lead to decreased food intake by experimental animals and reduced rates of growth and development. When such an effect is expected to occur in a long-term study, pair-feeding can be used to eliminate differences in food intake among

experimental groups; this is the preferred method for ensuring that differences in energy or nutrient intake have not caused the observed experimental results or complicated their interpretation. For example, a moderate restriction of energy intake may increase the life-span, decrease the background cancer rates, and decrease the potency of carcinogens in rodents, thereby potentially modulating the action of a chemical carcinogen. When pair-feeding studies are recommended to eliminate differences in food intake among experimental groups, animals should be single-caged and food consumption should be carefully and accurately determined for each animal in the study.

- When the test substance is added to the diet, accurate records of food consumption must be maintained to determine the administered dose and food intake must be equalized across control and experimental groups of animals. When the test substance is a carbohydrate, protein, or fiber that will be added to the diet in large quantities, it must replace a dietary ingredient or the nutrient and energy contents of the diet will be significantly diluted (see **Chapter VII B 1**). The nutrient and energy contents of control diets also must be adjusted to match those of experimental diets. One recommended strategy is to make the control and test diets isocaloric. If food consumption among groups of experimental animals has been equalized, then equal densities of metabolizable energy in the diets will equalize nutrient intake across the groups. [31]

- When oil is used as the gavage vehicle for fat-soluble test substances, the necessity of including a vehicle-control group in the study may introduce some problems. [32] If the quantity of oil administered daily by gavage contributes significantly to the total dietary energy of the animals, results for experimental and vehicle-control groups may be significantly different than results for the untreated control group. If a decision is made to administer a test substance by gavage, the volume of oil given as a vehicle should be limited to 0.3 to 0.4 ml/100 g of body weight and the use of a low-fat diet should be considered.

- Related issues are discussed in the following chapters: 1) control diets for test animals in **Chapter IV B 1 b-c**; 2) survivorship and recommendations concerning the duration of carcinogenicity bioassays in **Chapter IV C 6 a**; and 3) nutritional concerns for food substitutes (macro-additives) in **Chapter VII B**.

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Redbook 2000: IV.C.1 Short-Term Tests for Genetic Toxicity

July 2007

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter IV.C.1. Short-Term Tests for Genetic Toxicity

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Genetic changes known to be associated with adverse human health effects include gene mutations, chromosomal rearrangements or deletions, and loss or gain of whole chromosomes (aneuploidy) or chromosomal segments. Genotoxicity tests are *in vitro* and *in vivo* tests designed to detect compounds that induce genetic damage. Such tests include: (1) tests that directly assess the key types of genetic alterations (gene mutations and chromosomal effects) and (2) indirect genotoxicity tests that respond to types of DNA damage known to lead to these alterations. The latter category of tests may assess either DNA damage (*e.g.*, DNA adducts or DNA strand breakage) or cellular responses to DNA damage (*e.g.*, unscheduled DNA synthesis).

FDA recommends the use of a battery of short-term genetic toxicity tests for all when the cumulative estimated dietary intake exceeds 1.5 µg per person per day, corresponding to 0.5 parts per billion (ppb) in the total diet. The recommended tests directly measure gene mutations and/or chromosomal effects. The Agency uses such data, in the absence of long-term animal feeding studies, to determine whether or not a chemical should be considered to be a possible carcinogen. Such data may also indicate whether a chemical may have adverse heritable effects. When long-term animal feeding studies are available for the evaluation of carcinogenicity, genetic toxicity data may assist in the interpretation of the results of such studies.

Genetic Toxicity Test Battery

We consider it essential that chemicals be evaluated for their ability to induce both gene mutations and chromosomal aberrations. The most widely used test for gene mutations is performed using bacteria as the target cells. Tests for chemicals that induce gene mutations can also be performed in mammalian cells grown *in vitro*. Tests that detect the induction of chromosomal aberrations are performed using cells exposed to chemicals *in vitro* or *in vivo*. While the recommended battery consists of specific genetic toxicity tests, data from other systems that measure gene mutations, chromosomal effects, DNA damage, or cellular responses to DNA damage may be relevant to the overall genotoxicity evaluation of a chemical. Therefore, all available data relating to such endpoints in any test system should be submitted.

Recommended Tests

The recommended genetic toxicity test battery for food ingredients whose cumulative estimated daily intake exceeds 50 ppb in the diet (150 µg per person per day) generally includes:

a. a test for gene mutations in bacteria [2]

and

b. an *in vitro* test with cytogenetic evaluation of chromosomal damage using mammalian cells[3]
or
an *in vitro* mouse lymphoma thymidine kinase$^{+/-}$ gene mutation assay[4]
(the mouse lymphoma assay is preferred)
and

c. an *in vivo* test for chromosomal damage using mammalian hematopoietic cells[5].

The Agency prefers the mouse lymphoma tk$^{+/-}$ assay in item "b" because this assay measures heritable genetic damage arising by several mechanisms in living cells and is capable of detecting chemicals that induce either gene mutations or heritable chromosomal events, including genetic events associated with carcinogenesis. In performing the mouse lymphoma tk$^{+/-}$ assay, either the soft agar or the microwell method is acceptable.

When the cumulative estimated daily intake of a food ingredient is 50 ppb or less but greater than 0.5 ppb, then the recommended genetic toxicity test battery generally includes items "a" and "b" in the above list since there are few chemicals that are uniquely genotoxic when tested *in vivo* for chromosomal damage.

Guidance for performing a test for gene mutations in bacteria[6] and an *in vivo* micronucleus assay[7] (which is an acceptable *in vivo* test for chromosomal damage using mammalian hematopoietic cells) is presented here. This guidance is based directly on the guidelines published by the Organization for Economic Cooperation and Development[8] (OECD) or those published by the United States Environmental Protection Agency[9] (US EPA), which are virtually identical to each other. The reports of the International Conference on Ha rmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use[10] (ICH) were used as the basis for the general recommendations for genetic toxicity testing and were also referred to in drafting the guidelines on specific test systems. The guidance for an *in vivo* test for chromosomal damage using mammalian hematopoietic cells (micronucleus assay) was also based on the report of the 1998 International Workshop on Genotoxicity Test Procedures (Hayashi, *et al.*, *Environmental and Molecular Mutagenesis*, 2000, in press). The FDA has modified these previous guidelines as appropriate. For specific guidance on the conduct of genotoxicity tests for which guidance is not included here, the above referenced ICH, OECD, or US EPA guidelines should be consulted.

Types of Genetic Damage

The mutation assays specified in the standard battery are capable of detecting different spectra of genetic damage. The tests for gene mutations in bacteria detect point mutations which involve substitution, addition, or deletion of one or a few DNA base pairs. Point mutations may also be detected in *in vitro* mammalian cell mutagenicity assays. The mouse lymphoma tk$^{+/-}$ assay specified in the battery also detects large deletions, translocations, mitotic recombination/gene conversion and aneuploidy in addition to point mutations, making this the assay with the broadest spectrum of detectable genetic damage in the battery. The *in vitro* test with cytogenetic evaluation of chromosomal damage using mammalian cells detects structural chromosomal aberrations. The *in vivo* tests for chromosomal damage using mammalian hematopoietic cells detect structural chromosomal aberrations in the case of the mammalian bone marrow chromosome aberration test and structural damage to chromosomes or damage to the mitotic apparatus in the case of the mammalian erythrocyte micronucleus test.

It should also be noted that, although the mouse lymphoma tk$^{+/-}$ assay and the *in vitro* test with cytogenetic evaluation of chromosomal damage using mammalian cells both detect structural chromosomal damage, the mouse lymphoma tk$^{+/-}$ assay detects damage that is heritable but not lethal to the cells, while the *in vitro* test with cytogenetic evaluation of chromosomal damage detects additional types of damage that are lethal to the cells. Additionally, the mouse lymphoma tk$^{+/-}$ assay is the only assay in the battery capable of detecting gene conversion/mitotic recombination.

Thus, the tests included in the battery were chosen to complement each other in terms of the specific types of genetic damage detected. It is not expected that chemicals will always give uniformly positive or negative results in the various assays. A chemical may, for example, be positive only in the bacterial mutagenicity assay. Such a result may, nevertheless, be relevant to potential human health effects, including carcinogenicity. Conversely, it has been shown that some chemicals are negative in the bacterial assay but positive in other assays. These chemicals are likely operating by mechanisms that cause chromosomal mutations (large deletions, translocations, gene conversion/mitotic recombination and/or aneuploidy), while the bacterial assay detects only point and other very small-scale gene mutations.

In addition to the mouse lymphoma $tk^{+/-}$ assay, other *in vitro* mammalian gene mutation assays, which employ other cell lines including the CHO, AS52 and V79 lines of Chinese hamster cells and the TK6 human lymphoblastoid cells, are sometimes used. In these cell lines the most commonly used genetic endpoints measure mutation at either hypoxanthine-guanine phosphoribosyl transferase (*hprt*), a transgene of xanthine-guanine phosphoribosyl transferase (*xprt*), or thymidine kinase (*tk*). The *tk*, *hprt*, and *xprt* mutation tests detect different spectra of genetic events. Differences in sequences in the genes will lead to different spectra of point mutations that can be detected. In addition, the autosomal location of *tk* and *xprt* genes appears to allow for the detection of genetic events (*e.g.*, chromosomal exchange events) that are not detected at the *hprt* locus on the X-chromosome. This is because genetic damage that involves essential genes adjacent to the *hprt* locus on the X-chromosome will likely be lethal to the cell, while damage to essential genes on an autosome will be compensated for by intact genes on the homologous chromosome (which lacks functional *tk* or *xprt*). Also, the lack of a homologous chromosome in the case of the *hprt* gene may preclude mutations that arise *via* homologous recombination. The *tk* locus in mouse lymphoma cells (the mouse lymphoma $tk^{+/-}$ assay) is the preferred target for mammalian cell genotoxicity assays because of the wealth of data which exists to support this locus and assay and because of the broad spectrum of damage detected at this locus.

Modifications of the Test Battery

a. Limited Effectiveness of Bacterial Tests

There are circumstances where the performance of the bacterial reverse mutation test does not provide sufficient information for the assessment of genotoxicity. This may be the case for compounds that are highly toxic to bacteria (*e.g.*, some antibiotics) and compounds thought or known to interfere with mammalian cell-specific systems (*e.g.*, topoisomerase inhibitors, nucleoside analogues, or certain inhibitors of DNA metabolism). In these cases, usually two *in vitro* mammalian cell tests should be performed using two different cell types and two different endpoints, *i.e.*, gene mutation and chromosomal damage. Test approaches currently accepted for the assessment of mammalian cell gene mutation include tests for mutation: 1) at the *tk* locus using mouse lymphoma L5178Y cells or human lymphoblastoid TK6 cells; 2) at the *hprt* locus using CHO cells, V79 cells, or L5178Y cells; or 3) at the *gpt* locus using AS52 cells. When such additional tests are performed because of the high level of toxicity of the test chemical to bacteria, it is still important to perform the bacterial reverse mutation test because some antibacterial agents, albeit highly toxic to the tester strains, are genotoxic at very low, sub-lethal concentrations in the bacterial reverse mutation test (*e.g.*, nitrofuran antibiotics).

b. Compounds bearing structural alerts for genotoxic activity

Structurally alerting compounds are usually detectable in the standard test battery. However, compounds bearing structural alerts that have given negative results in the standard test battery may necessitate limited additional testing. The choice of additional test(s) or protocol modification(s) depends on the chemical nature, the known reactivity and metabolism data on the structurally alerting compound under question. For some classes of compounds with specific structural alerts, it is established that protocol modifications/additional tests are necessary for optimum detection of genotoxicity (*e.g.*, molecules containing an azo-group requiring testing of azo reduction products, glycosides requiring testing of hydrolysis products, compounds such as nitroimidazoles requiring nitroreduction for activation, compounds such as phenacetin requiring another rodent S9 for metabolic activation). When the standard test battery gives negative results with a chemical that falls within a class known to require special test conditions, then additional testing with appropriate test modifications should be performed.

c. Limitations to the use of standard *in vivo* tests

There are compounds for which standard *in vivo* tests do not provide additional useful information. This includes compounds for which data from studies on toxicokinetics or pharmacokinetics indicate that they are not systemically absorbed and therefore are not available for the target tissues in standard *in vivo* genotoxicity tests. In cases where sufficient target tissue exposure cannot be achieved, it may be appropriate to base the evaluation only on *in vitro* testing.

d. Evidence for tumor response

Additional genotoxicity testing in appropriate models may be conducted for food ingredients that were negative in the standard test battery but which have shown effects in carcinogenicity bioassay(s) with no clear evidence for a non-genotoxic mechanism. To help understand the mechanism of action, additional testing can

include modified conditions for metabolic activation in *in vitro* tests or can include *in vivo* tests measuring genetic damage in target organs of tumor induction (*e.g.*, liver UDS test, ^{32}P-postlabelling, mutation induction in transgenes, or molecular characterization of genetic changes in tumor-related genes).

e. Structurally unique chemical classes

On rare occasions, a completely novel compound in a unique structural chemical class will be introduced. When such a compound will not be tested in chronic rodent carcinogenicity bioassays, more extensive genotoxicity evaluation may be indicated.

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Redbook 2000: IV.C.1.a Bacterial Reverse Mutation Test

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This guidance represents the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

I. Purpose

A. The bacterial reverse mutation test uses amino acid-requiring strains of *Salmonella typhimurium* (*S. typhimurium*) and *Escherichia coli* (*E. coli*) to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs.[(3),(9),(16)] The principle of this bacterial reverse mutation test is that it detects chemicals that induce mutations which revert mutations present in the tester strains and restore the functional capability of the bacteria to synthesize an essential amino acid. The revertant bacteria are detected by their ability to grow in the absence of the amino acid required by the parent tester strain.

B. Point mutations are the cause of many human genetic diseases and there is substantial evidence that point mutations in oncogenes and tumor suppressor genes of somatic cells are involved in tumor formation in humans and experimental animals. The bacterial reverse mutation test is rapid, inexpensive and relatively easy to perform. Many of the tester strains have several features that make them more sensitive for the detection of mutations, including responsive DNA sequences at the reversion sites, increased cell permeability to large molecules and elimination of DNA repair systems or enhancement of error-prone DNA repair processes. The specificity of the tester strains can provide some useful information on the types of mutations that are induced by genotoxic agents. A very large data base of results for a wide variety of chemical structures is available for bacterial reverse mutation tests and well-established procedures have been developed for testing chemicals with different physicochemical properties, including volatile compounds.

II. Definitions

Reverse mutation test in either *Salmonella typhimurium* or *Escherichia coli* detects mutation in an amino acid requiring strain (histidine or tryptophan, respectively) to produce a strain whose growth is independent of an outside supply of the amino acid.

Point mutations are changes in one or a small number of base pairs in a DNA sequence. Point mutations may result from base pair substitutions or from small insertions or deletions.

Base pair substitution mutagens are agents that cause a base change in DNA. In a reversion test this change may occur at the site of the original mutation, or at a second site in the bacterial genome.

Frameshift mutagens are agents that cause the addition or deletion of one or more base pairs in the DNA, thus changing the reading frame in the RNA.

III. Initial Considerations

A. The bacterial reverse mutation test utilizes prokaryotic cells, which differ from mammalian cells in such factors as uptake, metabolism, chromosome structure and DNA repair processes. Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. *In vitro* metabolic activation systems cannot mimic entirely the mammalian *in vivo* conditions. The test therefore does not provide direct information on the mutagenic and carcinogenic potency of a substance in mammals.

B. The bacterial reverse mutation test is commonly employed as an initial screen for genotoxic activity and, in particular, for point mutation-inducing activity. An extensive data base has demonstrated that many chemicals that are positive in this test also are genotoxic in other tests. There are examples of mutagenic agents which are not detected by this test; reasons for this shortcoming can be ascribed to the specific nature of the endpoint detected, differences in metabolic activation, or differences in bioavailability.

C. There are circumstances in which the bacterial reverse mutation test may not provide sufficient information for the assessment of genotoxicity. This may be the case for compounds that are excessively toxic to bacteria (*e.g.*, some antibiotics) and compounds thought or known to interfere with the mammalian cell replication system (*e.g.*, topoisomerase inhibitors, nucleoside analogues, or inhibitors of DNA metabolism). For these cases, usually two *in vitro* mammalian cell tests should be performed using two different cell types and two different endpoints, *i.e.*, gene mutation and chromosomal damage (as discussed in section a. under "Modifications of Test Battery," in IV.C.1.[2]). Nevertheless, it is still important to perform the bacterial reverse mutation test.

D. Although most compounds that are positive in this test are mammalian carcinogens, the correlation is not absolute; it varies with chemical class. There are carcinogens that are not detected by this test because they act through other, presumably nongenotoxic mechanisms or mechanisms absent in bacterial cells or fail because of inadequate metabolic activation.

IV. Test Method

A. Principle

1. Bacterial mutagenicity tests are generally conducted using one of two basic methods. In both of these procedures, bacterial cultures are exposed to the test substance in the presence and in the absence of an exogenous metabolic activation system. In the plate incorporation method,[(3),(9),(14),(16)] these components are combined in molten overlay agar and plated immediately onto minimal agar medium. In the preincubation method,[(2),(8),(9),(16),(18),(34)] the treatment mixture is incubated and then mixed with the overlay agar before plating onto minimal agar medium. For both techniques, after 2 or 3 days of incubation, revertant colonies are counted and compared to the number of spontaneous revertant colonies on solvent control plates.
2. Several procedures for performing the bacterial reverse mutation test have been described in addition to the plate incorporation method and the preincubation method. These additional procedures include the fluctuation method,[(10),(12)] and the suspension method.[(31)] Suggestions for procedures for the testing of gases or vapors have also been described.[(4)]
3. The procedures described in this document pertain primarily to the plate incorporation and preincubation methods. Either method is acceptable for conducting experiments both with and without metabolic activation, although some compounds may be detected more efficiently using the preincubation method. These compounds belong to chemical classes that include short chain aliphatic nitrosamines, divalent metals, aldehydes, azo dyes and diazo compounds, pyrollizidine alkaloids, allyl compounds and nitro compounds.[(9)] It is also recognized that certain classes of mutagens are not always detected using standard procedures such as the plate incorporation method or preincubation method. These should be regarded as "special cases" and it is strongly recommended that alternative procedures be used for their detection. The following "special cases" could be identified (together with literature citations describing examples of procedures that could be used for their detection): azo dyes and diazo compounds,[(9),(18),(26),(34)] gases and volatile chemicals,[(4),(13),(21),(28),(35)] and glycosides.[(5),(20),(23),(30)] Deviations from standard procedures need to be scientifically justified. In the cases of azo compounds (which are reduced in the intestine to free aromatic amines) and glycosides (which are hydrolyzed in the intestine to a sugar and an aglycone), it is preferable to test the free aromatic amine or aglycone metabolites, if available, by standard techniques rather than using the modified methods in the references cited above.
4. There are cases in which test substances derived from plant or animal tissues may contain amino acids (histidine in the case of the *S. typhimurium* tester strains and tryptophan for the *E. coli* WP2 strains), or

peptides that can serve as a source of these amino acids, at levels that interfere with the conduct of these standard mutation assay procedures.[1],[27] While there are alternative bacterial mutagenicity testing procedures that are not affected by the presence of amino acids in test samples (*e.g.*, see references[11],[22],[24],[29]), such procedures have not been standardized, widely used, and well validated. If a test substance derived from biological material causes an increase in mutant colonies in a bacterial mutagenicity test, the possibility that such an increase may be due solely to the presence of histidine or tryptophan in the test substance should be evaluated. Experiments designed for such an evaluation might involve, for example, the testing of amino acid-free extracts of the test substance, with appropriate controls to show that the procedures used are capable of detecting mutagens added to the test substance.

B. Description

1. Preparations

a. Bacteria

i. Fresh cultures of bacteria should be grown up to the late exponential or early stationary phase of growth (approximately 10^9 cells per ml). Cultures in late stationary phase should not be used. Excessive aeration of overnight cultures should be avoided. It has been recommended that overnight shaking of cultures in flasks not exceed 120 rpm.[16] The cultures used in the experiment should contain a high titer of viable bacteria. The titer may be demonstrated either from historical control data on growth curves, or in each assay through the determination of viable cell numbers by a plating experiment.

ii. The culture temperature should be 37°C.

iii. At least five strains of bacteria should be used. These should include four strains of *S. typhimurium* (TA1535; TA1537 or TA97a or TA97; TA98; and TA100) that have been shown to be reliable and reproducibly responsive among laboratories. These four *S. typhimurium* strains have GC base pairs at the primary reversion site and it is known that they may not detect certain oxidizing mutagens, crosslinking agents and hydrazines. Such substances may be detected by *E. coli* WP2 strains or *S. typhimurium* TA102[33] which have an AT base pair at the primary reversion site. Therefore the recommended combination of strains is:

- *S. typhimurium* TA1535
- *S. typhimurium* TA1537 or TA97 or TA97a
- *S. typhimurium* TA98
- *S. typhimurium* TA100
- *E. coli* WP2 *uvr*A, or *E. coli* WP2 *uvr*A (pKM101), or *S. typhimurium* TA102.

If there is reason to believe that the test substance may be a crosslinking mutagen, then the test battery should include strain TA102, or a DNA repair-proficient strain of *E. coli* (*e.g.*, *E. coli* WP2 or *E. coli* WP2 (pKM101)) should be added.

iv. Established procedures for stock culture preparation, marker verification and storage should be used. The amino acid requirement for growth should be demonstrated for each frozen stock culture preparation (histidine for *S. typhimurium* strains, and tryptophan for *E. coli* strains). Other phenotypic characteristics should be similarly checked, namely: the presence or absence of R-factor plasmids where appropriate (*i.e.*, ampicillin resistance in strains TA98, TA100, TA97a, TA97, and WP2 *uvr*A (pKM101), and ampicillin + tetracycline resistance in strain TA102); the presence of characteristic mutations (*i.e., rfa* mutation in *S. typhimurium* through sensitivity to crystal violet, and *uvr*A mutation in *E. coli* or *uvr*B mutation in *S. typhimurium*, through sensitivity to ultraviolet light).[9],[16] The strains should also yield spontaneous revertant colony counts within the frequency ranges expected from the laboratory's historical control data and preferably within the range reported in the literature.

b. Medium

An appropriate minimal agar (*e.g.*, containing Vogel-Bonner minimal medium E and glucose) and an overlay agar containing histidine and biotin (for *S. typhimurium*) or tryptophan (for *E. coli*), to allow for a few cell divisions, should be used.[3],[10],[16]

c. Metabolic Activation

Bacteria should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a cofactor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents (usually rats) treated with enzyme-inducing agents such as Aroclor 1254[3],[16] or a combination of phenobarbitone and beta-naphthoflavone.[7],[19],[25],[30] The post-mitochondrial supernatant fraction is usually used at concentrations in the range from 10 to 30 percent v/v in the S9 mix. The choice and concentration of a metabolic activation system may depend upon the class of chemical being tested. In some cases it may be appropriate to utilize more than one concentration of post-mitochondrial fraction. For azo dyes and diazo compounds, using a reductive metabolic activation system may be more appropriate.[18],[26]

Liver S9 should be prepared using aseptic techniques so that subsequent filter-sterilization is not required. Filtration of the S9 or S9 mix may lead to loss of enzyme activity.[16] Each batch of S9, whether produced by the testing laboratory or obtained commercially, should be tested for sterility and discarded if contaminated.

d. Test Substance/Preparation

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted as appropriate prior to treatment of the bacteria. Liquid test substances may be added directly to the test systems and/or diluted prior to treatment. Fresh preparations should be employed unless stability data demonstrate the acceptability of storage.

2. Test Conditions

a. Solvent/Vehicle

The solvent/vehicle should not be suspected of chemical reaction with the test substance and the concentration used should be compatible with the survival of the bacteria and the S9 activity.[17] If other than well-established solvent/vehicles are used, their inclusion should be supported by data indicating their compatibility. It is recommended that wherever appropriate, an aqueous solvent/vehicle be used. When testing water-unstable substances, the organic solvents used should be free of water.

b. Exposure Concentrations

i. Among the criteria to be taken into consideration when determining the highest amount of test substance to be used are cytotoxicity and solubility in the final treatment mixture. It may be useful to determine toxicity and insolubility in a preliminary experiment. Cytotoxicity may be detected by a reduction in the number of revertant colonies or by a clearing or diminution of the background lawn. However, preliminary toxicity tests in which survival of cells in diluted cultures is determined may give erroneous results.[32] The cytotoxicity of a substance may be altered in the presence of metabolic activation systems.

If the doses of the test substance are limited by toxicity, then toxicity should be evident in all preliminary and final assays at one or more doses, and no toxicity should be evident at three or more doses in each assay, in each bacterial strain, both with and without metabolic activation. Insolubility should be assessed as precipitation in the final mixture under the actual test conditions and evident to the unaided eye in the tube or on the plate. The recommended maximum test concentration for soluble noncytotoxic substances is 5 mg/plate or 5 µl/plate. For noncytotoxic substances that are not soluble at 5 mg/plate or 5 µl/plate, one or more concentrations tested should be insoluble in the final treatment mixture. Test substances that are cytotoxic below 5 mg/plate or 5 µl/plate should be tested up to a cytotoxic concentration. If precipitate is present on any of the plates, it may interfere with automatic counting of the colonies. In such a situation, all plates in that series of doses and controls should be counted by hand.

In some cases, toxic levels of a test chemical may kill almost all the cells but permit those that survive to utilize the histidine in the medium and to grow into visible colonies, even though they have not undergone mutations from histidine-requiring (His^-) to histidine-independent (His^+) or, in the case of *E. coli*, from tryptophan-requiring (Trp^-) to tryptophan independent (Trp^+). This phenomenon may result in an increase in colony counts at one or more toxic doses although the chemical may not be mutagenic. In such cases, careful observation of the plates will usually reveal a clear or almost clear background lawn and unusually small "pinpoint" colonies resulting from toxicity. When the nature of such colonies remains in question, representative colonies from the plates of interest can be streaked onto minimal agar plates (supplemented with biotin (for *Salmonella*) but not histidine or tryptophan); colonies from solvent control plates are also streaked

as controls. If the cells streaked from the questionable plates do not grow into colonies and those streaked from the solvent control plates do grow, then it can be concluded that the questionable colonies seen were made up of His^- (or Trp^-) cells and that the increase in colony counts is not an indication of mutagenicity of the test chemical. If the cells do grow, this demonstrates that they were mutants and that the chemical is mutagenic.

ii. At least five different analyzable concentrations of the test substance should be used with approximately half log (*i.e.,* 10) intervals between test points for an initial experiment. Smaller intervals may be appropriate when a concentration-response is being investigated.

iii. Testing above the concentration of 5 mg/plate or 5 µl/plate may be considered when evaluating substances containing substantial amounts of potentially mutagenic impurities.

c. Controls

i. Concurrent negative (solvent or vehicle) and strain-specific positive controls, both with and without metabolic activation, should be included in each assay. Positive control chemicals and concentrations that demonstrate the effective performance of each assay should be selected.

ii. For assays employing a metabolic activation system, the positive control reference substance(s) should be selected on the basis of the type of bacteria strains used. The following chemicals are examples of suitable positive controls for assays with metabolic activation:

Chemical	CAS Number
9,10-Dimethylanthracene	781-43-1
7,12-Dimethylbenzanthracene	57-97-6
Congo Red (for the reductive metabolic activation method)	573-58-0
Benzo(a)pyrene	50-32-8
2-Acetamidofluorene	53-96-3
Cyclophosphamide (monohydrate)	50-18-0 (6055-19-2)
2-Aminoanthracene*	613-13-8

*2-Aminoanthracene should not be used as the sole indicator of the efficacy of the S9 mix. If 2-aminoanthracene is used, each batch of S9 should also be characterized with a mutagen that requires metabolic activation by microsomal enzymes, *e.g.*, benzo(a)pyrene, dimethylbenzanthracene.

iii. For assays performed without metabolic activation system, examples of strain-specific positive controls are:

Chemical	CAS Number	Strain
Sodium azide	26628-22-8	TA1535 and TA100
Nitrofurantoin	67-20-9	TA100
2-Nitrofluorene or 4-nitro- 1,2-phenylenediamine	607-57-8 or 99-56-9	TA 98
9-Aminoacridine or ICR 191	90-45-9 or 17070-45-0	TA1537, TA97 and TA97a
Cumene hydroperoxide	80-15-9	TA102
Mitomycin C	50-07-7	WP2 *uvr*A and TA102
N-Methyl-N'-nitro- N-nitrosoguanidine or 4-nitroquinoline 1-oxide	70-25-7 or 56-57-5	WP2, WP2 *uvr*A and WP2 *uvr*A (pKM101)
Furylfuramide (AF-2)	3688-53-7	Plasmid-containing strains

iv. Other appropriate positive control reference substances may be used. The use of chemical class-related positive control chemicals may be considered, when available.

v. Negative controls, consisting of solvent or vehicle alone, without test substance, and otherwise treated in the same way as the treatment groups, should be included. In addition, untreated controls should also be used unless there are historical control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent.

C. Procedure

1. Treatment with Test Substance

a. For the plate incorporation method,[3],[9],[14],[16] without metabolic activation, usually 0.05 ml or 0.1 ml of the test solutions and 0.1 ml of fresh bacterial culture (containing approximately 10^8 viable cells) are mixed with 2.0 ml of overlay agar (0.5 ml of sterile buffer may also be included). For the assay with metabolic activation, usually 0.5 ml of metabolic activation mixture containing an adequate amount of post-mitochondrial fraction (in the range from 10 to 30 percent v/v in the metabolic activation mixture) are mixed with the overlay agar (2.0 ml), together with the bacteria and test substance/test solution. The contents of each tube are mixed and poured over the surface of a minimal agar plate. The overlay agar is allowed to solidify before incubation.

b. For the preincubation method,[9],[16],[18],[34] the test substance/test solution (usually 0.05 ml or 0.1 ml) is preincubated with the tester strain (0.1 ml, containing approximately 10^8 viable cells) and sterile buffer (0.5 ml) or the metabolic activation system (0.5 ml) usually for 20 min. or more at 30-37°C prior to mixing with the overlay agar (2.0 ml) and pouring onto the surface of a minimal agar plate. Tubes are usually aerated during preincubation by using a shaker.

c. For an adequate estimate of variation, triplicate plating should be used at each dose level. The use of duplicate plating is acceptable when scientifically justified. The occasional loss of a plate does not necessarily invalidate the assay.

d. Gaseous or volatile substances should be tested by appropriate methods, such as in sealed vessels.[4],[13],[28],[35]

2. Incubation

All plates in a given assay should be incubated at 37°C for 2 or 3 days. After the incubation period, the number of revertant colonies per plate is counted.

V. Data and Reporting

A. Treatment of Results

1. Data should be presented as the number of revertant colonies per plate. The number of revertant colonies on both negative (solvent control, and untreated control if used) and positive control plates should also be given.
2. Individual plate counts, the mean number of revertant colonies per plate and the standard deviation should be presented for each dose of the test substance and positive and negative (untreated and/or solvent) controls.
3. There is no need to verify a clear positive response. Marginally or weakly positive results should be verified by additional testing. An attempt should be made to clarify repeatedly equivocal results by further testing using a modification of experimental conditions. Study parameters that might be modified include the concentration spacing, the method of treatment (plate incorporation or liquid preincubation), and metabolic activation conditions such as the mammalian source species for the S9 or the concentration of S9 in the S9 mix. Nevertheless, it is recognized that results may remain equivocal or questionable even after repeat testing with modified protocols.

   The results of a range-finding test may supply sufficient data to provide reassurance that a reported clearly negative result is correct. Preliminary range-finding tests performed on all bacterial strains, with and without metabolic activation, with appropriate positive and negative controls, and with quantification of mutants, may be considered a sufficient replication of a subsequent complete test whose results are clearly negative. Alternatively, if negative results are to be confirmed by additional complete testing, modification of protocols, as described above for repeats of equivocal tests, is recommended.

B. Evaluation and Interpretation of Results

1. There are several criteria for determining a positive result, such as a concentration-related increase over the range tested and/or a reproducible increase at one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation system.[6] Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results.[15] However, statistical significance should not be the only determining factor for a positive response.
2. A test substance for which the results do not meet the above criteria is considered nonmutagenic in this

test.

3. Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.
4. Positive results from the bacterial reverse mutation test indicate that a substance induces point mutations by base substitutions and/or frameshifts in the genome of either *Salmonella typhimurium* and/or *Escherichia coli*. Negative results indicate that under the test conditions, the test substance is not mutagenic in the tested species.

C. Test Report

The test report should include the following information:

1. Test Substance

- Identification data, including name and CAS no., if known.
- Physical nature and purity.
- Physicochemical properties relevant to the conduct of the study.
- Stability of the test substance, if known.

2. Solvent/Vehicle

- Justification for choice of solvent/vehicle.
- Solubility and stability of the test substance in solvent/vehicle, if known.

3. Dosing Solutions

- Times dosing solutions were prepared and used (or interval between preparation and usage), and storage conditions.
- Data that verify the concentration of the dosing solution, if available.

4. Strains

- Strains used.
- Number of cells per culture.
- Strain characteristics.

5. Test Conditions

- Amount of test substance per plate (µg/plate, mg/plate, or µl/plate) with rationale for selection of dose and number of plates per concentration.
- Media used.
- Source, type and composition of metabolic activation system, including concentration of S9 in S9 mix and acceptability criteria.
- Treatment procedures.

6. Results

- Signs of toxicity.
- Signs of precipitation.
- Individual plate counts.
- The mean number of revertant colonies per plate and standard deviation.
- Dose-response relationship, where possible.
- Statistical analyses, if any.
- Concurrent negative (solvent/vehicle) and positive control data, with ranges, means and standard deviations.

- Historical negative (solvent/vehicle) and positive control data, with *e.g.*, ranges, means and standard deviations.

7. Discussion of the results.

8. Conclusion.

VI. References

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1. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/default.htm
2. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/ucm078321.htm

Redbook 2000: IV.C.1.b In vitro Mammalian Chromosomal Aberration Test

November 2003

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter IV.C.1.b. *In vitro* Mammalian Chromosomal Aberration Test

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I. Introduction

The purpose of the *in vitro* chromosomal aberration test is to identify agents that cause structural chromosomal aberrations in cultured mammalian cells [1, 2, 3]. Structural aberrations may be of two types, chromosome or chromatid. With the majority of chemical mutagens, induced aberrations are of the chromatid type, but chromosome type aberrations also occur. The *in vitro* chromosomal aberration test may employ cultures of established cell lines, cell strains or primary cell cultures. Chromosomal aberrations are the cause of many human genetic diseases and there is substantial evidence that chromosomal damage and related events causing alterations in oncogenes and tumor suppressor genes of somatic cells are involved in cancer induction in humans and experimental animals.

An increase in polyploidy may indicate that a chemical has the potential to induce numerical aberrations [25]. However, the protocol prescribed in this guidance document is not intended to provide an adequate method for the detection of agents that cause numerical chromosomal aberrations. Thus, a lack of polyploidy should not be considered adequate evidence that the test material does not have the potential to induce numerical aberrations, including aneuploidy.

This guidance document is based on the guidelines published by the Organization for Economic Cooperation and Development (OECD) and/or those published by the United States Environmental Protection Agency (US EPA). At the date of publication of this chapter, these documents are available at:

http://www.oecd.org [2] and http://www.epa.gov/ [3].

II. Good Laboratory Practice

Nonclinical laboratory studies must be conducted according to U.S. FDA good laboratory practice (GLP) regulations, issued under Part 58. Title 21. Code of Federal Regulations. This document may be obtained from the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C., 20402, (toll free 866-512-1800).

III. Definitions

Aneuploid: Having an abnormal number of chromosomes not an exact multiple of the haploid number.

Centromere: A region(s) of a chromosome with which spindle fibers are associated during cell division, allowing orderly movement of daughter chromosomes to the poles of the daughter cells.

Chromatid-type aberration: Structural chromosomal damage expressed as breakage of single chromatids or breakage and reunion between chromatids.

Chromosome-type aberration: Structural chromosomal damage expressed as breakage, or breakage and reunion, of both chromatids at an identical site.

Clastogen: An agent that induces chromosome breaks, an essential step in the formation of structural chromosomal aberrations.

Endoreduplication: A process in which after the S phase of DNA replication, the nucleus does not go into mitosis but starts another S phase. The result is chromosomes with 4, 8, 16,...chromatids.

Gap: An achromatic lesion smaller than the width of one chromatid, and with minimum misalignment of the chromatid(s).

Mitotic index: The ratio of cells in metaphase divided by the total number of cells observed in a population of cells; an indication of the degree of proliferation of that population.

Numerical aberration: A change in the number of chromosomes from the normal number characteristic of the cells utilized.

Polyploidy: A multiple of the haploid chromosome number (n) greater than the diploid number (i.e., 3n, 4n and so on).

Structural aberration: A change in chromosomal structure detectable by microscopic examination of the metaphase stage of cell division, observed as intrachanges, or interchanges, or deletions and fragments.

IV. Initial Considerations

Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. This metabolic activation system cannot mimic entirely the mammalian *in vivo* metabolic and pharmacokinetic conditions. Care should be taken to avoid extreme conditions of pH or osmolality which would lead to positive results which do not reflect intrinsic mutagenicity [4, 5].

This test is used to screen for possible mammalian mutagens and carcinogens. Many compounds that are positive in this test are mammalian carcinogens; however, correlation is dependent on chemical class and there is increasing evidence that there are carcinogens that are not detected by this test or other genetic toxicity tests because they appear to act through mechanisms other than direct DNA damage.

V. Principle of the Test Method

Cell cultures are exposed to the test substance both with and without metabolic activation. At predetermined intervals after exposure of cell cultures to the test substance, they are treated with a metaphase-arresting substance (e.g., Colcemid® or colchicine), harvested, stained and metaphase cells are analyzed microscopically for the presence of chromosomal aberrations.

VI. Description of the Method

A. Preparations

1. Cells

A variety of mammalian cell lines, strains or primary cell cultures, including human cells, may be used (e.g., Chinese hamster fibroblasts, human or other mammalian peripheral blood lymphocytes). Cells are selected for use in the assay on the basis of experience with the cell type, growth ability in culture, stability of the karyotype, chromosome number, chromosomal morphological diversity, and spontaneous frequency of chromosomal aberrations. Established cell lines and strains should be checked routinely for stability in the modal chromosome number.

2. Media and Culture Conditions

Appropriate culture media, and incubation conditions (culture vessels, CO_2 concentration, temperature and humidity) should be used in maintaining cultures. Cultures should be monitored routinely for the absence of mycoplasma contamination and should not be used if contaminated. The normal cell cycle time for the cells and culture conditions used should be known.

3. Preparation of Cultures

Established cell lines and strains: cells are propagated from stock cultures, seeded in an appropriate culture medium at a density such that the cultures will not reach confluency before the time of harvest, and incubated at 37°C.

Lymphocytes: whole blood treated with an anti-coagulant (e.g., heparin) or separated lymphocytes are added to an appropriate culture medium containing a mitogen (e.g., phytohemagglutinin) and incubated at 37°C. Lymphocytes from different individuals may respond differently to culture conditions or the test materials. Therefore, lymphocytes from at least two healthy donors should be used.

4. Preparation of Doses

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted if appropriate prior to treatment of the cells. Liquid test substances may be added directly to the test systems and/or diluted prior to treatment. The volume of the test material plus solvent or vehicle should be the same in all cultures, including negative and vehicle controls. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

B. Test Conditions

1. Solvent/Vehicle

The solvent/vehicle should not interfere in any way with the performance of the test, e.g., by reacting chemically with the test substance, by affecting the metabolism of the test substance by S9, by altering the response of the cell to the test substance, or by inducing changes in the cell. The suitability of non-standard vehicles/solvents must be demonstrated according to these criteria. It is recommended that wherever possible, the use of an aqueous solvent/vehicle be considered first. When testing water-unstable substances, the organic solvents used should be free of water. Water can be removed by adding a molecular sieve.

2. Metabolic Activation

Cells should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a co-factor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rats treated with enzyme-inducing agents such as Aroclor 1254 [6, 7, 8, 9], or a mixture of phenobarbitone and ß-naphthoflavone [10, 11, 12]. The post-mitochondrial fraction is usually used at concentrations in the range from 1-10% v/v in the final test medium. The condition of a metabolic activation system may depend upon the class of chemical being tested. In some cases it may be appropriate to utilize more than one concentration of post-mitochondrial fraction. A number of developments, including the construction of genetically engineered cell lines expressing specific activating enzymes, may provide the potential for endogenous activation. The prudent use of engineered cell lines in place of exogenous metabolic activation should be scientifically justified (e.g., by the relevance of the cytochrome P450 isoenzyme for the metabolism of the test substance).

3. Controls

Concurrent positive and negative (solvent or vehicle) controls both with and without metabolic activation should be included in each experiment.

Positive controls should employ a known clastogen at exposure levels expected to give a reproducible and detectable increase over background which demonstrates the sensitivity of the test system. Positive control concentrations should be chosen so that the effects are clear but do not immediately reveal the identity of the coded positive control slide to the reader. When metabolic activation is used, the positive control chemical should be the one that requires activation to give a clastogenic response. Examples of positive control substances include:

Metabolic activation condition	Chemical and CAS No.
Absence of exogenous metabolic activation	Methyl methanesulfonate [CAS no. 66-27-3] Ethyl methanesulfonate [CAS no. 62-50-0] Ethylnitrosourea [CAS no. 759-73-9] Mitomycin C [CAS no. 56-57-7] 4-Nitroquinoline-N-Oxide [CAS no. 56-57-5]
Presence of exogenous metabolic activation	Benzo(a)pyrene [CAS no. 50-32-8] Cyclophosphamide (monohydrate)* [CAS no. 50-18-0 (CAS no. 6055-19-2)]

*There are literature reports (compiled in reference 26) showing that, in some studies but not in others, cyclophosphamide can be clastogenic *in vitro* in the absence of metabolic activation. If cyclophosphamide is used as a positive control, it should also be tested in the absence of S9 to demonstrate its S9-dependence in the cell line being used in that laboratory.

4. Dose Levels

Among the criteria to be considered when determining the highest concentration are cytotoxicity, solubility in the test system, and changes in pH or osmolality.

It may be useful to determine cytotoxicity and solubility in a preliminary dose-range finding assay using the same treatment regimen and metabolic activation to be used in the definitive chromosomal aberration study. Cytotoxicity should be determined using an appropriate indication of cell integrity and growth, such as degree of confluency, viable cell counts, plating efficiency, or mitotic index. Cytotoxicity should also be determined with and without metabolic activation in the definitive assay.

Analyzable cells must be obtained from a minimum of three test concentrations. Where cytotoxicity occurs, these concentrations should cover a range from the maximum to little or no toxicity; this will usually mean that the concentrations should be separated by no more than a factor between 2 and the square root of 10. At the time of harvesting, the highest concentration should show a reduction in degree of confluency, cell count, or plating efficiency, of at least 50%. The use of mitotic index as a measure of cytotoxicity is acceptable for mixed cell cultures, such as whole blood cultures where peripheral lymphocytes are used as the genotoxic target cell. In these types of cultures other toxicity estimators are not possible or are technically impractical. A reduction in mitotic index can indicate a cytostatic rather than cytotoxic response and may be affected by the length of time between treatment and harvest. It is therefore not recommended as a measure of cytotoxicity in experiments with cell lines in which other measures of cytotoxicity are possible. Information on cell cycle kinetics, such as average generation time (AGT), could be used as supplementary information. AGT, however, is an overall average that does not always reveal the existence of delayed subpopulations, and even slight increases in average generation time can be associated with very substantial delay in the time of optimal yield of aberrations.

For relatively non-cytotoxic compounds the maximum concentration should be 5 µl/ml, 5 mg/ml, or 0.01M, whichever is the lowest.

For relatively insoluble substances that are not toxic at soluble concentrations, the highest dose tested should be a concentration above the limit of solubility in the culture medium at the end of the treatment period. It has been suggested that in some cases (e.g., when toxicity occurs only at insoluble concentrations) it may be advisable to test at more than one concentration with visible precipitation. In considering this recommendation, caution should be exercised to minimize conditions that complicate quantification of results. It may be useful to assess solubility at the beginning and the end of the treatment, as solubility can change during the course of exposure in the test system due to presence of cells, S9, serum etc. Insolubility can be detected by using the unaided eye. The precipitate should not interfere with the scoring.

In some cases, it may be possible to use lower chemical concentrations and to increase the treatment time (without metabolic activation) so that the test can be performed under conditions where the chemical is soluble. Fresh chemical preparations should be employed unless stability data demonstrate the acceptability of storage.

C. Treatment

Proliferating cells are treated with the test substance in the presence and absence of a metabolic activation system. Treatment of lymphocytes should commence at about 48 hours after mitogenic stimulation.

Duplicate cultures should normally be used at each concentration, and are strongly recommended for negative/solvent control cultures. Where minimal variation between duplicate cultures can be demonstrated [13, 14] from historical data, it may be acceptable for single cultures to be used at each concentration.

Gaseous or volatile substances should be tested by appropriate methods, such as in sealed culture vessels [15, 16].

D. Harvest of Cultures

In the first experiment, cells should be exposed to the test substance both with and without metabolic activation for 3-6 hours, and sampled at a time equivalent to about 1.5 normal cell cycle length after the beginning of treatment [12]. If this protocol gives negative results both with and without activation, an additional experiment without activation should be done, with continuous treatment until sampling at a time equivalent to about 1.5 normal cell cycle lengths. Certain chemicals may be more readily detected by treatment/sampling times longer than 1.5 cycle lengths[12]. Negative results with metabolic activation need to be confirmed by additional testing. Modification of the test protocol, such as variation of S9 source or concentration, should be considered for this confirmatory test.

E. Chromosome Preparation

Cell cultures are treated with Colcemid® or colchicine usually for one to three hours prior to harvesting. Each cell culture is harvested and processed separately for the preparation of chromosomes. Chromosome preparation involves hypotonic treatment of the cells, fixation and staining [27]. Hypotonic treatment should be adjusted to provide optimal separation of chromosomes but without loss of chromosomes. Staining should allow accurate discrimination of chromosome structures [27].

F. Analysis

All slides, including those of positive and negative controls, should be independently coded before microscopic analysis. Since fixation procedures often result in the disruption of a proportion of metaphase cells with loss of chromosomes, the cells scored should contain a number of centromeres equal to the modal number ± 2 for all cell types. At least 200 well-spread metaphases should be scored per concentration and control equally divided among the duplicates, if applicable. This number can be reduced when high numbers of aberrations are observed. Though the purpose of the test is to detect structural chromosomal aberrations, it is important to record polyploidy and endoreduplication.

VII. Data and Reporting

A. Treatment Results

The experimental unit is the cell, and therefore the percentage of cells with structural chromosomal aberration (s) should be evaluated. Different types of structural chromosomal aberrations should be listed with their numbers and frequencies for experimental and control cultures. Gaps are recorded separately and reported but generally not included in the total aberration frequency. Various schemes involving varying degrees of detail have been used to classify chromosomal aberrations. For most tests, it is adequate to classify aberrations into four main categories; chromosome breaks, chromosome exchanges, chromatid breaks and chromatid exchanges. In addition, other events such as polyploidy, endoreduplication, heavily damaged cells (for example, more than ten aberrations in one cell), and cells with shattered or pulverized chromosomes should also be recorded.

Concurrent measures of cytotoxicity for all treated and negative control cultures in the main aberration experiment(s) should also be recorded.

Individual culture data should be provided. Additionally, all data should be summarised in tabular form.

There is no requirement for verification of a clear positive response. Marginally or weakly positive results should be verified by additional testing. Equivocal results should be clarified by further testing, preferably using modification of experimental conditions. The need to confirm negative results has been discussed in section V.D. "Harvest of Cultures". Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments. Study parameters that might be modified include the concentration spacing and the metabolic activation conditions.

B. Evaluation and Interpretation of Results

There are several criteria for determining a positive result, such as a concentration-related increase or a reproducible increase in the number of cells with chromosomal aberrations at a single test concentration. Statistical methods should be used as an aid in evaluating the test results [3, 13] but should not be the only determining factor for a positive response. Biological relevance should be considered.

An increase in the number of polyploid cells may indicate that the test substance has the potential to interfere with the mitotic processes and to induce numerical chromosomal aberrations. An increase in the number of cells with endoreduplicated chromosomes may indicate that the test substance has the potential to inhibit cell cycle progression [17, 18]. Induction of numerical aberrations should be confirmed.

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

Positive results from the *in vitro* chromosomal aberration test indicate that the test substance induces structural chromosomal aberrations in cultured mammalian somatic cells. Negative results indicate that, under the test conditions, the test substance does not induce chromosomal aberrations in cultured mammalian somatic cells.

There are several conditions under which positive data may be a result of the test conditions rather than any intrinsic clastogenicity of the test material. Changes in pH or osmolality have been shown to induce aberrations [5, 19]. There is also evidence that some chemicals induce an increase in structural aberrations only at high levels of cytotoxicity [20, 21]. In this case, the aberrations may be the result of disruption of cellular processes that would not be expected at lower doses, and may therefore have no relevance to clastogenic risk at physiologically relevant concentrations. However, before it can be concluded that the test agent presents no clastogenic hazard, it must be demonstrated that the material is not genotoxic in any other *in vitro* assays, it does not interact directly with the DNA, it is not a topoisomerase inhibitor, it is not related structurally to known clastogens, and that the concentrations inducing aberrations *in vitro* cannot be achieved *in vivo*.

Caution should also be exercised when interpreting data from studies using Chinese hamster cells. Certain chemicals appear to induce a high frequency of damage at a specific location, or fragile site, on the long arm of the X chromosome [22, 23, 24]. In tests where it can be documented that a large percentage of aberrations are at this site and there is no significant increase in other aberrations, the relevance of this phenomenon to effects in human cells is unclear.

C. Test Report

The test report should include the following information:

1. Test Substance

- identification data and CAS no., if known;
- physical nature and purity;
- physicochemical properties relevant to the conduct of the study;
- stability of the test substance, if known.

2. Solvent/Vehicle

- justification for choice of solvent/vehicle.
- solubility and stability of the test substance in solvent/vehicle, if known.

3. Dosing Solutions

- Time interval between stock solution and dosing solution preparation and use, and storage conditions.
- Data that verify concentration of the dosing solution, if available.

4. Test cultures

- type and source of cells;
- karyotype features and suitability of the cell type used;
- absence of mycoplasma, if applicable;
- information on cell cycle length;
- sex of blood donors, whole blood or separated lymphocytes, mitogen used;
- number of passages, if applicable;
- methods for maintenance of cell cultures if applicable;
- modal number of chromosomes.

5. Test Conditions

- identity of metaphase arresting substance, its concentration and duration of cell exposure;
- rationale for selection of concentrations and number of cultures including, e.g., cytotoxicity data and solubility limitations, if available;
- composition of media, CO_2 concentration if applicable;
- concentration of test substance;
- volume of vehicle and test substance added;
- incubation temperature;
- incubation time;
- duration of treatment;
- cell density at seeding, if appropriate;
- type and composition of metabolic activation system, including acceptability criteria;
- positive and negative controls;
- methods of slide preparation;
- criteria for scoring aberrations;
- number of metaphases analyzed;
- methods for the measurements of toxicity;
- criteria for considering studies as positive, negative or equivocal.

6. Results

- signs of toxicity, e.g., degree of confluency, cell cycle data, cell counts, mitotic index;
- signs of precipitation;
- data on pH and osmolality of the treatment medium, if determined;
- definition for aberrations, including gaps;
- number of cells with chromosomal aberrations and type of chromosomal aberrations given separately for each treated and control culture;
- changes in ploidy if seen;
- dose-response relationship, where possible;
- statistical analyses, if any;
- concurrent negative (solvent/vehicle) and positive control data;
- historical negative (solvent/vehicle) and positive control data, with ranges, means and standard deviations.

7. Discussion of the Results

8. Conclusion

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Redbook 2000: IV.C.1.c Mouse Lymphoma Thymidine Kinase Gene Mutation Assay

October 2001; Updated April 2006

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter IV.C.1.c. Mouse Lymphoma Thymidine Kinase Gene Mutation Assay

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I. Introduction

An *in vitro* mammalian cell gene mutation test can be used to detect gene alterations induced by chemical substances. While there are a number of cell lines that can be used, the L5178Y $TK^{+/-}$-3.7.2C mouse lymphoma cell line using the thymidine kinase (TK) gene is the cell line and assay of choice. The mouse lymphoma assay (MLA) was chosen because of a body of research indicating that many types of genetic alterations are detected. The assay detects mutations known to be important in the etiology of cancer and other human genetically mediated illnesses. There is evidence that the assay detects gene mutations (point mutations) and chromosomal events (deletions, translocations, mitotic recombination/gene conversion and aneuploidy) (Applegate et. al., 1990; Hozier, et al., 1981; Moore, et al., 1985; Sawyer, et al., 1989; Sawyer, et al., 1985). The efficiency of detection of all of these mutational events is still under investigation.

Other *in vitro* mammalian gene mutation assays exist including those that use either Chinese hamster cell lines (CHO, AS52, and V79) or human lymphoblastoid cells (TK6). In these cell lines the most commonly used genetic endpoints measure mutation at either the hypoxanthine-guanine phosphoribosyl transferase (HPRT), a transgene of xanthine-guanine phosphoribosyl transferase (XPRT), or TK. The TK, HPRT and XPRT mutation tests detect different spectra of genetic events. The autosomal location of TK allows for the detection of genetic events that are not detected at the HPRT locus on the X-chromosome (Moore et al., 1989).

The various mutation assays are capable of detecting different spectra of genetic damage. Thus, it is not expected that a chemical will give uniformly positive or negative results in the various assays. In particular, the bacterial Salmonella assay detects only point and other very small-scale gene mutations. Furthermore, the *in vitro* mammalian assays using the HPRT locus are unable to detect chemicals that do not induce point mutations yet are clastogenic (Moore et. al, 1989). These chemicals are likely operating by mechanisms that cause chromosomal mutations (deletions, translocations, gene conversion/mitotic recombination and/or aneuploidy).

Additional information is provided in Chapter IV. C. 1. [2]

II. Definitions

Forward mutation: A mutation that converts a wild-type allele to a mutant allele.

Base-pair-substitution mutagens: Substances that cause substitution of one or a small number of base pairs in the DNA.

Frameshift mutagens: Substances that cause insertion or deletion of a nucleotide pair or pairs, causing a disruption of the triplet translational reading frame.

Phenotypic expression time: The time after treatment during which the genetic alteration is fixed within the genome and any preexisting gene products are depleted to the point that the phenotypic trait is altered.

Mutant frequency: The number of mutant cells observed divided by the number of viable cells.

Relative survival (RS): The relative cloning efficiency of the test culture plated immediately after the cell treatment and compared to the cloning efficiency of the negative control (Cole, et al., 1986)

Relative suspension growth (RSG): The relative total two day suspension growth of the test culture compared to the total two-day suspension growth of the vehicle control. (Clive and Spector, 1975).

Relative total growth (RTG): RTG is used as the measure of treatment-related cytotoxicity in the MLA. It is a measure of relative (to the vehicle control) growth of test cultures both during the two-day expression and mutant selection cloning phases of the assay. The RSG of each test culture is multiplied by the relative cloning efficiency of the test culture at the time of mutant selection and expressed relative to the cloning efficiency of the vehicle control.

III. Initial Considerations

The established cell line L5178Y $TK^{+/-}$-3.7.2C mouse lymphoma is used for the assay. The assay requires the use of an exogenous source of metabolic activation. There are two versions of the assay, one using soft agar cloning to enumerate mutants (Clive, et al., 1979 and Turner et al., 1984) and one using liquid media and microwell plates (Cole, et al., 1986). Both versions of the assay are equally acceptable (Moore, et al., 2000).

IV. Principle of the Test Method

Cells deficient in thymidine kinase (TK) due to the mutation $TK^{+/-}$ to $TK^{-/-}$ are resistant to the cytostatic effects of the pyrimidine analogue trifluorothymidine (TFT). TK proficient cells are sensitive to TFT, which causes the inhibition of cellular metabolism and halts further cell division. Thus, mutant cells are able to proliferate in the presence of TFT, whereas normal cells, which contain the TK enzyme, are not.

Mouse lymphoma cells are grown and treated with test agents in suspension culture. Treatment should be done both with and without exogenous metabolic activation. Following treatment, cells are cultured to allow phenotypic expression prior to mutant selection. Cytotoxicity is measured and used to determine the appropriate dose range of the test chemical. Mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect the mutant cells, and in medium without the selective agent to determine cloning efficiency (viability). After a suitable incubation time, the colonies are counted. The mutant frequency is derived from the number of mutant colonies in the selective medium and the viability.

V. Description of the Method

A. Preparations

1. Cells

Because the assay was developed and characterized using the $TK^{+/-}$ -3.7.2C subline of L5178Y cells, it is important that the assay be conducted using $TK^{+/-}$ -3.7.2C cells (Mitchell, et al., 1997). It is advisable for all laboratories to karyotype the cells or paint the chromosome 11s to assure that there are two normal looking chromosome 11s and to identify any other irregularities. The karyotype for the $TK^{+/-}$-3.7.2.C cells has been published (Sawyer, et al., 1985, 1989, 2006). The modal chromosome number for the L5178Y/$TK^{+/-}$-3.7.2C cell line is 40. There is one metacentric chromosome (t12;13) that should be counted as one chromosome. Karyotyping and/or chromosome 11 painting should be performed when establishing a master stock. Cell cultures need to be monitored for doubling times. Normal doubling times are generally between 8 and 10 hr. Population doubling time should be checked when setting down master stocks. Cell cultures should always be maintained under conditions that ensure that they are growing in log phase. As a general guidance, if a laboratory is continually growing cells, the culture should be maintained for no longer than 3 months.

2. Media and Culture Conditions

Appropriate culture media and incubation conditions (culture vessels, temperature, CO_2 concentration and humidity) should be used. It is particularly important that culture conditions be chosen that ensure optimal

growth of cells during the expression period and colony forming ability of both mutant and non-mutant cells. For the MLA it is also important that the culture conditions ensure optimal growth of the small colony TK mutants. Both Fischer's Medium for Leukemic Cells of Mice and RPMI 1640 media have been successfully used with the MLA.

The osmolalilty and pH of the medium should be confirmed to be in the physiological range (300 ± 20 mOsm and pH 7.0 ± 0.4). Each lot of horse serum should be tested for its ability to support optimal cell growth in suspension culture (low and high cell densities), high plating efficiency and small colony mutant recovery (Turner et al, 1984).

3. Preparation of Cultures

Cells are propagated from stock cultures, seeded in culture medium and incubated at 37^{O}C. Prior to use, the culture needs to be cleansed of pre-existing mutant cells. This is accomplished using methotrexate to select against TK-deficient cells. Thymidine, hypoxanthine and guanine are added to the culture to ensure optimal growth of the TK-competent cells (Turner et al., 1984).

4. Metabolic Activation

Cells should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a co-factor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 or a combination of phenobarbitone and β-naphthoflavone (Mitchell, et al., 1997). The post-mitochondrial fraction is usually used at concentrations in the range from 1-10% v/v in the final test medium. The choice and condition of a metabolic activation system may depend upon the class of chemical being tested. In some cases it may be appropriate to utilize more than one concentration of post-mitochondrial fraction. The use of alternative metabolic activation systems such as primary hepatocytes (Brock, et al., 1987 and Oglesby, et al., 1989) should be justified.

5. Test Substance/Preparations

Liquid test substances may be added directly to the test system or diluted prior to treatment. Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted if appropriate prior to treatment of the cells. It should be noted that problems may occur, particularly with suspension cultures, if the test substance precipitates either prior to addition to the cultures or during the treatment. It is generally best to avoid testing chemicals using conditions where they are either insoluble or become insoluble during the treatment. In some cases, it may be possible to use lower chemical concentrations and to increase the treatment time (without metabolic activation) so that the test can be performed under conditions where the chemical is soluble. Fresh chemical preparations should be employed unless stability data demonstrate the acceptability of storage.

The properties of the test substance should be considered carefully if a base analogue or a compound related to the selective agent is tested in the MLA. For example, any suspected selective toxicity by the test substance for mutant and non-mutant cells should be investigated. If mutants and non-mutant cells are differentially sensitive to the test agent, the preexisting spontaneous mutants may be selectively increased in frequency during the treatment. The resultant increase in mutant frequency would be due to that selection of pre-existing spontaneous mutants rather than from the induction of new mutations. This possibility needs to be considered and investigated when the test chemical is structurally related to TFT.

B. Test Conditions

1. Solvent/vehicle

The solvent/vehicle should be chosen to maximize the solubility of the test agent. However, it should not be suspected of chemical reaction with the test substance and should be compatible with the survival of the cells and the S9 activity. If other than well-known solvent/vehicles are used, their inclusion should be supported by data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle be considered first. When testing water-unstable substances, the organic solvents used should be free of water. Water can be removed using a molecular sieve. Organic solvents that have been used with the MLA include DMSO, acetone and ethyl alcohol.

2. Exposure Conditions

Criteria to be considered when determining the highest test concentration include cytotoxicity, solubility in the test system, and changes in pH or osmolality. For relatively non-cytotoxic compounds the maximum

concentration should be 5 mg/ml, 5 l/ml or 0.01 M, whichever is lowest.

Relatively insoluble substances should be tested up to or beyond their limit of solubility under the culture conditions, although care should be exercised to minimize conditions that complicate quantification of results. Evidence of insolubility should be determined in the final treatment medium to which cells are exposed. It may be useful to assess solubility at the beginning and end or the treatment, as solubility can change during the course of exposure in the test system due to the presence of cells, S9, serum etc. Insolubility may be assessed using the naked eye.

In the MLA where the cells are grown in suspension, testing compounds in the precipitating range is problematical with respect to defining the exposure period. After the defined exposure period, the cells are normally pelleted by centrifugation and are then resuspended in fresh medium without the test compound. If a precipitate is present, the compound will be carried through to the later stages of the assay making control of exposure impossible. Therefore, for the MLA it is reasonable to use the lowest precipitating concentration as the highest concentration tested or the highest test concentration used in the data evaluation. This should help to minimize conditions that could complicate quantification of results.

3. Cytotoxicity

Cytotoxicity should be determined for each individual test and control culture. For the soft agar version of the MLA, this has generally been done using the relative total growth (RTG) which was originally defined by Clive and Spector (1975). This measure includes the relative growth in suspension during the expression time and the relative cloning efficiency at the time that mutants are selected. The microwell version of the assay was developed using the relative survival (RS) as the cytotoxicity measure. The RS is determined by the relative plating efficiency of each culture when plated immediately after the exposure period. The RTG and the RS are different measures of cytotoxicity and, although there is no real justification that one measure is superior to the other, it is important that the same measure of cytotoxicity be used for both versions of the assay. Because the RS is not normally measured in the soft agar version of the assay and the RTG is measured in both versions, it is recommended that the RTG be used as the standard measure of cytotoxicity. This cytotoxicity value is used both to determine the required concentration range for an acceptable test and for establishing the highest concentration that is used for defining positive and negative responses (Moore, et al., 2000).

There are additional considerations in the calculation of the RTG between the two methods for the conduct of the MLA. In the agar method, the cells are exposed to the test chemical, the chemical is removed by centrifugation and resuspension in fresh medium. The first cell count takes place approximately 24 hrs after the initiation of the chemical exposure. On the first day following treatment, the cell density for each culture is readjusted, generally to 0.2 or 0.3 $\times$ 10^6 cells per ml of medium. Treated cultures with densities less than 0.2 or 0.3 $\times$ 10^6 cells per ml of medium are generally not adjusted in their density, and usually have sustained too much cytotoxicity to carry through the full experiment for mutant enumeration. For each treatment culture, the relative cell growth (compared to control) is calculated. On the second day following treatment, the cultures are again counted, adjusted in density and prepared to clone for mutant enumeration. The total two-day suspension growth of each culture is calculated and each treated culture is compared to the control. This value is referred to as the relative suspension growth (RSG). Cultures are cloned with and without selective medium to enumerate mutants and to calculate the mutant frequency (number of mutants per 10^6 cloneable cells). The relative plating efficiency for each culture is determined (relative to the negative control) and multiplied by the RSG to obtain a relative total growth (RTG).

In the microwell method, most laboratories count the cell cultures immediately following exposure to the test chemical and adjust the density of the cultures. Following the end of treatment and the adjustment of cell density, the cell cultures are handled just like the cultures in the agar method. Following the two-day expression period, the cultures are plated in 96-well plates, with and without TFT selection.

As described above, handling of the cell cultures following treatment differs significantly between the two methods This difference impacts the calculation of the RSG and RTG. The RSG and the RTG, in the agar method, are calculated to include any differences that may occur in cell growth between the chemically treated and control cultures. However, in the microwell method, the cultures are generally adjusted in density following treatment and the RS, RSG and RTG calculated using the plating efficiency and cell growth that occurs following treatment. In other words, any differential growth that occurs between the negative controls and the treatment cultures during the treatment phase of the assay is not factored into the calculation.

To make the cytotoxicity measures obtained in the two versions equivalent, it is necessary for users of the microwell method to adjust their RS, RSG and RTG values to include the differential growth that can occur during treatment. This adjustment should be made by comparing the cell density in each treated culture with

that of the negative control immediately following treatment. By comparing the growth of each treated culture relative to the control, it is possible to calculate a relative growth during treatment factor that can then be used to adjust the RS, RSG and RTG. As an example, if following the treatment period, the negative control had a cell density of 0.6×10^6 cell/ml and the treated culture had a density of 0.3×10^6 cell/ml, then the relative growth during treatment for that treated culture is 0.5 (or 50%). If the RS for that culture is determined to be 0.4 then the adjusted RS would be calculated as the RS × the relative growth during treatment or 0.4 × 0.5=0.20 (or 20%). The RSG would be adjusted in the same manner. The adjusted RTG would be obtained by multiplying the adjusted RSG by the relative plating efficiency at the time of mutant selection.

4. Dose Selection

The selection and spacing of doses is a critical factor in the proper conduct of the MLA. It is desirable to have more than one data point that can be used to confirm a positive or negative response. The assay may be conducted using either single cultures per dose point or multiple cultures per dose point. The strategy for determining the number of doses and the selection and spacing of doses can vary based on the toxicity range of the test material being evaluated and degree by which the chemical does or does not increase mutant frequency. It is not necessary to have a prescribed number of analyzable cultures when the chemical is clearly positive. When the chemical is not mutagenic or is only weakly mutagenic, generally, at least 4 analyzable doses are required when duplicate cultures are used and 8 analyzable doses are required when single cultures are used.

For toxic test materials, the highest dose level should induce an 80% reduction in RTG. Dose levels that induce more than a 90% reduction in RTG are usually excluded from the evaluation. However, as noted below, there are circumstances where the data points obtained at less than 10% RTG can be useful in the final evaluation.

While it is generally advisable to obtain data points covering the entire 100 to 10% RTG range, the validity of a test does not always depend upon attaining such a complete dose-response. When a test material induces large increases in mutant frequency, it is generally sufficient to provide data points anywhere within the 100-10% RTG range. For test materials that are not mutagenic or that induce only weak mutagenic responses, it is advisable to place emphasis on selecting doses that are expected to produce higher toxicity. This increases the probability of obtaining data points that can be used to make a definitive evaluation, that is, data points in the approximately 10-20% RTG range.

Therefore, it is recommended that laboratories attempt to achieve a maximum dose with RTG values between 10-20%. However, as already indicated, if a chemical clearly satisfies the criteria for a positive response the result will still be valid even if there is no test concentration resulting in 10-20% RTG.

When the mutant frequency is elevated at one or more doses above the background frequency (yet has not reached a level to be determined positive), interpretation of the test, and therefore its validity, will depend upon at least one dose that results in an RTG within the 10-20% range. This may only be achievable by conducting a repeat experiment in which the dose range is modified to increase the probability of attaining data points within the 10-20% RTG range.

There are some circumstances under which a chemical may be determined to be nonmutagenic when there is no culture showing an RTG value between 10-20 % RTG. These situations are outlined as follows: (1) There is no evidence of mutagenicity (*e.g.*, no dose response, no mutant frequencies above those seen in the concurrent negative control or historical background ranges, etc.) in a series of data points within 100% to 20% RTG and there is at least one data point between 20 and 25% RTG. (2) There is no evidence of mutagenicity (*e.g.*, no dose response, no mutant frequencies above those seen in the concurrent negative control or historical background ranges, etc.) in a series of data points between 100% to 25% and there is also a negative data point between 10% and 1% RTG.

Significant increases in mutant frequencies seen only at RTG <10%, but with no evidence of mutagenicity at RTG >10%, do not constitute a positive result.

5. Controls

Concurrent positive and negative (solvent or vehicle) controls both with and without metabolic activation should be included in each experiment. When metabolic activation is used, the positive control chemical should be one that requires activation to give a mutagenic response.

Positive controls should induce mostly small colony TK mutants. One appropriate positive control in the absence of S9 metabolic activation is methyl methanesulfonate. Appropriate positive controls to be used with S9 activation include cyclophosphamide (monohydrate), benzo(a)pyrene, and 3-methylcholanthrene. Positive

control responses with and without S9 should be used for quality control measures and to demonstrate adequate detection of small colony mutants. Each laboratory must establish its own historical database for its positive and negative controls.

Negative controls, consisting of solvent or vehicle alone in the treatment medium, and treated in the same manner as the treatment groups should be included. In addition, untreated controls should also be used unless there are historical data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent.

C. Procedure

1. Treatment with test substance

Cells, growing in log phase, should be exposed to the test substance both with and without metabolic activation. Exposure should be for a suitable period of time (generally 3-4 hrs is used). However, it may also be advisable (particularly for chemicals demonstrating insolubility) to extend the treatment time (without metabolic activation) to 24 hrs. The International Conference on Harmonisation [3]has recommended that all chemicals that are negative following the standard 3-4 hr treatment be evaluated (without metabolic activation) using a 24-hr treatment.

Either duplicate or single treated cultures may be used at each concentration tested. In either event, the number of concentrations used must be sufficient to provide confidence in the evaluation. Particularly, in situations where the chemical is negative or weakly positive, it may be advisable to use single treated cultures, and increase the number of different concentrations evaluated in a single experiment. Because of the importance of the negative controls, it is recommended that duplicate negative (solvent) control cultures be used.

Gaseous or volatile substances should be tested by appropriate methods, such as in sealed culture vessels.

2. Expression time and measurement of mutant frequency

At the end of the exposure period, cells are washed and cultured to allow for the expression of the mutant phenotype.

Each locus has a defined minimum time requirement to allow near optimal phenotypic expression of newly induced mutants. For the TK locus that time is 2 days (Moore and Clive, 1982). Following the expression period, cells are grown in medium with and without selective agent for the determination of the numbers of mutants and cloning efficiency (used to calculate mutant frequency), respectively. This mutant selection can be accomplished using TFT selection (Moore-Brown et al., 1981) and either the soft agar or the microwell cloning method (Moore et al., 2000).

In the soft agar method, the mutant frequency (MF) is determined by counting the number of TFT resistant colonies and correcting the number of cells plated for selection by the plating efficiency (PE). That is, the MF = (number of mutants/number of cells plated) × PE. For the microwell method, the plating efficiency (PE) and the mutant frequency (MF) are calculated using the Poisson distribution. The plating efficiency (PE) in both the mutant selection plates and the viability plates is calculated as follows: From the zero term of the Poisson distribution, the probable number of clones/well (P) is equal to -ln(EW/TW), where EW = empty wells and TW = total wells. The PE = P/Number of cells plated per well. The mutant frequency is then calculated: MF = (PE (mutant)/PE(viable)) × 10^6.

3. Mutant Colony Sizing

If the test substance is positive in the MLA, mutant colony sizing should be performed on at least one of the test cultures (generally the highest acceptable positive concentration) and on the negative and positive controls. Colony sizing can be used to provide general information concerning the ability of the test chemical to cause point mutations and/or chromosomal events. If the test substance is negative, mutant colony sizing should be performed on the negative and positive controls. Colony sizing on the negative control is needed to demonstrate that large colonies are growing adequately. The test chemical cannot be determined to be negative if the positive control does not demonstrate the appropriate level of small mutant colony induction and detection.

VI. Data and Reporting

A. Treatment of results

Data should include cytotoxicity and plating efficiency determination, colony counts and mutant frequencies

for the treated and control cultures. In the case of a positive response, mutant colonies are scored using the criteria of small and large colonies on at least one concentration of the test substance (highest positive concentration) and on the negative and positive controls.

The molecular and cytogenetic nature of both large and small colony mutants has been explored in detail (Applegate, et al, 1990, Hozier et al., 1981, 1985 and Moore et al., 1985). Small and large colony mutants are distinguished by growth rate, and therefore they form colonies of differing size. Mutant cells that have suffered the most extensive genetic damage have prolonged doubling times and thus form small colonies. The induction of small colony mutants has been associated with chemicals that induce chromosomal aberrations. Less seriously affected mutant cells grow at rates similar to the parental cells and form large colonies.

The RS (if determined), RTG and RSG should be given. Mutant frequency should be expressed as number of mutant cells per number of surviving cells.

Individual culture data should be provided in tabular form that can be cross-referenced to the summary data. Additionally, all data should be summarized in tabular form.

While there is no requirement for verification of a clear positive response, a confirmatory experiment is often useful. Experiments that do not provide enough information to determine whether the chemical is positive or negative should be clarified by further testing preferably by modifying the test concentrations. Negative results using the short (3-4 hr) treatment should be confirmed by repeat testing using 24-hr treatment without metabolic activation. Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments for either equivocal or negative results. Study parameters that might be modified include the concentration spacing and the metabolic activation conditions.

B. Evaluation and interpretation of results

There are several criteria for determining a positive result, such as a concentration-related, or a reproducible increase in mutant frequency. The U.S. EPA MLA Gene-Tox Workgroup developed criteria for evaluating the published literature. These criteria for positive and negative (and other responses) can be used as guidance in interpreting data (Mitchell, et al., 1997). Statistical methods may be used as an aid in evaluating the test results. Statistical significance should not be the only determining factor for a positive response. Biological relevance of the results should also be considered. The MLA Workgroup of the International Workshop for Genotoxicity Testing recommends acceptance criteria the MLA and the use of a global evaluation factor combined with statistical analysis for the interpretation of MLA data (Moore et al., 2006).

Although most studies will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. In these situations, a chemical will give equivocal results in two or more very well conducted studies. In those situations, it is generally not useful to conduct a further study. Such chemicals are not negative and they should be considered to be borderline responses.

Positive results for the MLA indicate that the test substance induces mutations affecting the expression of the thymidine kinase gene in the cultured mammalian cells used. A positive concentration-related response that is reproducible is most meaningful. Negative results indicate that, under the test conditions, the test substance does not induce mutations affecting the thymidine kinase gene in the mouse lymphoma cells.

It should be noted that positive results that may not be relevant to the *in vivo* situation may arise *in vitro* from changes in pH, osmolality or high levels of cytotoxicity (Brusick, 1986, Mitchell, et al., 1997 and Scott, et al., 1991).

VII. Test Report

The test report should include the following information:

A. Test substance:

- identification data and CAS no., if known;
- physical nature and purity;
- physicochemical properties relevant to the conduct of the study;
- stability of the test substance-including both the "neat" sample and the sample in the solvent/vehicle/medium. This should be done both prior to and at the end of the treatment period.

B. Solvent/Vehicle:

- justification for choice of vehicle/solvent;
- solubility and stability of the test substance in solvent/vehicle, if known.

C. Cells:

- type and source of cells
- karyotype of cells
- number of cell cultures;
- methods for maintenance of cell cultures;
- absence of mycoplasma.

D. Test conditions:

- rationale for selection of concentrations and number of cell cultures including e.g., cytotoxicity
- data and solubility limitations, if available;
- composition of media, $C0_2$ concentration;
- concentration of test substance;
- volume of vehicle and test substance added;
- incubation temperature;
- incubation time;
- cell density during treatment;
- type and composition of metabolic activation system including acceptability criteria;
- positive and negative controls;
- length of expression period (including number of cells seeded, and subcultures and feeding schedules, if appropriate);
- selective agent(s);
- criteria for considering tests as positive, negative or equivocal;
- methods used to enumerate numbers of viable and mutant cells.
- definition of colonies of which size and type are considered (including criteria for "small' and "large" colonies, as appropriate).

E. Results:

- signs of toxicity;
- signs of precipitation;
- data on pH and osmolality during the exposure to the test substance, if determined;
- colony sizing (for positive test chemicals) and for the negative and positive control
- dose-response relationship, where possible;
- statistical analyses, if any;
- concurrent negative (solvent/vehicle) and positive control data;
- historical negative (solvent/vehicle) and positive control data with ranges, means and standard deviations; number of tests upon which the historical controls are based;
- mutant frequency;
- Raw data, including cell culture counts and colony counts

F. Discussion of the results:

G. Conclusion:

VIII. References

The following references should be consulted for additional background information on this test guideline.

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Redbook 2000: IV.C.1.d Mammalian Erythrocyte Micronucleus Test

July 2000

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter IV.C.1.d. Mammalian Erythrocyte Micronucleus Test

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I. Introduction

Micronuclei are cytoplasmic chromatin-containing bodies formed when acentric chromosome fragments or chromosomes lag during anaphase and fail to become incorporated into daughter cell nuclei during cell division. Because genetic damage that results in chromosome breaks, structurally abnormal chromosomes, or spindle abnormalities leads to micronucleus formation, the incidence of micronuclei serves as an index of these types of damage. It has been established that essentially all agents that cause double strand chromosome breaks (clastogens) induce micronuclei. Because enumeration of micronuclei is much faster and less technically demanding than is scoring of chromosomal aberrations, and because micronuclei arise from two important types of genetic damage (clastogenesis and spindle disruption), the micronucleus assay has been widely used to screen for chemicals that cause these types of damage.

This guidance addresses the most widely used *in vivo* micronucleus assay: the mammalian erythrocyte micronucleus assay. This *in vivo* micronucleus test is used for the detection of damage induced by the test substance to the chromosomes or the mitotic apparatus of erythroblasts by analysis of erythrocytes as sampled in bone marrow and/or peripheral blood cells of animals, usually rodents.

The purpose of the micronucleus test is to identify substances that cause cytogenetic damage which results in the formation of micronuclei containing lagging chromosome fragments or whole chromosomes.

When a bone marrow erythroblast develops into a polychromatic erythrocyte, the main nucleus is extruded; micronuclei that have been formed may remain behind in the otherwise enucleated cytoplasm. Visualization of micronuclei is facilitated in these cells using specific staining techniques and because they lack a main nucleus. An increase in the frequency of micronucleated polychromatic erythrocytes in treated animals is an indication of induced chromosome damage.

II. Definitions

Centromere (Kinetochore) is a region(s) of a chromosome with which spindle fibers are associated during cell division, allowing orderly movement of daughter chromosomes to the poles of the daughter cells.

Micronuclei are small nuclei, separate from and additional to the main nuclei of cells, produced during telophase of mitosis (meiosis) by lagging chromosome fragments or whole chromosomes.

Normochromatic erythrocyte is a mature erythrocyte that lacks ribosomes and can be distinguished from

immature, polychromatic erythrocytes by stains selective for ribosomes.

Polychromatic erythrocyte is an immature erythrocyte, in an intermediate stage of development, that still contains ribosomes and therefore can be distinguished from mature, normochromatic erythrocytes by stains selective for ribosomes.

III. Initial Considerations

The bone marrow of rodents is routinely used in this test since polychromatic erythrocytes are produced in that tissue. The measurement of micronucleated immature (polychromatic) erythrocytes in peripheral blood is equally acceptable in any species in which the inability of the spleen to remove micronucleated erythrocytes has been demonstrated, or which has shown an adequate sensitivity to detect agents that cause structural and/or numerical chromosome aberrations. Micronuclei can be distinguished by a number of criteria. These include identification of the presence or absence of a kinetochore or centromeric DNA in the micronuclei. The frequency of micronucleated immature (polychromatic) erythrocytes is the principal endpoint. The number of mature (normochromatic) erythrocytes in the peripheral blood that contain micronuclei among a given number of mature erythrocytes can also be used as the endpoint of the assay when animals are treated continuously for a period that exceeds the lifespan of the erythrocyte in the species under consideration (*e.g.*, 4 weeks in the mouse), provided that significant splenic selection against micronucleated erythrocytes does not occur in that species/strain. The consequences of splenic selection, if it occurs, should be fully addressed.

This mammalian *in vivo* micronucleus test is especially relevant to assessing mutagenic hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics and DNA-repair processes although these may vary among species, among tissues and among genetic endpoints. An *in vivo* assay is also useful for further investigation of a mutagenic effect detected by an *in vitro* system.

If there is evidence that the test substance, or a reactive metabolite, will not reach the target tissue, it is not appropriate to use this test.

IV. Principle of the Test Method

Animals are exposed to the test substance by an appropriate route. If bone marrow is used, the animals are sacrificed at appropriate times after treatment, the bone marrow extracted, and preparations made and stained.[16],[17],[18],[26],[32],[41] When peripheral blood is used, the blood is collected at appropriate times after treatment and smear preparations are made and stained.[4],[5],[14],[16],[27],[28],[29],[32] Preparations are analyzed for the presence of micronuclei.

V. Description of the Method

A. Preparations

1. Selection of Animal Species

Historically, mice or rats have been used routinely for this assay. If bone marrow is the tissue sampled, any appropriate mammalian species may be used (see section *III.* [2], above). As with any toxicology study, selection of the appropriate species should be justified. When peripheral blood is used, mice are recommended. However, any appropriate mammalian species may be used provided it is a species in which the spleen does not remove micronucleated erythrocytes or is a species which has shown an adequate sensitivity to detect agents that cause structural and/or numerical chromosome aberrations. Commonly used laboratory strains of young healthy animals should be employed. At the commencement of the study, the weight variation of animals should be minimal and not exceed ±20% of the mean weight of each sex.

2. Housing and Feeding Conditions

The temperature in the experimental animal room should be appropriate for the species used; for mice and rats this should be 22°C (±3°C). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this route. Animals may be housed individually, or caged in small groups of the same sex.

3. Preparation of the Animals

Healthy young adult animals should be randomly assigned to the control and treatment groups. The animals should be identified uniquely. The animals should be acclimated to the laboratory conditions for at least five days. Cages should be arranged in such a way that possible effects due to cage placement are minimized.

4. Preparation of Doses

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

B. Test Conditions

1. Solvent/Vehicle

The solvent/vehicle should not produce toxic effects at the dose levels used, and should not be suspected of chemical reaction with the test substance. If other than commonly employed solvents/vehicles are used, their use should be supported with reference data indicating their compatibility with the test [3] substance and the animals. It is recommended that, wherever appropriate, the use of an aqueous solvent/vehicle should be considered first.

2. Controls

Concurrent positive and negative (solvent/vehicle) controls should generally be included for each sex in each test conducted with rodents. However, when the micronucleus assay is conducted as part of a general toxicity study according to GLP guidelines, then verification of appropriate dosing will be performed by chemical analysis. In such cases, concurrent treatment of animals with a positive control agent may not be necessary and control of staining and scoring procedures may be accomplished by including appropriate reference samples obtained previously from animals that are not part of the current experiment. In studies with higher species, such as primates or dogs, positive controls may be omitted provided that an acceptable response to positive control substances of the species used has been demonstrated previously by the testing laboratory. In all cases, concurrent negative controls are an obligatory study component. Except for treatment with the test substance, animals in the control groups should be handled in an identical manner to animals of the treatment groups.

Positive controls should produce micronuclei *in vivo* at exposure levels expected to give a detectable, statistically significant increase over background. Positive control doses should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. It is acceptable that the positive control be administered by a route different from the test substance and sampled at only a single time. In addition, the use of chemical class-related positive control chemicals may be considered, when available. Examples of positive control substances include:

Chemical	CAS Number
Ethyl methanesulphonate	62-50-0
Ethyl nitrosourea	759-73-9
Mitomycin C	50-07-7
Cyclophosphamide (monohydrate)	50-18-0 (6055-19-2)
Triethylenemelamine	51-18-3

Negative control animals, treated with solvent or vehicle alone and otherwise treated in the same way as the treatment groups, should be included for every sampling time, except that under appropriate circumstances it may be possible to use an animal as its own control by comparing pre-treatment and post-treatment samples. If single sampling is applied for negative controls, the sampling time chosen should be justified. In addition, untreated controls should also be used unless there are (a) data available from the test laboratory, or (b) historical or published control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent/vehicle.

If peripheral blood is used, a pre-treatment sample may also be acceptable as a concurrent negative control, but only in the short peripheral blood studies (*e.g.*, 1-3 treatment(s)) when the resulting data are in the expected range for the historical control and when the absence of a solvent effect has been demonstrated.

VI. Procedure for Rats and Mice

The following sections provide guidance for procedures in mice and rats, the species used most commonly in

this assay.

A. Number and Sex of Animals

Each treated and control group should include at least 5 analyzable animals per sex.[12] If at the time of the study there are data available from studies in the same species and using the same route of exposure that demonstrate that there are no substantial differences between sexes in toxicity, then testing in a single sex will be sufficient.

B. Treatment Schedule

Several different treatment schedules (*i.e.*, 1, 2, or more treatments at 24 hr intervals) can be recommended. Samples from extended dose regimens are acceptable as long as a positive effect has been demonstrated for this study or, for a negative study, as long as toxicity has been demonstrated or the limit dose (see section "D", below) has been used, and dosing continued until the time of sampling. This is based on studies showing that repeated exposures of mice and rats of up to subchronic duration produced effects of a magnitude similar to those obtained with the traditional acute assay.[1],[2],[8],[11],[19],[21],[22],[23],[25],[29],[37],[38],[44],[48],[50] However, because there is some concern that sensitivity might be reduced in longer term studies due to a failure to achieve a true MTD, or because adaptation may occur, it is currently considered that the duration treatment should be limited to four weeks until the sensitivity of the assay is confirmed when longer treatments are used.[12]

Test substances may also be administered as a split dose, *i.e.*, two or more treatments on the same day separated by no more than a few hours, to facilitate administering a large volume of material or to minimize fluctuations in blood levels of the test article.

Two ways in which the test may be performed are:

- Animals are treated with the test substance once, or twice at an interval of not more than 24 hours. Samples of bone marrow are taken at least twice between 24 and 48 hr after the last dose, with appropriate interval(s) between samples. The use of sampling times earlier than 24 hours after treatment should be justified. Samples of peripheral blood are taken at least twice between 36 and 72 hours after the last treatment, with appropriate interval(s) between samples. When a positive response is recognized at one sampling time, additional sampling is not required.
- If three or more daily treatments are used (*e.g.*, three or more treatments at 24 hour intervals), samples may be collected once no later than 24 hours following the final treatment for the bone marrow and once no later than 40 hours following the final treatment for the peripheral blood.[12], [20]

Additional sampling times may be used, when relevant and scientifically justified.

C. Dose Levels

If a dose range finding study is performed because there are no suitable data available, it should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen to be used in the main study.[7] If there is toxicity, three dose levels should be used for the first sampling time. These dose levels should cover a range from clear toxicity to little or no toxicity. At the later sampling time only the highest dose needs to be used. The highest dose is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality. Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens) may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis. The highest dose may also be defined as a dose that produces some indication of toxicity of the bone marrow (*e.g.*, a reduction in the proportion of immature erythrocytes among total erythrocytes in the bone marrow or peripheral blood).

D. Limit Test

If no observable toxic effects result from a single treatment with one dose level of at least 2000 mg/kg body weight, or from two treatments on the same day, and if genotoxicity would not be expected based upon data from structurally related substances, then a full study using 3 dose levels may not be necessary. For studies of a longer duration, the limit dose is 2000 mg/kg/body weight/day for treatment up to 14 days, and 1000 mg/kg/body weight/day for treatment longer than 14 days. Expected human exposure may indicate the need for a higher dose level to be used in the limit test.

E. Administration of Doses

The test substance is usually administered by gavage using a stomach tube or a suitable intubation cannula,

or by intraperitoneal injection. Other routes of exposure may be acceptable where they can be justified. The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. The volume should not exceed 2 ml/100g body weight. The use of volumes higher than these must be justified. Except for irritating or corrosive substances, which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels.

F. Bone Marrow/Blood Preparation

Bone marrow cells are usually obtained from the femurs or tibias immediately following sacrifice. Commonly, cells are removed from femurs or tibias, prepared and stained using established methods. Peripheral blood is obtained from the tail vein or other appropriate blood vessel. Blood cells are immediately stained supravitally [(4),(5),(14)] or smear preparations are made and then stained. The use of a DNA specific stain (*e.g.*, acridine orange[(15)] or Hoechst 33258 plus pyronin-Y[(30)]) can eliminate some of the artifacts associated with using a non-DNA specific stain. This advantage does not preclude the use of conventional stains (*e.g.*, Giemsa). Additional systems (*e.g.*, cellulose columns to remove nucleated cells[(36)]) can also be used provided that these systems have been shown to work adequately for micronucleus preparation in the laboratory.

G. Analysis

The proportion of immature among total (immature + mature) erythrocytes is determined for each animal by counting a total of at least 200 erythrocytes for bone marrow and 1000 erythrocytes for peripheral blood.[(9)] All slides, including those of positive and negative controls, should be independently coded before microscopic analysis. At least 2000 immature erythrocytes per animal are scored for the incidence of micronucleated immature erythrocytes. Additional information may be obtained by scoring mature erythrocytes for micronuclei. When analyzing slides, the proportion of immature erythrocytes among total erythrocytes should not be less than 20% of the control value. When animals are treated continuously for 4 weeks or more, at least 2000 mature erythrocytes per animal can also be scored for the incidence of micronuclei. Systems for automated analysis (image analysis or flow cytometric analysis of cell suspensions) are acceptable alternatives to manual evaluation if appropriately justified and validated relative to classical microscopic scoring.[(12)]

VII. Procedure for Species Other than Rats and Mice

Published information based on studies in mice, rats, hamsters, swine, dogs, nonhuman primates, and humans[(3),(6),(18),(28),(31),(32),(39),(40),(45)] indicate that spontaneous and induced frequencies of micronucleated erythrocytes are similar in most mammalian species, and suggests that measurement of the incidence of micronucleated immature erythrocytes in the bone marrow is appropriate for assessing chromosomal or spindle damage in those species studied to date. Appearance and disappearance of micronucleated erythrocytes in the bone marrow is a function of the kinetics of erythrogenesis and the lifespan of erythrocytes in each species, and therefore dosing and sampling regimens must be modified in accordance with the appropriate parameters of erythrocyte kinetics for each species. Species other than mice or rats may be used when appropriate, but the following information should be included:

- Justification of the selected species, and the dosing and sampling schedules used in relation to the kinetics of erythropoiesis and the lifespan of erythrocytes in the species used;
- Evidence that the spontaneous micronucleus frequency is consistent with published information, and/or consistent within the laboratory conducting the study;
- Evidence that known genotoxicants produce an increase in micronucleus frequency in the species used, and reference values for the magnitude of the response induced;
- Impact of splenic selective removal of micronucleated cells from peripheral blood (when the latter serves as the tissue being monitored).

VIII. Data and Reporting

A. Treatment Results

Individual animal data should be presented in tabular form. The experimental unit is the animal. The number of immature erythrocytes scored, the number of micronucleated immature erythrocytes, and the number of immature among total erythrocytes should be listed separately for each animal analyzed. When animals are treated continuously for 4 weeks or more, the data on mature erythrocytes should also be given if it is collected. The proportion of immature among total erythrocytes and, if considered applicable, the percentage of micronucleated mature erythrocytes should be given for each animal. If there is no evidence for a

difference in response between the sexes, the data from both sexes may be combined for statistical analysis.

B. Evaluation and Interpretation of Results

There are several criteria for determining a positive result, such as a dose-related increase in the number of micronucleated cells or a clear increase in the number of micronucleated cells in a single dose group at a single sampling time. Statistical methods should be used to evaluate the test results.[24],[35] The statistical criteria for a positive, negative, or equivocal result should be stated clearly in the protocol. Since biological factors may modify the interpretation, statistical significance should not be the only determining factor for reaching a conclusion. Equivocal results should be clarified by further testing, using a modification of experimental conditions if appropriate.

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

Positive results in the micronucleus test indicate that a substance induces micronuclei, which are the result of chromosomal damage or damage to the mitotic apparatus in the erythroblasts of the test species. Negative results indicate that, under the test conditions, the test substance does not produce chromosomal or spindle damage leading to the formation of micronuclei in the immature erythrocytes of the test species.

The likelihood that the test substance or its metabolites reach the general circulation or specifically the target tissue (*e.g.*, systemic toxicity) should be discussed. The demonstration of adequate target tissue exposure in a negative micronucleus assay is a particularly important consideration when there is positive evidence of genotoxicity in one or more other test systems.

C. Test Report

The test report should also include the following information:

1. Test Substance

- identification data and CAS no., if known
- physical nature and purity
- physicochemical properties relevant to the conduct of the study
- stability of the test substance, if known

2. Solvent/Vehicle

- justification for choice of vehicle
- solubility and stability of the test substance in the solvent/vehicle, if known

3. Dosing solutions

- times dosing solutions were prepared and used (or interval between preparation and usage), and storage conditions
- data that verifies the concentration of the dosing solution, if available

4. Test animals

- species/strain used, including justification
- number, age and sex of animals
- source, housing conditions, diet, *etc.*
- individual weight of the animals at the start of the test, including body weight range, mean and standard deviation for each group
- information regarding the potential influence of splenic selection on the incidence of micronucleated cells in the peripheral blood, if applicable

5. Test Conditions

- positive and negative (vehicle/solvent) control data
- data from range-finding study, if conducted
- rationale for dose level selection

- details of test substance preparation
- details of the administration of the test substance
- rationale for route of administration and dosing regimen
- methods for verifying that the test substance reached the general circulation and/or target tissue, if applicable
- conversion from diet/drinking water test substance concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable
- details of food and water quality
- detailed description of treatment and sampling schedules
- methods of slide preparation
- methods for measurement of toxicity
- criteria for scoring micronucleated immature erythrocytes and, if appropriate and applicable, mature erythrocytes
- number of cells analyzed per animal
- criteria for considering studies as positive, negative or equivocal

6. Results

- signs of toxicity
- proportion of immature erythrocytes among total erythrocytes
- number of micronucleated immature erythrocytes among total immature erythrocytes, given separately for each animal
- if appropriate and applicable, number of micronucleated mature erythrocytes among total mature erythrocytes, given separately for each animal
- mean ± standard deviation of micronucleated immature and, if applicable, mature erythrocytes per group
- dose-response relationship, where possible
- statistical analyses and justification for method applied, with appropriate literature citation
- concurrent and historical negative control data
- concurrent and historical positive control data

7. Discussion of the Results

8. Conclusion

XI. Addendum: Identification of Micronuclei Derived from Acentric Fragments *vs.* Centromeric Chromosomes

Micronuclei can be formed by acentric fragments or entire chromosomes lagging in mitosis. These latter micronuclei were first recognized by their large size,[49] by C-banding[47] or by measurement of DNA content.[46] However, these methods were not very reliable. Therefore, two molecular cytogenetic methods were developed to identify the presence of centromeres in micronuclei and thereby differentiate between micronuclei of clastogenic and aneugenic origin:[12] 1) immunofluorescent CREST-staining and 2) fluorescence *in situ* hybridization (FISH) with pancentromeric DNA-probes. When it is mechanistically important to determine the presence of the kinetochore or centromere in the micronuclei, these methods can be applied.

The CREST method applied to the bone marrow micronucleus test is described in detail by Miller and Adler.[33] Cells on slides (normal bone marrow smears) are fixed, dehydrated, incubated in two steps with SDS and Triton-X, and then stained with antibody. DNA is counterstained by Hoechst 33258.

With FISH, the minor satellite DNA-probe which hybridizes close to the centromere[43] is used to identify the centromeric region, if present. A method for FISH with the centromeric DNA-probes has been described by Pinkel *et al.*[34] This method can be applied to flow sorted micronuclei-containing erythrocytes[10] or to isolated micronuclei obtained from peripheral blood samples.[13]

In control slides, the rate of labeled micronuclei containing the centromeric region is about 50%.[43] Approximately 70% of micronuclei induced by known aneugens (colchicine and vinblastine) are labeled,[33] whereas those induced by clastogens (hydroquinone and mitomycin C) only show 5-15% labeled micronuclei.

[33] To characterize the relative clastogenic vs aneugenic activity of a chemical, it is useful to use as an index the number of micronucleated polychromatic erythrocytes per 1000 polychromatic erythrocytes that contain the centromeric region.[42]

The main deficiency in the FISH methods described to date is that they do not differentiate between normochromatic and polychromatic erythrocytes.[12] Thus, only preparations in which a large fraction of the micronuclei present are induced by the test article are suitable for analysis. For example, peripheral blood samples from experiments with acute exposures in adult animals are essentially never suitable for analysis because the target cell population (immature erythrocytes) is only 3-5% of the erythrocytes in the sample.

In conclusion, CREST- or FISH-labeling are considered reliable methods to detect aneugenic properties of chemicals in the *in vivo* micronucleus assay, provided attention is paid to ensuring that appropriate samples are used for analysis.[12] However, the complexity of the current methods limits their use to those cases in which a chemical is suspected of causing spindle impairment (*e.g.*, due to the presence of large micronuclei, the induction of polyploidy, *etc.*) or when there are other specific reasons to obtain this mechanistic information.

X. References

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Draft

Chapter IV. Guidelines for Toxicity Tests

IV C 2. Acute Oral Toxicity Tests

Acute toxicity tests can provide preliminary information on the toxic nature of a material for which no other toxicology information is available. Such information can be used to:

- deal with cases of accidental ingestion of a large amount of the material (*e.g.*, for poison control information);
- determine possible target organs that should be scrutinized and/or special tests that should be conducted in repeated-dose toxicity tests; [1] and
- select doses for short-term and subchronic toxicity tests when no other toxicology information is available.

In most acute toxicity tests, each test animal is administered a single (relatively high) dose of the test substance, observed for 1 or 2 weeks for signs of treatment-related effects, then necropsied. Some acute toxicity tests (such as the "classical" LD_{50} test) are designed to determine the mean lethal dose of the test substance. The median lethal dose (or LD_{50}) is defined as the dose of a test substance that is lethal for 50% of the animals in a dose group. LD_{50} values have been used to compare relative acute hazards of industrial chemicals, especially when no other toxicology data are available for the chemicals. However, many important observations of toxicity are not represented by LD_{50} values or by slopes of dose-response curves for lethality. For example, information about morbidity and pathogenesis may have more toxicological significance than mortality, and these endpoints also should be evaluated in short term toxicity tests.

The Agency does not recommend that petitioners determine the median lethal dose (or LD_{50}) for direct food additives or color additives used in food. However, if a petitioner decides to conduct an acute oral toxicity test, alternative test protocols can provide useful information about the acute toxicity of a substance.[3] These protocols generally use fewer animals, and are thus more cost efficient, than tests designed to determine LD_{50}s.[2] The following guidelines should help the petitioner design acute oral toxicity tests when the petitioner has decided that such information is useful:

- The main focus of the acute toxicity test should be on observing the symptoms and recovery of the test animals, rather than on determining the median lethal dose (LD_{50}) of the substance.
- The rat often is used as the animal model in acute toxicity tests, but other species also may be used.
- Often only one sex is studied in an acute toxicity test; generally, the female is assumed to be more sensitive to the acute toxic effects of chemicals than the male.[1]
- Before deciding on the dose of a test compound that will be used in studying its acute toxicity, the compound's chemical and physical characteristics (including molecular weight, partition coefficient, and the toxicity of related chemicals) should be considered; otherwise, oral toxicity--including lethality--caused by relatively large doses of a chemical may have no biological relevance to the chemical's effects at lower doses. [1,5]

The following brief descriptions of oral toxicity tests may help the petitioner choose a test that meets his needs; detailed information about each type of test is available in the referenced material.

a. Limit Tests

To determine the acute toxicity of a new food additive that is not expected to be particularly toxic, 5 gm (or ml) of the compound/kg body weight of the test animal should be administered orally by gavage to several (perhaps 5) animals that have been fasted (overnight for rats, 4 hours for mice). Test animals should be observed closely for up to 14 days; symptoms of toxicity and recovery should be noted. Gross and histopathological examination of the test animals at the end of the study may help identify toxic effects on target organs. If no animals die as a result of this dose, there is no need to test higher dosages. The acute toxicity of the compound can then be expressed as being greater than 5 gm (or ml)/kg body weight of the test animal. This method is called the "limit test." In general, 5 gm or 5 ml of the test substance/kg body weight is the practical upper limit for the amount of test material that can be administered in one oral gavage dose to a rodent.

If there are deaths following administration of an acute dose of 5 gm/kg body weight, then a lower dose should be administered to several (perhaps 5) animals and the results evaluated as discussed above. For compounds expected to be acutely toxic at 5 gm/kg body weight, it would be wise to select a lower initial "limit" dose.

b. Dose-Probing Tests

Dose-probing acute toxicity protocols may have value when the petitioner has no preliminary information about the test substance that would help him select appropriate doses for toxicity studies. In a dose-probing acute toxicity test, one animal per each of 3 widely spaced dosages should be used and a sufficient observation period should follow administration of the doses. Subsequent toxicity studies may be based on the results of the dose-probing study. [1] Variations of dose-probing acute toxicity studies are described in the literature. [6,7] Other methods of determining appropriate doses for longer-term toxicity studies include a simple test wherein 3 or 4 doses are each administered to 1 or 2 test animals and the animals are observed for up to 14 days. If some of the animals die, one can estimate an approximate median lethal dose, termed ALD. [8]

c. Up-and-Down Tests

The "up-and-down" procedure involves dosing animals one at a time: First one animal at one dose, then another animal one or two days later at a higher dose (if the first animal survives) or a lower dose (if the first animal dies). This process continues until the approximate LD_{50} has been determined. One disadvantage to this test is the length of the study. Each animal should be observed for at least seven days after dosing so that delayed deaths can be recorded. However, this method usually requires only six or eight test animals as compared with the 40 to 50 test animals that may be used in the "classical" LD_{50} test. [9-11]

d. Pyramiding Tests

Pyramiding studies involve a minimum number of animals: Two animals are given successively increasing doses of the test substance on alternate days until an acutely toxic dose or some practical upward limit is reached. This test does not yield a lethality curve and often is used to assess acute toxicity in non-rodents. This test, although more like a short-term, repeated dose toxicity study than a true acute toxicity study, can provide useful preliminary information on the toxic nature of a new material for which no other toxicology information is available.

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Redbook 2000: IV.C.3.a Short-Term Toxicity Studies with Rodents

November 2003

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter IV.C.3.a. Short-Term Toxicity Studies with Rodents

Return to *Redbook 2000* table of contents [1]

This guidance represents the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

Short-term toxicity studies with rodents are generally conducted for 14 or 28 days (one month). Results of these studies (1) can help predict appropriate doses of the test substance for future subchronic or chronic toxicity studies, (2) can be used to determine NOELs for some toxicology endpoints, and (3) allow future studies in rodents to be designed with special emphasis on identified target organs. Guidelines for short-term toxicity studies with non-rodents are discussed in IV.C.3b. Sponsors/submitters of petitions/notifications are encouraged to also become familiar with the Guidance for Reporting Results of Toxicity Studies (Chapter IV.B.2.), Pathology Considerations in Toxicity Studies (Chapter IV.B.3.), and Statistical Considerations in Toxicity Studies (Chapter IV.B.4.) during the development of study design.

Scientifically justified changes to the 1993 draft "Redbook II" version of this section have been made following consultation with other authoritative guidelines and publications[1-8].

I. Good Laboratory Practice

Nonclinical laboratory studies must be conducted according to U.S. FDA good laboratory practice (GLP) regulations, issued under Part 58. Title 21. Code of Federal Regulations. This document may be obtained from the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C., 20402, (toll free 866-512-1800).

II. Test Animals

A. Care, Maintenance and Housing:

Recommendations about the care, maintenance, and housing of animals contained in NIH publication 85-23, "Guide for the Care and Use of Laboratory Animals"[8], and the DHEW publication no. 78-23 should be followed unless they conflict with specific recommendations in these guidelines.

B. Selection of Rodent Species, Strains and Sex:

These guidelines are for studies with rodents (usually rats and mice); if other species are used, modifications of these guidelines may be necessary. Both male and female test animals, that are healthy and have not been subjected to previous experimental procedures should be used.

It is important to consider the test animals' general sensitivity and the responsiveness of particular organs and tissues of test animals to toxic chemicals when selecting rodent species, strains, and substrains for toxicity studies.The selection of the use of inbred, out-bred, or hybrid rodent strains for toxicity tests should be based upon the scientific questions to be answered. Additionally, it is important that test animals come from well-characterized and healthy colonies. FDA encourages petitioners and notifiers to consult with Agency scientists before toxicity testing is begun if they have questions about the appropriateness of a particular species, strain, or substrain.

C. Age:

Testing should be performed on young animals, with dosing beginning as soon as possible after weaning, but no later than 6 to 8 weeks of age, and following an acclimation period of at least 5 days.

D. Number and Sex:

Equal numbers of males and females of each species and strain should be used for the test. For short-term toxicity studies of 30 days duration or less, experimental and control groups should have at least 10 rodents per sex per group. The number of animals that survive until the end of the study must be sufficient to permit a meaningful evaluation of toxicological effects.

E. Infected Animals:

Generally, it is not possible to treat animals for infection during the course of a study without risking interaction between the compound used for treatment and the test substance. This interaction may confound or complicate the interpretation of study results.

F. Animal Identification:

Test animals should be characterized by reference to their species, strain (and substrain), sex, age, and weight. Each animal must be assigned a unique identification number (e.g., ear tag, implanted identification chip, tatoo).

G. Caging:

Animals should be housed one per cage (single-caged). This recommendation reflects three principal concerns:

- The amount of feed consumed by each animal in the study cannot be determined when more than one animal is housed in each cage. This information is necessary in the determination of feed efficiency (relationship of feed consumed to body weight gained).
- Minimizing the possibility of confounding analyses and determining whether decreases in body weight gain are due to decreased palatability or substance mediated toxicity.
- Organs and tissues from moribund and dead animals which are single-caged would not be lost due to cannibalism.

H. Diet:

In general, feed and water should be provided *ad libitum* to rodents in toxicity studies, and the diets for these studies should meet the nutritional requirements of the species[4-7]for normal growth and reproduction. Unless special circumstances apply which justify otherwise, care should be taken to ensure that the diets of the compound treated groups of animals are isocaloric (equivalent in caloric density) with and contain the same

levels of nutrients (e.g., fiber, micronutrients) as the diets of the control group. Unrecognized or inadequately controlled dietary variables may result in nutritional imbalances or caloric deprivation that could confound interpretation of the toxicity study results (e.g., lifespan, background rates of tumor incidences) and alter the outcome and reproducibility of the studies.

The following issues are important to consider when establishing diets for animals in toxicity studies:

When the test substance has no caloric value and constitutes a substantial amount of the diet (e.g., more than 5%), both caloric and nutrient densities of the high dose diet would be diluted in comparison to the diets of the other groups. As a consequence, some high dose animals may receive higher test article doses than expected because animals fed such diluted diets *ad libitum* may eat more than animals in other dosed groups to compensate for the differences in energy and nutrient content of the high dose diets. Such circumstances make it especially important that feed consumption of these animals be as closely and accurately monitored as possible in order to determine whether changes observed could be due to overt toxicity of the test substance or to a dietary imbalance. To further aid in this assessment, two control groups can be used; one group would be fed the undiluted control diet and a second group would be fed the control diet supplemented with an inert filler (e.g., methylcellulose) at a percentage equal to the highest percentage of the test substance in the diet.

When the vehicle for the test substance is expected to have caloric and/or nutritional values, which are greater than that of the control ration, an adjustment in the caloric and/or nutritional components may be necessary.

When administration of the test substance is expected to have an effect on feed intake because of its unpleasant taste or texture, paired feeding can be used to eliminate the differences in consumption between control and compound treated groups. When a paired feeding study design is to be employed, pairs of litter-mate weanling rats of the same sex and approximate size are selected and fed the control or the experimental diet. Animals should be single-caged so that feed consumption can be determined daily, and the control animal is then fed an amount of food equal to that which the paired experimental animal ate on the preceding day. If the test substance is non-nutritive and composes a significant proportion of the diet, the pair-fed control animals should be fed an amount of its feed such that it consumes a nutritionally equivalent amount of diet as the paired experimental animal. Additionally, the study should include a second group of control animals fed *ad libitum* to ensure that the impact on any observed experimental result is due to differences in energy or nutrient intake.

When the test substance interferes with the absorption of nutrients, leading to nutritional deficiencies or changes in nutrient ratios, this can confound assessment of the toxicological endpoints under consideration. For example, fat soluble vitamins may preferentially partition with a mineral oil or fat substitute which is largely unabsorbed, such that a potential deficiency in these vitamins may result. This potential may be eliminated by additional nutrient fortification of the feed for those groups receiving the test substance. Appropriate levels of nutrient fortification should be determined experimentally.

It may be preferable to use a semi-purified diet prepared with known amounts of well-characterized ingredients for short-term and subchronic toxicity studies because of batch to batch variations in diet composition (e.g., fiber, mineral, vitamins, isoflavones) in some of the commonly used laboratory animal chows. The use of these semi-purified diets, however, may not be advisable in long-term and reproductive studies due to inadequate historical data related to their influences on animal survival and toxicological endpoints. For example, loss of necessary but unidentified micronutrients in the semipurified diet may interfere with normal reproduction.

Related issues are discussed in the section on Diets for Toxicity Studies in Chapter IV.B.5. in the 1993 draft "Redbook II"

I. Assignment of Control and Compound Treated Animals:

Animals should be assigned to control and compound treated groups in a stratified random manner; this will help minimize bias and assure comparability of pertinent variables across compound treated and control groups (for example: mean body weights and body weight ranges). If other characteristics are used as the basis for randomization then that characterization should be described and justified.

Animals in all groups should be placed on study on the same day; if this is not possible because of the large number of animals in a study, animals may be placed on study over several days. If the latter recommendation is followed, a preselected portion of the control and experimental animals should be placed on the study each day in order to maintain concurrence.

J. Mortality:

Excessive mortality due to poor animal management is unacceptable and may be cause to repeat the study. For example, under normal circumstances, mortality in the control group should not exceed 10%.

K. Autolysis:

Adequate animal husbandry practices should result in considerably less than 10% of animals and tissues or organs lost to a study because of autolysis. Autolysis in excess of this standard may be cause to repeat the study.

L. Necropsy:

Necropsy should be performed soon after an animal is sacrificed or found dead, so that loss of tissues due to autolysis is minimized. When necropsy cannot be performed immediately, the animal should be refrigerated at a temperature that is low enough to prevent autolysis but not so low as to cause cell damage. If histopathological examination is to be conducted, tissue specimens should be taken from the animals and placed in appropriate fixatives when the necropsy is performed.

III. Test Substance

The test substance used in toxicity studies should be the same substance that the petitioner/notifier intends to market. A single lot of test substance should be used throughout the study, when possible. Alternatively, lots that are similar in purity and composition should be used.

A. Identity:

The identity of the test substance or mixture of substances to be tested should be known. We urge petitioners/notifiers to consult with the Agency in determination of test compound and to provide a Chemical Abstract Service (CAS) Registry Number or Numbers.

B. Composition/Purity:

The composition of the test substance should be known including the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials.

C. Conditions of Storage:

The test sample should be stored under conditions that maintain its stability, quality, and purity until the studies are complete.

D. Expiration Date:

The expiration date of the test material should be known and easily available. Test materials should not be used past their expiration date.

IV. Experimental Design

A. Duration of Testing:

Animals should be exposed to the test substance 7 days per week for the duration of the study (from 2 to 4 consecutive weeks).

B. Route of Administration:

The route of administration of the test substance should approximate that of normal human exposure, if possible. For food ingredients (e.g., food and color additives) the oral route of administration is preferred. A justification should be provided when other routes are used. The same method of administration should be used for all test animals throughout the study.

The test substance should be administered in one of the following ways:

- **In the diet**, if human exposure to the test substance is likely to be through consumption of solid foods or a combination of solid and liquid foods. If the test substance is added to the diet, animals should not be able to selectively consume either basal diet or test substance in the diet on the basis of color, smell,

or particle size. If the compound is mixed with ground feed and pelleted, nothing in the pelleting process should affect the test substance (for example, heat-labile substances may be destroyed during pellet production by a steam process). When the test substance is administered in the diet, dietary levels should be expressed as mg of the test substance per kg of feed.

- **Dissolved in the drinking water**, if the test substance is likely to be ingested in liquid form (for example, in soft drinks or beer), or if administration in the diet is inappropriate for other reasons. The amount of test substance administered in drinking water should be expressed as mg of test substance per ml of water.
- **By encapsulation or oral intubation (gavage)**, if the two previous methods are unsatisfactory or if human exposure is expected to be through daily ingestion of single, large doses instead of continual ingestion of small doses. If the test substance is administered by gavage, it should be given at approximately the same time each day. The maximum volume of solution that can be given by gavage in one dose depends on the test animal's size; for rodents, the volume ordinarily should not exceed 1 ml/100 g body weight. If the gavage vehicle is oil (see Chapter IV.B.5.b. in the 1993 draft "Redbook II"), then the volume should be no more than 0.4 ml/100 g of body weight, and the use of a low-fat diet should be considered. If the test substance must be given in divided doses, all doses should be administered within a 6 hour period. Doses of test substance administered by gavage should be expressed as mg of test substance per ml of gavage vehicle. Finally, the petitioner/notifier should provide information that can allow the reviewer to conclude that administration of the test compound by encapsulation or gavage is equivalent in all toxicologically important respects to administration in the diet or drinking water. Alternatively, metabolic information on both modes of administration should be provided so that appropriate interpretation of data can be accomplished.

C. Dose groups:

A minimum of three dose levels of the test substance, per sex, should be used, however 4 or 5 dose levels of the test substance is preferred. A concurrent control group should be included.

1. Selection of Treatment Doses:

Dose selection for toxicity studies should be based on information related to the toxicity of the test substance.

A minimum of three dose levels of the test substance and a concurrent control group should be used in toxicity studies. When designing and conducting toxicity studies the following should be considered: 1) the high dose should be sufficiently high to induce toxic responses in test animals; 2) the low dose should not induce toxic responses in test animals; and 3) the intermediate dose should be sufficiently high to elicit minimal toxic effects in test animals (such as alterations in enzyme levels or slight decreases in body weight gains). No dose should cause an incidence of fatalities that prevents meaningful evaluation of the data. Administration of the test substance to all dose groups should be done concurrently.

2. Controls:

A concurrent control group of test animals is required. The control group in dietary studies should be fed the basal diet. Exceptions to this and other related information, including a discussion regarding pair-feeding, was provided above in section "II Test Animals, H. Diet".

A carrier or vehicle for the test substance should be given to control animals at a volume equal to the maximum volume of carrier or vehicle given to any dosed group of animals. Sufficient toxicology information should be available on the carrier or vehicle to ensure that its use will not compromise the results of the study. If there is insufficient information about the toxic properties of the vehicle used to administer the test substance, an additional control group that is not exposed to the carrier or vehicle should be included. In all other respects, animals in the control group should be treated the same as animals in dosed groups. (See additional information in section "II Test Animals, H. Diet" above.)

D. Computerized systems

Computerized systems that are used in the generation, measurement, or assessment of data should be developed, validated, operated, and maintained in ways that are compliant with Good Laboratory Practice principles.[9]

V. Observations and Clinical Tests

A. Observations of Test Animals:

Routine cage-side observations should be made on all animals at least once or twice a day throughout the study for general signs of pharmacologic and toxicologic effects, morbidity and mortality. The usual interval between observations should be at least 6 hours. Individual records should be maintained for each animal and the time of onset and the characteristics and progression of any effects should be recorded, preferably using a scoring system.

An expanded set of clinical evaluations, performed inside and outside of the cage, should be carried out in short-term and subchronic toxicity studies in rodents and non-rodents, in one-year non-rodent toxicity studies, and reproductive toxicity studies in rodents to enable detection not only of general pharmacologic and toxicologic effects but also of neurologic disorders, behavioral changes, autonomic dysfunctions, and other signs of nervous system toxicity. Specific information about the systematic clinical tests/observations is contained in Chapter IV.C.10. This expanded set of clinical examinations (Chapter IV.C.10.), conducted inside and outside the cage, should be age appropriate and performed on all animals at least once prior to initiation of treatment, and periodically during treatment. Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g., lacrimation, pilorection, pupil size, unusual respiratory pattern). Additionally, changes in gait, posture and response to handling, as well as the presence of clonic or tonic movements, stereotypes (e.g., excessive grooming, repetitive circling) or bizarre behavior (e.g., self-mutilating, walking backwards) should be recorded. Tumor development, particularly in long-term studies, should be followed: the time of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumor should be recorded. During the course of a study, toxic and pharmacologic signs may suggest the need for additional clinical tests or expanded post-mortem examinations.

B. Body Weight and Feed Intake Data:

Test animals should be weighed at least once a week. Feed consumption (or water consumption if the test substance is administered in the drinking water) should be measured every week during the short-term toxicity test. Petitioners/notifiers should also attempt to quantify spillage of feed by test animals, and to determine if spillage is greater with test diets than with control diets. Appropriate discussions of feed spillage should be included in the study report.

C. Clinical Testing:

Ophthalmological examination, hematology profiles, clinical chemistry tests, and urinalyses should be performed as described in the following sections:

1. **Opthalmological Examination:** This examination should be performed by a qualified individual on all animals before the study begins and on control and high-dose animals at the end of the study. If the results of examinations at termination indicate that changes in the eyes may be associated with administration of the test substance, ophthalmological examinations should be performed on all animals in the study.
2. **Hematology**: For rodents, hematologic tests should be performed on 10 animals of each sex per group. Sampling of test animals should be conducted during the first two weeks on study (receiving treatment) and at the end of the study. The determination of the first sampling time point should be based on the expected time of initial toxicological effects on the organ systems. Ideally, samples should be obtained from the same animals during the study and at termination. Blood should be drawn at approximately the same time each sampling day and blood samples should be analyzed individually, and not pooled.
    1. The following determinations are recommended:
        1. hematocrit
        2. hemoglobin concentration
        3. erythrocyte count
        4. total and differential leukocyte counts
        5. mean corpuscular hemoglobin
        6. mean corpuscular volume
        7. mean corpuscular hemoglobin concentration
        8. and a measure of clotting potential (such as clotting time, prothrombin time, thromboplastin

time, or platelet count).

Test compounds may have an effect on the hematopoietic system and therefore appropriate measures should be employed so that evaluations of reticulocyte counts and bone marrow cytology may be performed if warranted. Reticulocyte counts should be obtained for each animal using automated reticulocyte counting capabilities, or from air-dried blood smears. Bone marrow slides should be prepared from each animal for evaluating bone marrow cytology. These slides would only need to be examined microscopically if effects on the hematopoietic system were noted.

3. **Clinical Chemistry**: Ideally, the same animals should be sampled at blood collection time points. For rodents, clinical chemistry tests should be performed on 10 animals of each sex per group. Sampling of test animals should be conducted during the first two weeks on study (receiving treatment) and at the end of the study. The determination of the first sampling time point should be based on the expected time of initial toxicological effects on the organ systems. Blood samples should be drawn at the end of the fasting time and before feeding. Fasting duration should be appropriate for the species and the analytical tests to be performed. Blood should be drawn at approximately the same time on each sampling day, and blood samples should be analyzed individually, and not pooled.

   Clinical chemistry tests that are appropriate for all test substances include measurements of electrolyte balance, carbohydrate metabolism, and liver and kidney function. Specific determinations should include:

   1. Hepatocellular evaluation: select at least 3 of the following 5
      1. alanine aminotransferase (SGPT, ALT)
      2. aspartate aminotransferase (SGOT, AST)
      3. sorbitol dehydrogenase
      4. glutamate dehydrogenase
      5. total bile acids
   2. Hepatobiliary evaluation: select at least 3 of the following 5
      1. alkaline phosphatase
      2. bilirubin (total)
      3. gamma-glutamyl transpeptidase (GG transferase)
      4. 5' nucleotidase
      5. total bile acids
   3. Other markers of cell changes or cellular function
      1. albumin
      2. calcium
      3. chloride
      4. cholestrol(total)
      5. cholinesterase
      6. creatinine
      7. globulin (calculated)
      8. glucose (in fasted animals)
      9. phosphorous
      10. potassium
      11. protein (total)
      12. sodium
      13. triglycerides (fasting)
      14. urea nitrogen

   1. However, when adequate volumes of blood cannot be obtained from test animals, the following determinations should generally be given priority. FDA understands that the specific nature of the test compound may warrant the consideration of alternative tests. Appropriate justification for alternative tests should be presented in study reports.

1. alanine aminotransferase
2. alkaline phosphatase
3. chloride
4. creatinine
5. gamma-glutamyl transpeptidase (GG transferase)
6. glucose (in fasted animals)
7. potassium
8. protein (total)
9. sodium
10. urea nitrogen

Additional clinical chemistry tests may be recommended to extend the search for toxic effects attributable to a test substance. The selection of specific tests will be influenced by observations on the mechanism of action of the test substance. Clinical chemistry determinations that may be recommended to ensure adequate toxicological evaluation of the test substance include analyses of acid/base balance, hormones, lipids, methemoglobin, and proteins.

In spite of standard operating procedures and equipment calibration, it is not unusual to observe considerable variation in the results of clinical chemistry analyses from day to day[10]. Ideally, clinical chemistry analyses for all dose groups should be completed during one day. If this is not possible, analyses should be conducted in such a way as to minimize potential variability.

4. **Urinalyses**: Timed urine volume collection should be conducted during the last week of the study. For rodents, these tests should be performed on 10 animals of each sex in each group. The volume of urine collected, specific gravity, pH, glucose, and protein should be determined as well as conducting a microscopic evaluation of urine for sediment and presence of blood/blood cells.[11]
5. **Neurotoxicity Screening/Testing** : Screening for neurotoxic effects should be routinely carried out in all short-term toxicity studies with rodents (preferably rats). The neurotoxicity screen should be age appropriate and would typically include: (1) a specific histopathological examination of tissue samples representative of major areas of the brain, spinal cord, and peripheral nervous system (see organs and tissues listed below under VI.C. Preparation of Tissues for Microscopic Examination) and (2) a functional battery of quantifiable observations and manipulative tests selected to detect signs of neurological, behavioral, and physiological dysfunctions. This functional battery is also referred to as an expanded set of clinical evaluations and is described more fully in section V.A. Observations of Test Animals in this Chapter and in Chapter IV.C.10. Neurotoxicity Studies.

   Short-term toxicity study reports should include an assessment of the potential for the test substance to adversely affect the structural or functional integrity of the nervous system. This assessment should evaluate data from the neurotoxicity screen and other toxicity data from the study, as appropriate. Based on this assessment, the petitioner should make an explicit statement about whether or not the test substance presents a potential neurotoxic hazard and if additional neurotoxicity testing is deemed appropriate. FDA recommends that additional neurotoxicity testing not be undertaken without first consulting with the Agency.
6. **Immunotoxicity**: Results from tests that are included in the list of primary indicators for immune toxicity (see Chapter V.C. of the 1993 draft "Redbook II") should also be evaluated as part of an immunotoxicity screen. Reports of short-term toxicity tests should include an assessment of the potential for the test substance to adversely affect the immune system. This assessment should evaluate data from the list of primary indicators included in the immunotoxicity screen and other toxicity data from the study, as appropriate. Based on this assessment, the petitioner/notifier should make an explicit statement about whether or not the test substance presents a potential immunotoxic hazard which requires further immunotoxicity testing. Additional immunotoxicity tests are discussed in Chapter V. C. of the 1993 draft "Redbook II", but should not be undertaken without first consulting with the Agency.

VI. Necropsy and Microscopic Examination

A. Gross Necropsy

All test animals should be subjected to complete gross necropsy, including examination of external surfaces, orifices, cranial, thoracic and abdominal cavities, carcass, and all organs. The gross necropsy should be performed by, or under the direct supervision of, a qualified pathologist, preferably the pathologist who will later perform the microscopic examination (see below).

B. Organ Weight

Organs that should be weighed include the adrenals, brain, epididymides, heart, kidneys, liver, spleen, testes, thyroid/parathyroid, thymus, ovaries and uterus. Organs should be carefully dissected and trimmed to remove fat and other contiguous tissue and then be weighed immediately to minimize the effects of drying on organ weight.

C. Preparation of Tissues for Microscopic Examination

Generally, the following tissues should be fixed in 10% buffered formalin (or another generally recognized fixative) and sections prepared and stained with hematoxylin and eosin (or another appropriate stain) in preparation for microscopic examination. Lungs should be inflated with fixative prior to immersion in fixative.

1. adrenals
2. aorta
3. bone (femur)
4. bone marrow (sternum)
5. brain (at least 3 different levels)
6. cecum
7. colon
8. corpus and cervix uteri
9. duodenum
10. epididymis
11. esophagus
12. eyes
13. gall bladder (if present)
14. Harderian gland
15. heart
16. ileum
17. jejunum
18. kidneys
19. liver
20. lung (with main-stem bronchi)
21. lymph nodes (1 related to route of administration and 1 from a distant location)
22. mammary glands
23. nasal turbinates
24. ovaries and fallopian tubes
25. pancreas
26. pituitary
27. prostate
28. rectum
29. salivary gland
30. sciatic nerve
31. seminal vesicle
32. skeletal muscle
33. skin

34. spinal cord (3 locations: cervical, mid-thoracic, lumbar)
35. spleen
36. stomach
37. testes
38. thymus (or thymic region)
39. thyroid/parathyroid
40. trachea
41. urinary bladder
42. vagina
43. Zymbal's gland
44. all tissues showing abnormality

D. Microscopic Evaluation

All gross lesions should be examined microscopically. All tissues from the animals in the control and high dose groups should be examined. If treatment related effects are noted in certain tissues, then the next lower dose level tested of those specific tissues should be examined. Successive examination of the next lower dose level continues until no effects are noted. In addition, all tissues from animals which died prematurely or were sacrificed during the study should be examined microscopically to assess any potential toxic effects.

E. Histopathology of Lymphoid Organs

Histopathological evaluation of the lymphoid organs should be performed as described in the section on immunotoxicity testing (see Chapter V. C.) for all animals.

VII. References

1. EPA Health Effects Test Guidelines OPPTS 870.3050, 28-Day Oral Toxicity in Rodents, July 2000
2. OECD Guideline For The Testing Of Chemicals , Repeated Dose 28-day Oral Toxicity Study in Rodents, 407, July 1995
3. Kurt Weingand et al, Harmonization of Animal Clinical Pathology Testing in Toxicity and Safety Studies, Fundamental and Applied Toxicology 29, pp 198-201, 1996
4. Nutrient Requirements of Laboratory Animals, Fourth Revised Edition, Subcommittee on Laboratory Animal Nutrition, Committee on Animal Nutrition, Board on Agriculture, National Research Council, 1995.
5. Nutrient Requirements of Dogs, Revised, Committee on Animal Nutrition, National Research Council, 1985.
6. Nutrient Requirements of Swine: 10th Revised Edition, Subcommittee on Swine Nutrition, Committee on Animal Nutrition, National Research Council, 1998.
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11. Ragan, H.A. and Weller, R.E., "Markers of Renal Function and Injury" in *The Clinical Chemistry of Laboratory Animals*, Second Edition 1999, Taylor & Francis, Philadelphia, PA, pp. 520-533

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Redbook 2000: IV.C.3.b Short-Term Toxicity Studies with Non-Rodents

November 2003

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter IV.C.3.b. Short-Term Toxicity Studies with Non-Rodents

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Short-term toxicity studies with non-rodents (usually dogs) are generally conducted for 14 or 28 days (one month). Results of these studies (1) can help predict appropriate doses of the test substance for future subchronic or chronic toxicity studies, (2) can be used to determine NOELs for some toxicology endpoints, and (3) allow future studies in rodents and non-rodents to be designed with special emphasis on identified target organs. Guidelines for short-term toxicity studies with rodents are discussed in IV.C.3a. Sponsors/submitters of petitions/notifications are encouraged to also become familiar with the Guidance for Reporting Results of Toxicity Studies (Chapter IV.B.2.), Pathology Considerations in Toxicity Studies (Chapter IV.B.3.), and Statistical Considerations in Toxicity Studies (Chapter IV.B.4.) during the development of study design.

Scientifically justified changes to the 1993 draft "Redbook II" version of this section have been made following consultation with other authoritative guidelines and publications[1-7].

I. Good Laboratory Practice

Nonclinical laboratory studies must be conducted according to U.S. FDA good laboratory practice (GLP) regulations, issued under Part 58. Title 21. Code of Federal Regulations. This document may be obtained from the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C., 20402, (toll free 866-512-1800).

II. Test Animals

A. Care, Maintenance and Housing:

Recommendations about the care, maintenance, and housing of animals contained in NIH publication 85-23, "Guide for the Care and Use of Laboratory Animals"[7], and the DHEW publication no. 78-23 should be followed unless they conflict with specific recommendations in these guidelines.

B. Selection of Species, Strains and Sex:

These guidelines are for studies with non-rodents (usually dogs); if other species are used, modifications of these guidelines may be necessary. Both male and female test animals, that are healthy and have not been subjected to previous experimental procedures should be used.

It is important to consider the test animals' general sensitivity and the responsiveness of particular organs and tissues of test animals to toxic chemicals when selecting species and strains for toxicity studies. Test animals should be selected that are likely to survive the recommended duration of the study. It is important that test animals come from well-characterized and healthy colonies. FDA encourages petitioners and notifiers to consult with Agency scientists before toxicity testing is begun if they have questions about the appropriateness of a particular species or strain.

C. Age:

Testing should be performed on young animals, with dosing beginning as soon as possible after weaning, and following an acclimation period of at least 5 days. Testing should begin when dogs are no older than 4 to 6 months of age.

D. Number and Sex:

Equal numbers of males and females of each species and strain should be used for the test. For short-term toxicity studies of 30 days duration or less, experimental and control groups should have at least 4 dogs per sex per group. If the study will be used to determine appropriate doses for longer-term dog studies, but will not be used to determine a NOEL for the test substance, experimental and control groups may have 2 dogs per sex per group. The number of animals that survive until the end of the study must be sufficient to permit a meaningful evaluation of toxicological effects.

E. Infected Animals:

Generally, it is not possible to treat animals for infection during the course of a study without risking interaction between the compound used for treatment and the test substance. This interaction may confound or complicate the interpretation of study results.

F. Animal Identification:

Test animals should be characterized by reference to their species, strain (and substrain), sex, age, and weight. Each animal must be assigned a unique identification number (e.g., ear tag, implanted identification chip, tatoo).

G. Caging:

Animals should be housed one per cage or run (single-caged). This recommendation reflects three principal concerns:

- The amount of feed consumed by each animal in the study cannot be determined when more than one animal is housed in each cage. This information is necessary in the determination of feed efficiency (relationship of feed consumed to body weight gained).
- Minimizing the possibility of confounding analyses and determining whether decreases in body weight gain are due to decreased palatability or substance mediated toxicity.
- Organs and tissues from moribund and dead animals which are single-caged would not be lost due to cannibalism.

H. Diet:

Diets for short-term studies should meet the nutritional requirements of the species[3-6]for normal growth and reproduction. In general, water should be provided *ad libitum* . Unless special circumstances apply which justify otherwise, care should be taken to ensure that the diets of the compound treated groups of animals are isocaloric (equivalent in caloric density) with and contain the same levels of nutrients (e.g., fiber,

micronutrients) as the diets of the control group. Unrecognized or inadequately controlled dietary variables may result in nutritional imbalances or caloric deprivation that could confound interpretation of the toxicity study results (e.g., lifespan, background rates of tumor incidences) and alter the outcome and reproducibility of the studies.

The following issues are important to consider when establishing diets for animals in toxicity studies:

When the test substance has no caloric value and constitutes a substantial amount of the diet (e.g., more than 5%), both caloric and nutrient densities of the high dose diet would be diluted in comparison to the diets of the other groups. It is important that feed consumption of animals be as closely and accurately monitored as possible in order to determine whether changes observed could be due to overt toxicity of the test substance or to a dietary imbalance. To further aid in this assessment, two control groups can be used; one group would be fed the undiluted control diet and a second group would be fed the control diet supplemented with an inert filler (e.g., methylcellulose) at a percentage equal to the highest percentage of the test substance in the diet.

When the vehicle for the test substance is expected to have caloric and/or nutritional values, which are greater than that of the control ration, an adjustment in the caloric and/or nutritional components may be necessary.

When administration of the test substance is expected to have an effect on feed intake because of its unpleasant taste or texture, paired feeding can be used to eliminate the differences in consumption between control and compound treated groups. When a paired feeding study design is to be employed, pairs of animals of the same sex, age, and approximate size are selected and fed the control or the experimental diet. Animals should be single-caged so that feed consumption can be determined daily, and the control animal is then fed an amount of food equal to that which the paired experimental animal ate on the preceding day. If the test substance is non-nutritive and composes a significant proportion of the diet, the pair-fed control animals should be fed an amount of its feed such that it consumes a nutritionally equivalent amount of diet as the paired experimental animal. Additionally, the study should include a second group of control animals fed the normal ration amount to ensure that the impact on any observed experimental result is due to differences in energy or nutrient intake.

When the test substance interferes with the absorption of nutrients, leading to nutritional deficiencies or changes in nutrient ratios, this can confound assessment of the toxicological endpoints under consideration. For example, fat soluble vitamins may preferentially partition with a mineral oil or fat substitute which is largely unabsorbed, such that a potential deficiency in these vitamins may result. This potential may be eliminated by additional nutrient fortification of the feed for those groups receiving the test substance. Appropriate levels of nutrient fortification should be determined experimentally.

It may be preferable to use a semi-purified diet prepared with known amounts of well-characterized ingredients for short-term toxicity studies because of batch to batch variations in diet composition (e.g., fiber, mineral, vitamins, isoflavones) in some of the commonly used laboratory animal chows. The use of these semi-purified diets, however, may not be advisable in long-term and reproductive studies due to inadequate historical data related to their influences on animal survival and toxicological endpoints. For example, loss of necessary but unidentified micronutrients in the semipurified diet may interfere with normal reproduction.

FDA recommends reviewing the diet section in IV.C.3.a. when non-rodents (e.g., rabbits) are to be fed *ad libitum*. Related issues are discussed in the section on Diets for Toxicity Studies in Chapter IV.B.5. in the 1993 draft "Redbook II"

I. Assignment of Control and Compound Treated Animals:

Animals should be assigned to control and compound treated groups in a stratified random manner; this will help minimize bias and assure comparability of pertinent variables across compound treated and control groups (for example: mean body weights and body weight ranges). If other characteristics are used as the basis for randomization then that characterization should be described and justified.

Animals in all groups should be placed on study on the same day.

J. Mortality:

Excessive mortality due to poor animal management is unacceptable and may be cause to repeat the study.

K. Autolysis:

Adequate animal husbandry practices should be employed so that tissues and/or organs are not lost to a study because of autolysis.

L. Necropsy:

Necropsy should be performed soon after an animal is sacrificed or found dead, so that loss of tissues due to autolysis is minimized. When necropsy cannot be performed immediately, the animal should be refrigerated at a temperature that is low enough to prevent autolysis but not so low as to cause cell damage. If histopathological examination is to be conducted, tissue specimens should be taken from the animals and placed in appropriate fixatives when the necropsy is performed.

III. Test Substance

The test substance used in toxicity studies should be the same substance that the petitioner/notifier intends to market. A single lot of test substance should be used throughout the study, when possible. Alternatively, lots that are similar in purity and composition should be used.

A. Identity:

The identity of the test substance or mixture of substances to be tested should be known. We urge petitioners/notifiers to consult with the Agency in determination of test compound and to provide a Chemical Abstract Service (CAS) Registry Number or Numbers.

B. Composition/Purity:

The composition of the test substance should be known including the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials.

C. Conditions of Storage:

The test sample should be stored under conditions that maintain its stability, quality, and purity until the studies are complete.

D. Expiration Date:

The expiration date of the test material should be known and easily available. Test materials should not be used past their expiration date.

IV. Experimental Design

A. Duration of Testing:

Animals should be exposed to the test substance 7 days per week for the duration of the study (from 2 to 4 consecutive weeks).

B. Route of Administration:

The route of administration of the test substance should approximate that of normal human exposure, if possible. For food ingredients (e.g., food and color additives) the oral route of administration is preferred. A justification should be provided when other routes are used. The same method of administration should be used for all test animals throughout the study.

The test substance should be administered in one of the following ways:

- **In the diet**, if human exposure to the test substance is likely to be through consumption of solid foods or a combination of solid and liquid foods. If the test substance is added to the diet, animals should not be able to selectively consume either basal diet or test substance in the diet on the basis of color, smell, or particle size. If the compound is mixed with ground feed and pelleted, nothing in the pelleting process should affect the test substance (for example, heat-labile substances may be destroyed during pellet production by a steam process). When the test substance is administered in the diet, dietary levels should be expressed as mg of the test substance per kg of feed.
- **Dissolved in the drinking water**, if the test substance is likely to be ingested in liquid form (for example, in soft drinks or beer), or if administration in the diet is inappropriate for other reasons. The amount of test substance administered in drinking water should be expressed as mg of test substance per ml of water.
- **By encapsulation or oral intubation (gavage)**, if the two previous methods are unsatisfactory or if

human exposure is expected to be through daily ingestion of single, large doses instead of continual ingestion of small doses. If the test substance is administered by gavage, it should be given at approximately the same time each day. The maximum volume of solution that can be given by gavage in one dose depends on the test animal's size. Doses of test substance administered by gavage should be expressed as mg of test substance per ml of gavage vehicle. Finally, the petitioner/notifier should provide information that can allow the reviewer to conclude that administration of the test compound by encapsulation or gavage is equivalent in all toxicologically important respects to administration in the diet or drinking water. Alternatively, metabolic information on both modes of administration should be provided so that appropriate interpretation of data can be accomplished. (See additional information in Chapter IV.B.5. in the 1993 draft "Redbook II".)

C. Dose groups:

A minimum of three dose levels of the test substance, per sex, should be used, however 4 or 5 dose levels of the test substance is preferred. A concurrent control group should be included.

1. Selection of Treatment Doses:

Dose selection for toxicity studies should be based on information related to the toxicity of the test substance.

A minimum of three dose levels of the test substance and a concurrent control group should be used in toxicity studies. When designing and conducting toxicity studies the following should be considered: 1) the high dose should be sufficiently high to induce toxic responses in test animals; 2) the low dose should not induce toxic responses in test animals; and 3) the intermediate dose should be sufficiently high to elicit minimal toxic effects in test animals (such as alterations in enzyme levels or slight decreases in body weight gains). No dose should cause an incidence of fatalities that prevents meaningful evaluation of the data. Administration of the test substance to all dose groups should be done concurrently.

2. Controls:

A concurrent control group of test animals is required. The control group in dietary studies should be fed the basal diet. Exceptions to this and other related information, including a discussion regarding pair-feeding, was provided above in section "II Test Animals, H. Diet".

A carrier or vehicle for the test substance should be given to control animals at a volume equal to the maximum volume of carrier or vehicle given to any dosed group of animals. Sufficient toxicology information should be available on the carrier or vehicle to ensure that its use will not compromise the results of the study. If there is insufficient information about the toxic properties of the vehicle used to administer the test substance, an additional control group that is not exposed to the carrier or vehicle should be included. In all other respects, animals in the control group should be treated the same as animals in dosed groups. (See additional information in section "II Test Animals, H. Diet" above.)

D. Computerized systems

Computerized systems that are used in the generation, measurement, or assessment of data should be developed, validated, operated, and maintained in ways that are compliant with Good Laboratory Practice principles. [8]

V. Observations and Clinical Tests

A. Observations of Test Animals:

Routine cage-side observations should be made on all animals at least once or twice a day throughout the study for general signs of pharmacologic and toxicologic effects, morbidity and mortality. The usual interval between observations should be at least 6 hours. Individual records should be maintained for each animal and the time of onset and the characteristics and progression of any effects should be recorded, preferably using a scoring system.

An expanded set of clinical evaluations, performed inside and outside of the cage, should be carried out in short-term and subchronic toxicity studies in rodents and non-rodents, in one-year non-rodent toxicity studies, and reproductive toxicity studies in rodents to enable detection not only of general pharmacologic and toxicologic effects but also of neurologic disorders, behavioral changes, autonomic dysfunctions, and other signs of nervous system toxicity. Specific information about the systematic clinical tests/observations is contained in Chapter IV.C.10. This expanded set of clinical examinations (Chapter IV.C.10), conducted inside

and outside the cage, should be age appropriate and performed on all animals at least once prior to initiation of treatment, and periodically during treatment. Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions or other evidence of autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern). Additionally, changes in gait, posture and response to handling, as well as the presence of clonic or tonic seizures, stereotypes (e.g., excessive grooming, repetitive circling) or bizarre behavior (e.g., self-mutilating, walking backwards) should be recorded. Tumor development, particularly in long-term studies, should be followed: the time of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumor should be recorded. During the course of a study, toxic and pharmacologic signs may suggest the need for additional clinical tests or expanded post-mortem examinations.

B. Body Weight and Feed Intake Data:

Test animals should be weighed at least once a week. Feed consumption (or water consumption if the test substance is administered in the drinking water) should be measured every week during the short-term toxicity test. Petitioners/notifiers should also attempt to quantify spillage of feed by test animals, and to determine if spillage is greater with test diets than with control diets. Appropriate discussions of feed spillage should be included in the study report.

C. Clinical Testing:

Ophthalmological examination, hematology profiles, clinical chemistry tests, and urinalyses should be performed as described in the following sections:

1. **Opthalmological Examination:** This examination should be performed by a qualified individual on all animals before the study begins and on control and high-dose animals at the end of the study. If the results of examinations at termination indicate that changes in the eyes may be associated with administration of the test substance, ophthalmological examinations should be performed on all animals in the study.
2. **Hematology**: For non-rodents (e.g., dogs and mini swine) hematologic tests should be performed on all animals of each sex per group. Sampling of test animals should be conducted prior to initiation of treatment, during the first two weeks on study (receiving treatment), and at the end of the study. The determination of the second sampling time point should be based on the expected time of initial toxicological effects on the organ systems. Blood should be drawn at approximately the same time each sampling day, and blood samples should be analyzed individually, and not pooled.

   Test compounds may have an effect on the hematopoietic system and therefore appropriate measures should be employed so that evaluations of reticulocyte counts and bone marrow cytology may be performed if warranted. Reticulocyte counts should be obtained for each animal using automated reticulocyte counting capabilities, or from air-dried blood smears. Bone marrow slides should be prepared from each animal for evaluating bone marrow cytology. These slides would only need to be examined microscopically if effects on the hematopoietic system were noted.
3. **Clinical Chemistry**: For non-rodents (e.g., dogs and mini swine) hematologic tests should be performed on all animals of each sex per group. Sampling of test animals should be conducted prior to initiation of treatment, during the first two weeks on study (receiving treatment), and at the end of the study. The determination of the second sampling time point should be based on the expected time of initial toxicological effects on the organ systems. Blood samples should be drawn at the end of the fasting time and before feeding. Fasting duration should be appropriate for the species and the analytical tests to be performed. Blood should be drawn at approximately the same time on each sampling day, and blood samples should be analyzed individually, and not pooled.

   Clinical chemistry tests that are appropriate for all test substances include measurements of electrolyte balance, carbohydrate metabolism, and liver and kidney function. Specific determinations should include:

   1. Hepatocellular evaluation: select at least 3 of the following 5
      1. alanine aminotransferase (SGPT, ALT)
      2. aspartate aminotransferase (SGOT, AST)
      3. sorbitol dehydrogenase
      4. glutamate dehydrogenase
      5. total bile acids

2. Hepatobiliary evaluation: select at least 3 of the following 5
    1. alkaline phosphatase
    2. bilirubin (total)
    3. gamma-glutamyl transpeptidase (GG transferase)
    4. 5' nucleotidase
    5. total bile acids
3. Other markers of cell changes or cellular function
    1. albumin
    2. calcium
    3. chloride
    4. cholestrol(total)
    5. cholinesterase
    6. creatinine
    7. globulin (calculated)
    8. glucose (in fasted animals)
    9. phosphorous
    10. potassium
    11. protein (total)
    12. sodium
    13. triglycerides (fasting)
    14. urea nitrogen

1. However, when adequate volumes of blood cannot be obtained from test animals, the following determinations should generally be given priority. FDA understands that the specific nature of the test compound may warrant the consideration of alternative tests. Appropriate justification for alternative tests should be presented in study reports.

    1. alanine aminotransferase
    2. alkaline phosphatase
    3. chloride
    4. creatinine
    5. gamma-glutamyl transpeptidase (GG transferase)
    6. glucose (in fasted animals)
    7. potassium
    8. protein (total)
    9. sodium
    10. urea nitrogen

Additional clinical chemistry tests may be recommended to extend the search for toxic effects attributable to a test substance. The selection of specific tests will be influenced by observations on the mechanism of action of the test substance. Clinical chemistry determinations that may be recommended to ensure adequate toxicological evaluation of the test substance include analyses of acid/base balance, hormones, lipids, methemoglobin, and proteins.

In spite of standard operating procedures and equipment calibration, it is not unusual to observe considerable variation in the results of clinical chemistry analyses from day to day[9]. Ideally, clinical chemistry analyses for all dose groups should be completed during one day. If this is not possible, analyses should be conducted in such a way as to minimize potential variability.

4. **Urinalyses**: Timed urine volume collection should be conducted during the last week of the study. For non-rodents, the tests should be performed on all animals in the study. The volume of urine collected, specific gravity, pH, glucose, and protein should be determined as well as conducting a microscopic

evaluation of urine for sediment and presence of blood/blood cells.[10]

5. **Neurotoxicity Screening/Testing** : Screening for neurotoxic effects should be routinely carried out in all short-term toxicity studies with non-rodents (preferably dogs or miniature swine). The neurotoxicity screen should be age appropriate and would typically include: (1) a specific histopathological examination of tissue samples representative of major areas of the brain, spinal cord, and peripheral nervous system (see organs and tissues listed below under VI.C. Preparation of Tissues for Microscopic Examination) and (2) a functional battery of quantifiable observations and manipulative tests selected to detect signs of neurological, behavioral, and physiological dysfunctions. This functional battery is also referred to as an expanded set of clinical evaluations and is described more fully in section V.A. Observations of Test Animals in this chapter and in Chapter IV.C.10. Neurotoxicity Studies.

   Short-term toxicity study reports should include an assessment of the potential for the test substance to adversely affect the structural or functional integrity of the nervous system. This assessment should evaluate data from the neurotoxicity screen and other toxicity data from the study, as appropriate. Based on this assessment, the petitioner should make an explicit statement about whether or not the test substance presents a potential neurotoxic hazard and if additional neurotoxicity testing is deemed appropriate. FDA recommends that additional neurotoxicity testing not be undertaken without first consulting with the Agency.

6. **Immunotoxicity**: Results from tests that are included in the list of primary indicators for immune toxicity (see Chapter V.C. of the 1993 draft "Redbook II") should also be evaluated as part of an immunotoxicity screen. Reports of short-term toxicity tests should include an assessment of the potential for the test substance to adversely affect the immune system. This assessment should evaluate data from the list of primary indicators included in the immunotoxicity screen and other toxicity data from the study, as appropriate. Based on this assessment, the petitioner/notifier should make an explicit statement about whether or not the test substance presents a potential immunotoxic hazard which requires further immunotoxicity testing. Additional immunotoxicity tests are discussed in Chapter V.C. of the 1993 draft "Redbook II", but should not be undertaken without first consulting with the Agency.

VI. Necropsy and Microscopic Examination

A. Gross Necropsy

All test animals should be subjected to complete gross necropsy, including examination of external surfaces, orifices, cranial, thoracic and abdominal cavities, carcass, and all organs. The gross necropsy should be performed by, or under the direct supervision of, a qualified pathologist, preferably the pathologist who will later perform the microscopic examination (see below).

B. Organ Weight

Organs that should be weighed include the adrenals, brain, epididymides, heart, kidneys, liver, spleen, testes, thyroid/parathyroid, thymus, ovaries and uterus. Organs should be carefully dissected and trimmed to remove fat and other contiguous tissue and then be weighed immediately to minimize the effects of drying on organ weight.

C. Preparation of Tissues for Microscopic Examination

Generally, the following tissues should be fixed in 10% buffered formalin (or another generally recognized fixative) and sections prepared and stained with hematoxylin and eosin (or another appropriate stain) in preparation for microscopic examination. Lungs should be inflated with fixative prior to immersion in fixative.

1. adrenals
2. aorta
3. bone (femur)
4. bone marrow (sternum)
5. brain (at least 3 different levels)
6. cecum
7. colon
8. corpus and cervix uteri
9. duodenum

10. epididymis
11. esophagus
12. eyes
13. gall bladder (if present)
14. Harderian gland
15. heart
16. ileum
17. jejunum
18. kidneys
19. liver
20. lung (with main-stem bronchi)
21. lymph nodes (1 related to route of administration and 1 from a distant location)
22. mammary glands
23. nasal turbinates
24. ovaries and fallopian tubes
25. pancreas
26. pituitary
27. prostate
28. rectum
29. salivary gland
30. sciatic nerve
31. seminal vesicle (if present)
32. skeletal muscle
33. skin
34. spinal cord (3 locations: cervical, mid-thoracic, lumbar)
35. spleen
36. stomach
37. testes
38. thymus (or thymic region)
39. thyroid/parathyroid
40. trachea
41. urinary bladder
42. vagina
43. all tissues showing abnormality

D. Microscopic Evaluation

All gross lesions should be examined microscopically. All tissues from the animals in the control and high dose groups should be examined. If treatment related effects are noted in certain tissues, then the next lower dose level tested of those specific tissues should be examined. Successive examination of the next lower dose level continues until no effects are noted. In addition, all tissues from animals which died prematurely or were sacrificed during the study should be examined microscopically to assess any potential toxic effects.

E. Histopathology of Lymphoid Organs

Histopathological evaluation of the lymphoid organs should be performed as described in the section on immunotoxicity testing (see Chapter V.C. of the 1993 draft "Redbook II") for all animals.

VII. References

1. OECD Guideline For The Testing Of Chemicals , Repeated Dose 28-day Oral Toxicity Study in Rodents, 407, July 1995
2. Kurt Weingand et al., 1996. "Harmonization of Animal Clinical Pathology Testing in Toxicity and Safety Studies", *Fundamental and Applied Toxicology*, 29, pp 198-201.
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10. Ragan, H.A. and Weller, R.E., "Markers of Renal Function and Injury" in *The Clinical Chemistry of Laboratory Animals*, Second Edition 1999, Taylor & Francis, Philadelphia, PA, pp. 520-533

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Redbook 2000: IV.C.4.a Subchronic Toxicity Studies with Rodents

November 2003

Toxicological Principles for the Safety Assessment of Food Ingredients
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Chapter IV.C.4.a. Subchronic Toxicity Studies with Rodents

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Subchronic toxicity studies with rodents are generally conducted for 90 days (3 months), but they may be conducted for up to 12 months. Results of these studies (1) can help predict appropriate doses of the test substance for future chronic toxicity studies, (2) can be used to determine NOELs for some toxicology endpoints, and (3) allow future long-term toxicity studies in rodents and non-rodents to be designed with special emphasis on identified target organs. Subchronic toxicity studies usually cannot determine the carcinogenic potential of a test substance. Guidance specific to subchronic toxicity studies with non-rodents is presented in chapter IV.C.4.b. Sponsors/submitters of petitions/notifications are encouraged to also become familiar with the Guidance for Reporting Results of Toxicity Studies (Chapter IV.B.2.), Pathology Considerations in Toxicity Studies (Chapter IV.B.3.), and Statistical Considerations in Toxicity Studies (Chapter IV.B.4.) during the development of study design.

Scientifically justified changes to the 1993 draft "Redbook II" version of this section have been made following consultation with other authoritative guidelines and publications[1-8].

I. Good Laboratory Practice

Nonclinical laboratory studies must be conducted according to U.S. FDA good laboratory practice (GLP) regulations, issued under Part 58. Title 21. Code of Federal Regulations. This document may be obtained from the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C., 20402, (toll free 866-512-1800).

II. Test Animals

A. Care, Maintenance and Housing:

Recommendations about the care, maintenance, and housing of animals contained in NIH publication 85-23, "Guide for the Care and Use of Laboratory Animals"[8], and the DHEW publication no. 78-23 should be followed unless they conflict with specific recommendations in these guidelines.

B. Selection of Species, Strains and Sex:

These guidelines are for studies with rodents (usually rats and mice); if other species are used, modifications of these guidelines may be necessary. Both male and female test animals, that are healthy and have not been subjected to previous experimental procedures should be used.

It is important to consider the test animals' general sensitivity and the responsiveness of particular organs and tissues of test animals to toxic chemicals when selecting rodent species, strains, and substrains for toxicity studies. The selection of the use of inbred, out-bred, or hybrid rodent strains for toxicity studies should be based upon the scientific questions to be answered. Additionally, it is important that test animals come from well-characterized and healthy colonies. Because recent information suggests survivability problems exist for some strains of rats, test animals should be selected that are likely to achieve the recommended duration of the study. FDA encourages petitioners and notifiers to consult with Agency scientists before toxicity testing is begun if they have questions about the appropriateness of a particular species, strain, or substrain.

C. Age:

Testing should be performed on young animals, with dosing beginning as soon as possible after weaning, following an acclimation period of at least 5 days, and for rodents no later than 6 to 8 weeks of age.

D. Number and Sex:

Equal numbers of males and females of each species and strain should be used for the study. In general, for subchronic toxicity studies, experimental and control groups should have at least 20 rodents per sex per group. Ten rodents/sex/group may be acceptable for subchronic rodent studies when the study is considered to be range-finding in nature or when longer term studies are anticipated. These recommendations will help ensure that the number of animals that survive until the end of the study will be sufficient to permit a meaningful evaluation of toxicological effects.

If interim necropsies are planned, the number of animals per sex per group should be increased by the number scheduled to be sacrificed before completion of the study; for rodents, at least 10 animals per sex per group should be available for interim necropsy.

E. Infected Animals:

Generally, it is not possible to treat animals for infection during the course of a study without risking interaction between the compound used for treatment and the test substance. This interaction may confound or complicate the interpretation of study results.

F. Animal Identification:

Test animals should be characterized by reference to their species, strain (and substrain), sex, age, and weight. Each animal must be assigned a unique identification number (e.g., ear tag, implanted identification chip, tatoo).

G. Caging:

Animals should be housed one per cage (single-caged). This recommendation reflects three principal concerns:

- The amount of feed consumed by each animal in the study cannot be determined when more than one animal is housed in each cage. This information is necessary in the determination of feed efficiency (relationship of feed consumed to body weight gained).
- Minimizing the possibility of confounding analyses and determining whether decreases in body weight gain are due to decreased palatability or substance mediated toxicity.
- Organs and tissues from moribund and dead animals which are single-caged would not be lost due to

cannibalism.

H. Diet:

In general, feed and water should be provided *ad libitum* to animals in toxicity studies, and the diets for these studies should meet the nutritional requirements of the species[4-7] for normal growth and reproduction. Unless special circumstances apply which justify otherwise, care should be taken to ensure that the diets of the compound treated groups of animals are isocaloric (equivalent in caloric density) with and contain the same levels of nutrients (e.g., fiber, micronutrients) as the diets of the control group. Unrecognized or inadequately controlled dietary variables may result in nutritional imbalances or caloric deprivation that could confound interpretation of the toxicity study results (e.g., lifespan, background rates of tumor incidences) and alter the outcome and reproducibility of the studies.

The following issues are important to consider when establishing diets for animals in toxicity studies:

When the test substance has no caloric value and constitutes a substantial amount of the diet (e.g., more than 5%), both caloric and nutrient densities of the high dose diet would be diluted in comparison to the diets of the other groups. As a consequence, some high dose animals may receive higher test article doses than expected because animals fed such diluted diets *ad libitum* may eat more than animals in other dosed groups to compensate for the differences in energy and nutrient content of the high dose diets. Such circumstances make it especially important that feed consumption of these animals be as closely and accurately monitored as possible in order to determine whether changes observed could be due to overt toxicity of the test substance or to a dietary imbalance. To further aid in this assessment, two control groups can be used; one group would be fed the undiluted control diet and a second group would be fed the control diet supplemented with an inert filler (e.g., methylcellulose) at a percentage equal to the highest percentage of the test substance in the diet.

When the vehicle for the test substance is expected to have caloric and/or nutritional values, which are greater than that of the control ration, an adjustment in the caloric and/or nutritional components may be necessary.

When administration of the test substance is expected to have an effect on feed intake because of its unpleasant taste or texture, paired feeding can be used to eliminate the differences in consumption between control and compound treated groups. When a paired feeding study design is to be employed, pairs of litter-mate weanling rats of the same sex and approximate size are selected and fed the control or the experimental diet. Animals should be single-caged so that feed consumption can be determined daily, and the control animal is then fed an amount of food equal to that which the paired experimental animal ate on the preceding day. If the test substance is non-nutritive and composes a significant proportion of the diet, the pair-fed control animals should be fed an amount of its feed such that it consumes a nutritionally equivalent amount of diet as the paired experimental animal. Additionally, the study should include a second group of control animals fed *ad libitum* to ensure that the impact on any observed experimental result is due to differences in energy or nutrient intake.

When the test substance interferes with the absorption of nutrients, leading to nutritional deficiencies or changes in nutrient ratios, this can confound assessment of the toxicological endpoints under consideration. For example, fat soluble vitamins may preferentially partition with a mineral oil or fat substitute which is largely unabsorbed, such that a potential deficiency in these vitamins may result. This potential may be eliminated by additional nutrient fortification of the feed for those groups receiving the test substance. Appropriate levels of nutrient fortification should be determined experimentally.

It may be preferable to use a semi-purified diet prepared with known amounts of well-characterized ingredients for short-term and subchronic toxicity studies because of batch to batch variations in diet composition (e.g., fiber, mineral, vitamins, isoflavones) in some of the commonly used laboratory animal chows. The use of these semi-purified diets, however, may not be advisable in long-term and reproductive studies due to inadequate historical data related to their influences on animal survival and toxicological endpoints. For example, loss of necessary but unidentified micronutrients in the semipurified diet may interfere with normal reproduction.

Related issues are discussed in the section on Diets for Toxicity Studies in Chapter IV.B.5. in the 1993 draft "Redbook II"

I. Assignment of Control and Compound Treated Animals:

Animals should be assigned to control and compound treated groups in a stratified random manner; this will help minimize bias and assure comparability of pertinent variables across compound treated and control

groups (for example: mean body weights and body weight ranges). If other characteristics are used as the basis for randomization then that characterization should be described and justified.

Animals in all groups should be placed on study on the same day; if this is not possible because of the large number of animals in a study, animals may be placed on study over several days. If the latter recommendation is followed, a preselected portion of the control and experimental animals should be placed on the study each day in order to maintain concurrence.

J. Mortality:

Excessive mortality due to poor animal management is unacceptable and may be cause to repeat the study. For example, under normal circumstances, mortality in the control group should not exceed 10%.

K. Autolysis:

Adequate animal husbandry practices should result in considerably less than 10% of animals and tissues or organs lost to a study because of autolysis. Autolysis in excess of this standard may be cause to repeat the study.

L. Necropsy:

Necropsy should be performed soon after an animal is sacrificed or found dead, so that loss of tissues due to autolysis is minimized. When necropsy cannot be performed immediately, the animal should be refrigerated at a temperature that is low enough to prevent autolysis but not so low as to cause cell damage. If histopathological examination is to be conducted, tissue specimens should be taken from the animals and placed in appropriate fixatives when the necropsy is performed.

III. Test Substance

The test substance used in toxicity studies should be the same substance that the petitioner/notifier intends to market. A single lot of test substance should be used throughout the study, when possible. Alternatively, lots that are similar in purity and composition should be used.

A. Identity:

The identity of the test substance or mixture of substances to be tested should be known. We urge petitioners/notifiers to consult with the Agency in determination of test compound and to provide a Chemical Abstract Service (CAS) Registry Number or Numbers.

B. Composition/Purity:

The composition of the test substance should be known including the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials.

C. Conditions of Storage:

The test sample should be stored under conditions that maintain its stability, quality, and purity until the studies are complete.

D. Expiration Date:

The expiration date of the test material should be known and easily available. Test materials should not be used past their expiration date.

IV. Experimental Design

A. Duration of Testing:

Animals should be exposed to the test substance 7 days per week for a minimum of 90 consecutive days (3 months). Any other regime must be justified.

B. Route of Administration:

The route of administration of the test substance should approximate that of normal human exposure, if possible. For food ingredients (e.g., food and color additives) the oral route of administration is preferred. A

justification should be provided when other routes are used. The same method of administration should be used for all test animals throughout the study.

The test substance should be administered in one of the following ways:

- **In the diet**, if human exposure to the test substance is likely to be through consumption of solid foods or a combination of solid and liquid foods. If the test substance is added to the diet, animals should not be able to selectively consume either basal diet or test substance in the diet on the basis of color, smell, or particle size. If the compound is mixed with ground feed and pelleted, nothing in the pelleting process should affect the test substance (for example, heat-labile substances may be destroyed during pellet production by a steam process). When the test substance is administered in the diet, dietary levels should be expressed as mg of the test substance per kg of feed.
- **Dissolved in the drinking water**, if the test substance is likely to be ingested in liquid form (for example, in soft drinks or beer), or if administration in the diet is inappropriate for other reasons. The amount of test substance administered in drinking water should be expressed as mg of test substance per ml of water.
- **By encapsulation or oral intubation (gavage)**, if the two previous methods are unsatisfactory or if human exposure is expected to be through daily ingestion of single, large doses instead of continual ingestion of small doses. If the test substance is administered by gavage, it should be given at approximately the same time each day. The maximum volume of solution that can be given by gavage in one dose depends on the test animal's size; for rodents, the volume ordinarily should not exceed 1 ml/100 g body weight. If the gavage vehicle is oil (see Chapter IV.B.5.b. in the 1993 draft "Redbook II"), then the volume should be no more than 0.4 ml/100 g of body weight, and the use of a low-fat diet should be considered. If the test substance must be given in divided doses, all doses should be administered within a 6 hour period. Doses of test substance administered by gavage should be expressed as mg of test substance per ml of gavage vehicle. Finally, the petitioner/notifier should provide information that can allow the reviewer to conclude that administration of the test compound by encapsulation or gavage is equivalent in all toxicologically important respects to administration in the diet or drinking water. Alternatively, metabolic information on both modes of administration should be provided so that appropriate interpretation of data can be accomplished.

C. Dose groups:

At least three dose levels of the test substance should be used per sex (one dose level per group); ideally, 4 or 5 dose levels of the test substance should be used. A concurrent control group should be included. Information from acute (Chapter IV.C.2. in the 1993 draft "Redbook II") and short-term (Chapter IV.C.3.) toxicity studies can help determine appropriate doses for subchronic studies.

1. Selection of Treatment Doses:

Dose selection for toxicity studies should be based on information related to the toxicity of the test substance.

A minimum of three dose levels of the test substance and a concurrent control group should be used in toxicity studies. When designing and conducting toxicity studies the following should be considered: 1) the high dose should be sufficiently high to induce toxic responses in test animals; 2) the low dose should not induce toxic responses in test animals; and 3) the intermediate dose should be sufficiently high to elicit minimal toxic effects in test animals (such as alterations in enzyme levels or slight decreases in body weight gains). No dose should cause an incidence of fatalities that prevents meaningful evaluation of the data. Administration of the test substance to all dose groups should be done concurrently.

2. Controls:

A concurrent control group of test animals is required. The control group in dietary studies should be fed the basal diet. Exceptions to this and other related information, including a discussion regarding pair-feeding, was provided above in section "II Test Animals, H. Diet".

A carrier or vehicle for the test substance should be given to control animals at a volume equal to the maximum volume of carrier or vehicle given to any dosed group of animals. Sufficient toxicology information should be available on the carrier or vehicle to ensure that its use will not compromise the results of the study. If there is insufficient information about the toxic properties of the vehicle used to administer the test substance, an additional control group that is not exposed to the carrier or vehicle should be included. In all other respects, animals in the control group should be treated the same as animals in dosed groups. (See additional information in section "II Test Animals, H. Diet" above.)

D. Computerized systems

Computerized systems that are used in the generation, measurement, or assessment of data should be developed, validated, operated, and maintained in ways that are compliant with Good Laboratory Practice principles. [9]

V. Observations and Clinical Tests

A. Observations of Test Animals:

Routine cage-side observations should be made on all animals at least once or twice a day throughout the study for general signs of pharmacologic and toxicologic effects, morbidity and mortality. The usual interval between observations should be at least 6 hours. Individual records should be maintained for each animal and the time of onset and the characteristics and progression of any effects should be recorded, preferably using a scoring system.

An expanded set of clinical evaluations, performed inside and outside of the cage, should be carried out in short-term and subchronic toxicity studies in rodents and non-rodents, in one-year non-rodent toxicity studies, and reproductive toxicity studies in rodents to enable detection not only of general pharmacologic and toxicologic effects but also of neurologic disorders, behavioral changes, autonomic dysfunctions, and other signs of nervous system toxicity. Specific information about the systematic clinical tests/observations is contained in Chapter IV.C.10. This expanded set of clinical examinations (Chapter IV.C.10), conducted inside and outside the cage, should be age appropriate and performed on all animals at least once prior to initiation of treatment, and periodically during treatment. Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions or other evidence of autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern). Additionally, changes in gait, posture and response to handling, as well as the presence of clonic or tonic seizures, stereotypes (e.g., excessive grooming, repetitive circling) or bizarre behavior (e.g., self-mutilating, walking backwards) should be recorded. Tumor development, particularly in long-term studies, should be followed: the time of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumor should be recorded. During the course of a study, toxic and pharmacologic signs may suggest the need for additional clinical tests or expanded post-mortem examinations.

B. Body Weight and Feed Intake Data:

Test animals should be weighed at least once a week. Feed consumption (or water consumption if the test substance is administered in the drinking water) should be measured every week during the subchronic toxicity study. Petitioners should also attempt to quantify spillage of feed by test animals, and to determine if spillage is greater with test diets than with control diets. Appropriate discussions of feed spillage should be included in the study report.

C. Clinical Testing:

Ophthalmological examination, hematology profiles, clinical chemistry tests, and urinalyses should be performed as described in the following sections:

1. **Opthalmological Examination**: This examination should be performed by a qualified individual on all animals before the study begins and on control and high-dose animals at the end of the study. If the results of examinations at termination indicate that changes in the eyes may be associated with administration of the test substance, ophthalmological examinations should be performed on all animals in the study.
2. **Hematology**: Blood samples should be obtained from a minimum of 10 rodents of each sex per group at least three times during the study. Sampling of test animals should be conducted during the first two weeks on study (receiving treatment), monthly or midway through treatment (day 45), and at termination. The determination of the first sampling time point should be based on the expected time of initial toxicological effects on the organ systems. Ideally, the same rodents should be sampled during the study, and at termination. The collection of blood samples should occur at approximately the same time on each sampling day. If animals are fasted prior to sampling, then blood collection should occur at the conclusion of the fast and prior to feeding. Fasting duration should be appropriate for the species and the analytical tests to be performed. Hematologic tests should be performed on individual samples and not pooled.

1. The following determinations are recommended:
    1. hematocrit
    2. hemoglobin concentration
    3. erythrocyte count
    4. total and differential leukocyte counts
    5. mean corpuscular hemoglobin
    6. mean corpuscular volume
    7. mean corpuscular hemoglobin concentration
    8. and a measure of clotting potential (such as clotting time, prothrombin time, thromboplastin time, or platelet count).

Test compounds may have an effect on the hematopoietic system and therefore appropriate measures should be employed so that evaluations of reticulocyte counts and bone marrow cytology may be performed if warranted. Reticulocyte counts should be obtained for each animal using automated reticulocyte counting capabilities, or from air-dried blood smears. Bone marrow slides should be prepared from each animal for evaluating bone marrow cytology. These slides would only need to be examined microscopically if effects on the hematopoietic system were noted.

3. **Clinical Chemistry**: Blood samples should be obtained from a minimum of 10 rodents of each sex per group at least three times during the study. Sampling of test animals should be conducted during the first two weeks on study (receiving treatment), monthly or midway through treatment (day 45), and at termination. The determination of the first sampling time point should be based on the expected time of initial toxicological effects on the organ systems. Ideally, the same rodents should be sampled during the study, and at termination. The collection of blood samples should occur at approximately the same time on each sampling day. If animals are fasted prior to sampling, then blood collection should occur at the conclusion of the fast and prior to feeding. Fasting duration should be appropriate for the species and the analytical tests to be performed. Clinical chemistry tests should be performed on individual samples and not pooled.

   Clinical chemistry tests that are appropriate for all test substances include measurements of electrolyte balance, carbohydrate metabolism, and liver and kidney function. Specific determinations should include:

   1. Hepatocellular evaluation: select at least 3 of the following 5
       1. alanine aminotransferase (SGPT, ALT)
       2. aspartate aminotransferase (SGOT, AST)
       3. sorbitol dehydrogenase
       4. glutamate dehydrogenase
       5. total bile acids
   2. Hepatobiliary evaluation: select at least 3 of the following 5
       1. alkaline phosphatase
       2. bilirubin (total)
       3. gamma-glutamyl transpeptidase (GG transferase)
       4. 5' nucleotidase
       5. total bile acids
   3. Other markers of cell changes or cellular function
       1. albumin
       2. calcium
       3. chloride
       4. cholestrol(total)
       5. cholinesterase
       6. creatinine
       7. globulin (calculated)

8. glucose (in fasted animals)
9. phosphorous
10. potassium
11. protein (total)
12. sodium
13. triglycerides (fasting)
14. urea nitrogen

However, when adequate volumes of blood cannot be obtained from test animals, the following determinations should generally be given priority. FDA understands that the specific nature of the test compound may warrant the consideration of alternative tests. Appropriate justification for alternative tests should be presented in study reports.

1. alanine aminotransferase
2. alkaline phosphatase
3. chloride
4. creatinine
5. gamma-glutamyl transpeptidase (GG transferase)
6. glucose (in fasted animals)
7. potassium
8. protein (total)
9. sodium
10. urea nitrogen

Additional clinical chemistry tests may be recommended to extend the search for toxic effects attributable to a test substance. The selection of specific tests will be influenced by observations on the mechanism of action of the test substance. Clinical chemistry determinations that may be recommended to ensure adequate toxicological evaluation of the test substance include analyses of acid/base balance, hormones, lipids, methemoglobin, and proteins.

In spite of standard operating procedures and equipment calibration, it is not unusual to observe considerable variation in the results of clinical chemistry analyses from day to day[10]. Ideally, clinical chemistry analyses for all dose groups should be completed during one day. If this is not possible, analyses should be conducted in such a way as to minimize potential variability.

4. **Urinalyses**: Timed urine volume collection should be conducted during the last week of the study on at least 10 animals of each sex in each group. The volume of urine collected, specific gravity, pH, glucose, and protein should be determined as well as conducting a microscopic evaluation of urine for sediment and presence of blood/blood cells[11].
5. **Neurotoxicity Screening/Testing** : Screening for neurotoxic effects should be routinely carried out in all subchronic toxicity studies with rodents (preferably rats). The neurotoxicity screen should be age appropriate and would typically include: (1) specific histopathological examination of tissue samples representative of major areas of the brain, spinal cord, and peripheral nervous system (see organs and tissues listed below under VI.C. Preparation of Tissues for Microscopic Examination) and (2) a functional battery of quantifiable observations and manipulative tests selected to detect signs of neurological, behavioral, and physiological dysfunctions. This functional battery is also referred to as an expanded set of clinical evaluations and is described more fully in section V.A. Observations of Test Animals in this chapter and in Chapter IV.C.10. Neurotoxicity Studies.

   Subchronic toxicity study reports should include an assessment of the potential for the test substance to adversely affect the structural or functional integrity of the nervous system. This assessment should evaluate data from the neurotoxicity screen and other toxicity data from the study, as appropriate. Based on this assessment, the petitioner should make an explicit statement about whether or not the test substance presents a potential neurotoxic hazard and if additional neurotoxicity testing is deemed appropriate. FDA recommends that additional neurotoxicity testing not be undertaken without first consulting with the Agency.
6. **Immunotoxicity**: Results from tests that are included in the list of primary indicators for immune toxicity (see Chapter V.C. of the 1993 draft "Redbook II") should also be evaluated as part of an

immunotoxicity screen. Reports of subchronic toxicity studies should include an assessment of the potential for the test substance to adversely affect the immune system. This assessment should evaluate data from the list of primary indicators included in the immunotoxicity screen and other toxicity data from the study, as appropriate. Based on this assessment, the petitioner/notifier should make an explicit statement about whether or not the test substance presents a potential immunotoxic hazard which requires further immunotoxicity testing. Additional immunotoxicity tests are discussed in Chapter V. C. of the 1993 draft "Redbook II", but should not be undertaken without first consulting with the Agency.

VI. Necropsy and Microscopic Examination

A. Gross Necropsy

All test animals should be subjected to complete gross necropsy, including examination of external surfaces, orifices, cranial, thoracic and abdominal cavities, carcass, and all organs. The gross necropsy should be performed by, or under the direct supervision of, a qualified pathologist, preferably the pathologist who will later perform the microscopic examination (see below).

B. Organ Weight

Organs that should be weighed include the adrenals, brain, epididymides, heart, kidneys, liver, spleen, testes, thyroid/parathyroid, thymus, ovaries and uterus. Organs should be carefully dissected and trimmed to remove fat and other contiguous tissue and then be weighed immediately to minimize the effects of drying on organ weight.

C. Preparation of Tissues for Microscopic Examination

Generally, the following tissues should be fixed in 10% buffered formalin (or another generally recognized fixative) and sections prepared and stained with hematoxylin and eosin (or another appropriate stain) in preparation for microscopic examination. Lungs should be inflated with fixative prior to immersion in fixative.

1. adrenals
2. aorta
3. bone (femur)
4. bone marrow (sternum)
5. brain (at least 3 different levels)
6. cecum
7. colon
8. corpus and cervix uteri
9. duodenum
10. epididymis
11. esophagus
12. eyes
13. gall bladder (if present)
14. Harderian gland (if present)
15. heart
16. ileum
17. jejunum
18. kidneys
19. liver
20. lung (with main-stem bronchi)
21. lymph nodes (1 related to route of administration and 1 from a distant location)
22. mammary glands
23. nasal turbinates

24. ovaries and fallopian tubes
25. pancreas
26. pituitary
27. prostate
28. rectum
29. salivary gland
30. sciatic nerve
31. seminal vesicle (if present)
32. skeletal muscle
33. skin
34. spinal cord (3 locations: cervical, mid-thoracic, lumbar)
35. spleen
36. stomach
37. testes
38. thymus (or thymic region)
39. thyroid/parathyroid
40. trachea
41. urinary bladder
42. vagina
43. all tissues showing abnormality

D. Microscopic Evaluation

All gross lesions should be examined microscopically. All tissues from the animals in the control and high dose groups should be examined. If treatment related effects are noted in certain tissues, then the next lower dose level tested of those specific tissues should be examined. Successive examination of the next lower dose level continues until no effects are noted. In addition, all tissues from animals which died prematurely or were sacrificed during the study should be examined microscopically to assess any potential toxic effects.

E. Histopathology of Lymphoid Organs

Histopathological evaluation of the lymphoid organs should be performed as described in the section on immunotoxicity testing (see Chapter V. C. of the 1993 draft "Redbook II") for all animals.

VII. References

1. EPA Health Effects Test Guidelines OPPTS 870.3100, 90-Day Oral Toxicity in Rodents, August 1998
2. OECD Guideline For The Testing Of Chemicals , Repeated Dose 90-day Oral Toxicity Study in Rodents, 408, September 1998
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9. The Application of the Principles of GLP to Computerised Systems, Organisation for Economic

Cooperation and Development (OECD), Environmental Monograph No. 116, Paris, 1995.

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Links on this page:

1. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/default.htm

Redbook 2000: IV.C.4.b Subchronic Toxicity Studies with Non-Rodents

November 2003

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter IV.C.4.b. Subchronic Toxicity Studies with Non-Rodents

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This guidance represents the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

Subchronic toxicity studies with non-rodents (usually dogs) are generally conducted for 90 days (3 months), but they may be conducted for up to 12 months. Results of these studies (1) can help predict appropriate doses of the test substance for future chronic toxicity studies, (2) can be used to determine NOELs for some toxicology endpoints, and (3) allow future long-term toxicity studies in rodents and non-rodents to be designed with special emphasis on identified target organs. Subchronic toxicity studies usually cannot determine the carcinogenic potential of a test substance. Guidance specific to subchronic toxicity studies with rodents is presented in chapter IV.C.4b. Sponsors/submitters of petitions/notifications are encouraged to also become familiar with the Guidance for Reporting Results of Toxicity Studies (Chapter IV.B.2.), Pathology Considerations in Toxicity Studies (Chapter IV.B.3.), and Statistical Considerations in Toxicity Studies (Chapter IV.B.4.) during the development of study design.

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It is important to consider the test animals' general sensitivity and the responsiveness of particular organs and tissues of test animals to toxic chemicals when selecting species and strains for toxicity studies. Test animals should be selected that are likely to survive the recommended duration of the study. It is important that test animals come from well-characterized and healthy colonies. FDA encourages petitioners and notifiers to consult with Agency scientists before toxicity testing is begun if they have questions about the appropriateness of a particular species or strain.

C. Age:

Testing should be performed on young animals, with dosing beginning as soon as possible after weaning, and following an acclimation period of at least 5 days. Testing should begin when dogs are no older than 4 to 6 months of age.

D. Number and Sex:

Equal numbers of males and females of each species and strain should be used for the study. In general, for subchronic toxicity studies, experimental and control groups should have at least 4 dogs per sex per group. These recommendations will help ensure that the number of animals that survive until the end of the study will be sufficient to permit a meaningful evaluation of toxicological effects.

If interim necropsies are planned, the number of animals per sex per group should be increased by the number scheduled to be sacrificed before completion of the study.

E. Infected Animals:

Generally, it is not possible to treat animals for infection during the course of a study without risking interaction between the compound used for treatment and the test substance. This interaction may confound or complicate the interpretation of study results.

F. Animal Identification:

Test animals should be characterized by reference to their species, strain, sex, age, and weight. Each animal must be assigned a unique identification number (e.g., ear tag, implanted identification chip, tatoo).

G. Caging:

Animals should be housed one per cage or run (single-caged). This recommendation reflects three principal concerns:

- The amount of feed consumed by each animal in the study cannot be determined when more than one animal is housed in each cage. This information is necessary in the determination of feed efficiency (relationship of feed consumed to body weight gained).
- Minimizing the possibility of confounding analyses and determining whether decreases in body weight gain are due to decreased palatability or substance mediated toxicity.
- Organs and tissues from moribund and dead animals which are single-caged would not be lost due to cannibalism.

H. Diet:

Diets for subchronic studies should meet the nutritional requirements of the species [4-7]for normal growth and reproduction. In general, water should be provided *ad libitum* . Unless special circumstances apply which

justify otherwise, care should be taken to ensure that the diets of the compound treated groups of animals are isocaloric (equivalent in caloric density) with and contain the same levels of nutrients (e.g., fiber, micronutrients) as the diets of the control group. Unrecognized or inadequately controlled dietary variables may result in nutritional imbalances or caloric deprivation that could confound interpretation of the toxicity study results (e.g., lifespan, background rates of tumor incidences) and alter the outcome and reproducibility of the studies.

The following issues are important to consider when establishing diets for animals in toxicity studies:

When the test substance has no caloric value and constitutes a substantial amount of the diet (e.g., more than 5%), both caloric and nutrient densities of the high dose diet would be diluted in comparison to the diets of the other groups. It is important that feed consumption of animals be as closely and accurately monitored as possible in order to determine whether changes observed could be due to overt toxicity of the test substance or to a dietary imbalance. To further aid in this assessment, two control groups can be used; one group would be fed the undiluted control diet and a second group would be fed the control diet supplemented with an inert filler (e.g., methylcellulose) at a percentage equal to the highest percentage of the test substance in the diet.

When the vehicle for the test substance is expected to have caloric and/or nutritional values, which are greater than that of the control ration, an adjustment in the caloric and/or nutritional components may be necessary.

When administration of the test substance is expected to have an effect on feed intake because of its unpleasant taste or texture, paired feeding can be used to eliminate the differences in consumption between control and compound treated groups. When a paired feeding study design is to be employed, pairs of animals of the same sex, age, and approximate size are selected and fed the control or the experimental diet. Animals should be single-caged so that feed consumption can be determined daily, and the control animal is then fed an amount of food equal to that which the paired experimental animal ate on the preceding day. If the test substance is non-nutritive and composes a significant proportion of the diet, the pair-fed control animals should be fed an amount of its feed such that it consumes a nutritionally equivalent amount of diet as the paired experimental animal. Additionally, the study should include a second group of control animals fed the normal ration amount to ensure that the impact on any observed experimental result is due to differences in energy or nutrient intake.

When the test substance interferes with the absorption of nutrients, leading to nutritional deficiencies or changes in nutrient ratios, this can confound assessment of the toxicological endpoints under consideration. For example, fat soluble vitamins may preferentially partition with a mineral oil or fat substitute which is largely unabsorbed, such that a potential deficiency in these vitamins may result. This potential may be eliminated by additional nutrient fortification of the feed for those groups receiving the test substance. Appropriate levels of nutrient fortification should be determined experimentally.

It may be preferable to use a semi-purified diet prepared with known amounts of well-characterized ingredients for short-term and subchronic toxicity studies because of batch to batch variations in diet composition (e.g., fiber, mineral, vitamins, isoflavones) in some of the commonly used laboratory animal chows. The use of these semi-purified diets, however, may not be advisable in long-term and reproductive studies due to inadequate historical data related to their influences on animal survival and toxicological endpoints. For example, loss of necessary but unidentified micronutrients in the semipurified diet may interfere with normal reproduction.

FDA recommends reviewing the diet section in IV.C.4.a. when non-rodents (e.g., rabbits)are to be fed *ad libitum*. Related issues are discussed in the section on Diets for Toxicity Studies in Chapter IV.B.5. in the 1993 draft "Redbook II".

I. Assignment of Control and Compound Treated Animals:

Animals should be assigned to control and compound treated groups in a stratified random manner; this will help minimize bias and assure comparability of pertinent variables across compound treated and control groups (for example: mean body weights and body weight ranges). If other characteristics are used as the basis for randomization then that characterization should be described and justified.

Animals in all groups should be placed on study on the same day; if this is not possible because of the large number of animals in a study, animals may be placed on study over several days. If the latter recommendation is followed, a preselected portion of the control and experimental animals should be placed on the study each day in order to maintain concurrence.

J. Mortality:

Excessive mortality due to poor animal management is unacceptable and may be cause to repeat the study. For example, under normal circumstances, mortality in the control group should not exceed 10%.

K. Autolysis:

Adequate animal husbandry practices should result in considerably less than 10% of animals and tissues or organs lost to a study because of autolysis. Autolysis in excess of this standard may be cause to repeat the study.

L. Necropsy:

Necropsy should be performed soon after an animal is sacrificed or found dead, so that loss of tissues due to autolysis is minimized. When necropsy cannot be performed immediately, the animal should be refrigerated at a temperature that is low enough to prevent autolysis but not so low as to cause cell damage. If histopathological examination is to be conducted, tissue specimens should be taken from the animals and placed in appropriate fixatives when the necropsy is performed.

III. Test Substance

The test substance used in toxicity studies should be the same substance that the petitioner/notifier intends to market. A single lot of test substance should be used throughout the study, when possible. Alternatively, lots that are similar in purity and composition should be used.

A. Identity:

The identity of the test substance or mixture of substances to be tested should be known. We urge petitioners/notifiers to consult with the Agency in determination of test compound and to provide a Chemical Abstract Service (CAS) Registry Number or Numbers.

B. Composition/Purity:

The composition of the test substance should be known including the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials.

C. Conditions of Storage:

The test sample should be stored under conditions that maintain its stability, quality, and purity until the studies are complete.

D. Expiration Date:

The expiration date of the test material should be known and easily available. Test materials should not be used past their expiration date.

IV. Experimental Design

A. Duration of Testing:

Animals should be exposed to the test substance 7 days per week for a minimum of 90 consecutive days (3 months). Any other regime must be justified.

B. Route of Administration:

The route of administration of the test substance should approximate that of normal human exposure, if possible. For food ingredients (e.g., food and color additives) the oral route of administration is preferred. A justification should be provided when other routes are used. The same method of administration should be used for all test animals throughout the study.

The test substance should be administered in one of the following ways:

- **In the diet**, if human exposure to the test substance is likely to be through consumption of solid foods or a combination of solid and liquid foods. If the test substance is added to the diet, animals should not be able to selectively consume either basal diet or test substance in the diet on the basis of color, smell,

or particle size. If the compound is mixed with ground feed and pelleted, nothing in the pelleting process should affect the test substance (for example, heat-labile substances may be destroyed during pellet production by a steam process). When the test substance is administered in the diet, dietary levels should be expressed as mg of the test substance per kg of feed.

- **Dissolved in the drinking water**, if the test substance is likely to be ingested in liquid form (for example, in soft drinks or beer), or if administration in the diet is inappropriate for other reasons. The amount of test substance administered in drinking water should be expressed as mg of test substance per ml of water.
- **By encapsulation or oral intubation (gavage)**, if the two previous methods are unsatisfactory or if human exposure is expected to be through daily ingestion of single, large doses instead of continual ingestion of small doses. If the test substance is administered by gavage, it should be given at approximately the same time each day. The maximum volume of solution that can be given by gavage in one dose depends on the test animal's size. Doses of test substance administered by gavage should be expressed as mg of test substance per ml of gavage vehicle. Finally, the petitioner/notifier should provide information that can allow the reviewer to conclude that administration of the test compound by encapsulation or gavage is equivalent in all toxicologically important respects to administration in the diet or drinking water. Alternatively, metabolic information on both modes of administration should be provided so that appropriate interpretation of data can be accomplished.

C. Dose groups:

At least three dose levels of the test substance should be used per sex (one dose level per group); ideally, 4 or 5 dose levels of the test substance should be used. A concurrent control group should be included. Information from acute (Chapter IV.C.2. in the 1993 draft "Redbook II") and short-term (Chapter IV.C.3.) toxicity studies can help determine appropriate doses for subchronic studies.

1. Selection of Treatment Doses:

Dose selection for toxicity studies should be based on information related to the toxicity of the test substance.

A minimum of three dose levels of the test substance and a concurrent control group should be used in toxicity studies. When designing and conducting toxicity studies the following should be considered: 1) the high dose should be sufficiently high to induce toxic responses in test animals; 2) the low dose should not induce toxic responses in test animals; and 3) the intermediate dose should be sufficiently high to elicit minimal toxic effects in test animals (such as alterations in enzyme levels or slight decreases in body weight gains). No dose should cause an incidence of fatalities that prevents meaningful evaluation of the data. Administration of the test substance to all dose groups should be done concurrently.

2. Controls:

A concurrent control group of test animals is required. The control group in dietary studies should be fed the basal diet. Exceptions to this and other related information, including a discussion regarding pair-feeding, was provided above in section "II Test Animals, H. Diet".

A carrier or vehicle for the test substance should be given to control animals at a volume equal to the maximum volume of carrier or vehicle given to any dosed group of animals. Sufficient toxicology information should be available on the carrier or vehicle to ensure that its use will not compromise the results of the study. If there is insufficient information about the toxic properties of the vehicle used to administer the test substance, an additional control group that is not exposed to the carrier or vehicle should be included. In all other respects, animals in the control group should be treated the same as animals in dosed groups. (See additional information in section "II Test Animals, H. Diet" above.)

D. Computerized systems

Computerized systems that are used in the generation, measurement, or assessment of data should be developed, validated, operated, and maintained in ways that are compliant with Good Laboratory Practice principles. [9]

V. Observations and Clinical Tests

A. Observations of Test Animals:

Routine cage-side observations should be made on all animals at least once or twice a day throughout the

study for general signs of pharmacologic and toxicologic effects, morbidity and mortality. The usual interval between observations should be at least 6 hours. Individual records should be maintained for each animal and the time of onset and the characteristics and progression of any effects should be recorded, preferably using a scoring system.

An expanded set of clinical evaluations, performed inside and outside of the cage, should be carried out in short-term and subchronic toxicity studies in rodents and non-rodents, in one-year non-rodent toxicity studies, and reproductive toxicity studies in rodents to enable detection not only of general pharmacologic and toxicologic effects but also of neurologic disorders, behavioral changes, autonomic dysfunctions, and other signs of nervous system toxicity. Specific information about the systematic clinical tests/observations is contained in Chapter IV.C.10. This expanded set of clinical examinations (Chapter IV.C.10), conducted inside and outside the cage, should be age appropriate and performed on all animals at least once prior to initiation of treatment, and periodically during treatment. Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions or other evidence of autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern). Additionally, changes in gait, posture and response to handling, as well as the presence of clonic or tonic seizures, stereotypes (e.g., excessive grooming, repetitive circling) or bizarre behavior (e.g., self-mutilating, walking backwards) should be recorded. Tumor development, particularly in long-term studies, should be followed: the time of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumor should be recorded. During the course of a study, toxic and pharmacologic signs may suggest the need for additional clinical tests or expanded post-mortem examinations.

B. Body Weight and Feed Intake Data:

Test animals should be weighed at least once a week. Feed consumption (or water consumption if the test substance is administered in the drinking water) should be measured every week during the subchronic toxicity study. Petitioners should also attempt to quantify spillage of feed by test animals, and to determine if spillage is greater with test diets than with control diets. Appropriate discussions of feed spillage should be included in the study report.

C. Clinical Testing:

Ophthalmological examination, hematology profiles, clinical chemistry tests, and urinalyses should be performed as described in the following sections:

1. **Opthalmological Examination:** This examination should be performed by a qualified individual on all animals before the study begins and on control and high-dose animals at the end of the study. If the results of examinations at termination indicate that changes in the eyes may be associated with administration of the test substance, ophthalmological examinations should be performed on all animals in the study.
2. **Hematology**: Blood samples should be obtained from all animals of all groups at the following times: prior to initiation of treatment, during the first two weeks on study (receiving treatment), monthly or midway through treatment (day 45), and at termination. The determination of the second sampling time point should be based on the expected time of initial toxicological effects on the organ systems. The collection of blood samples should occur at approximately the same time on each sampling day. If animals are fasted prior to sampling, then blood collection should occur at the conclusion of the fast and prior to feeding. Fasting duration should be appropriate for the species and the analytical tests to be performed. Hematologic tests should be performed on all samples individually, and not pooled.
    1. The following determinations are recommended:
        1. hematocrit
        2. hemoglobin concentration
        3. erythrocyte count
        4. total and differential leukocyte counts
        5. mean corpuscular hemoglobin
        6. mean corpuscular volume
        7. mean corpuscular hemoglobin concentration
        8. and a measure of clotting potential (such as clotting time, prothrombin time, thromboplastin time, or platelet count).

    Test compounds may have an effect on the hematopoietic system and therefore appropriate measures

should be employed so that evaluations of reticulocyte counts and bone marrow cytology may be performed if warranted. Reticulocyte counts should be obtained for each animal using automated reticulocyte counting capabilities, or from air-dried blood smears. Bone marrow slides should be prepared from each animal for evaluating bone marrow cytology. These slides would only need to be examined microscopically if effects on the hematopoietic system were noted.

3. **Clinical Chemistry**: Blood samples should be obtained from all animals of all groups at the following times: prior to initiation of treatment, during the first two weeks on study (receiving treatment), monthly or midway through treatment (day 45), and at termination. The determination of the second sampling time point should be based on the expected time of initial toxicological effects on the organ systems. The collection of blood samples should occur at approximately the same time on each sampling day. If animals are fasted prior to sampling, then blood collection should occur at the conclusion of the fast and prior to feeding. Fasting duration should be appropriate for the species and the analytical tests to be performed. Clinical chemistry tests should be performed on all samples individually, and not pooled.

    1. Hepatocellular evaluation: select at least 3 of the following 5
        1. alanine aminotransferase (SGPT, ALT)
        2. aspartate aminotransferase (SGOT, AST)
        3. sorbitol dehydrogenase
        4. glutamate dehydrogenase
        5. total bile acids
    2. Hepatobiliary evaluation: select at least 3 of the following 5
        1. alkaline phosphatase
        2. bilirubin (total)
        3. gamma-glutamyl transpeptidase (GG transferase)
        4. 5' nucleotidase
        5. total bile acids
    3. Other markers of cell changes or cellular function
        1. albumin
        2. calcium
        3. chloride
        4. cholestrol(total)
        5. cholinesterase
        6. creatinine
        7. globulin (calculated)
        8. glucose (in fasted animals)
        9. phosphorous
        10. potassium
        11. protein (total)
        12. sodium
        13. triglycerides (fasting)
        14. urea nitrogen

    1. However, when adequate volumes of blood cannot be obtained from test animals, the following determinations should generally be given priority. FDA understands that the specific nature of the test compound may warrant the consideration of alternative tests. Appropriate justification for alternative tests should be presented in study reports.

        1. alanine aminotransferase
        2. alkaline phosphatase
        3. chloride

4. creatinine
5. gamma-glutamyl transpeptidase (GG transferase)
6. glucose (in fasted animals)
7. potassium
8. protein (total)
9. sodium
10. urea nitrogen

Additional clinical chemistry tests may be recommended to extend the search for toxic effects attributable to a test substance. The selection of specific tests will be influenced by observations on the mechanism of action of the test substance. Clinical chemistry determinations that may be recommended to ensure adequate toxicological evaluation of the test substance include analyses of acid/base balance, hormones, lipids, methemoglobin, and proteins.

In spite of standard operating procedures and equipment calibration, it is not unusual to observe considerable variation in the results of clinical chemistry analyses from day to day[10]. Ideally, clinical chemistry analyses for all dose groups should be completed during one day. If this is not possible, analyses should be conducted in such a way as to minimize potential variability.

4. **Urinalyses**: Timed urine volume collection should be conducted on all animals in the study, pretest, at 30 and 60 days, and during the last week of the study. The volume of urine collected, specific gravity, pH, glucose, and protein should be determined as well as conducting a microscopic evaluation of urine for sediment and presence of blood/blood cells[11].
5. **Neurotoxicity Screening/Testing** : Screening for neurotoxic effects should be routinely carried out in all subchronic toxicity studies with non-rodents (preferably dogs or miniature swine). The neurotoxicity screen should be age appropriate and would typically include: (1) specific histopathological examination of tissue samples representative of major areas of the brain, spinal cord, and peripheral nervous system (see organs and tissues listed below under VI.C. Preparation of Tissues for Microscopic Examination) and (2) a functional battery of quantifiable observations and manipulative tests selected to detect signs of neurological, behavioral, and physiological dysfunctions. This functional battery is also referred to as an expanded set of clinical evaluations and is described more fully in section V.A. Observations of Test Animals in this chapter and in Chapter IV.C.10. Neurotoxicity Studies.

   Subchronic toxicity study reports should include an assessment of the potential for the test substance to adversely affect the structural or functional integrity of the nervous system. This assessment should evaluate data from the neurotoxicity screen and other toxicity data from the study, as appropriate. Based on this assessment, the petitioner should make an explicit statement about whether or not the test substance presents a potential neurotoxic hazard and if additional neurotoxicity testing is deemed appropriate. FDA recommends that additional neurotoxicity testing not be undertaken without first consulting with the Agency.
6. **Immunotoxicity**: Results from tests that are included in the list of primary indicators for immune toxicity (see Chapter V.C. of the 1993 draft "Redbook II") should also be evaluated as part of an immunotoxicity screen. Reports of subchronic toxicity studies should include an assessment of the potential for the test substance to adversely affect the immune system. This assessment should evaluate data from the list of primary indicators included in the immunotoxicity screen and other toxicity data from the study, as appropriate. Based on this assessment, the petitioner/notifier should make an explicit statement about whether or not the test substance presents a potential immunotoxic hazard which requires further immunotoxicity testing. Additional immunotoxicity tests are discussed in Chapter V. C. of the 1993 draft "Redbook II", but should not be undertaken without first consulting with the Agency.

VI. Necropsy and Microscopic Examination

A. Gross Necropsy

All test animals should be subjected to complete gross necropsy, including examination of external surfaces, orifices, cranial, thoracic and abdominal cavities, carcass, and all organs. The gross necropsy should be performed by, or under the direct supervision of, a qualified pathologist, preferably the pathologist who will later perform the microscopic examination (see below).

B. Organ Weight

Organs that should be weighed include the adrenals, brain, epididymides, heart, kidneys, liver, spleen, testes, thyroid/parathyroid, thymus, ovaries and uterus. Organs should be carefully dissected and trimmed to remove fat and other contiguous tissue and then be weighed immediately to minimize the effects of drying on organ weight.

C. Preparation of Tissues for Microscopic Examination

Generally, the following tissues should be fixed in 10% buffered formalin (or another generally recognized fixative) and sections prepared and stained with hematoxylin and eosin (or another appropriate stain) in preparation for microscopic examination. Lungs should be inflated with fixative prior to immersion in fixative.

1. adrenals
2. aorta
3. bone (femur)
4. bone marrow (sternum)
5. brain (at least 3 different levels)
6. cecum
7. colon
8. corpus and cervix uteri
9. duodenum
10. epididymis
11. esophagus
12. eyes
13. gall bladder (if present)
14. Harderian gland (if present)
15. heart
16. ileum
17. jejunum
18. kidneys
19. liver
20. lung (with main-stem bronchi)
21. lymph nodes (1 related to route of administration and 1 from a distant location)
22. mammary glands
23. nasal turbinates
24. ovaries and fallopian tubes
25. pancreas
26. pituitary
27. prostate
28. rectum
29. salivary gland
30. sciatic nerve
31. seminal vesicle (if present)
32. skeletal muscle
33. skin
34. spinal cord (3 locations: cervical, mid-thoracic, lumbar)
35. spleen
36. stomach
37. testes
38. thymus (or thymic region)

39. thyroid/parathyroid
40. trachea
41. urinary bladder
42. vagina
43. all tissues showing abnormality

D. Microscopic Evaluation

All gross lesions should be examined microscopically. All tissues from the animals in the control and high dose groups should be examined. If treatment related effects are noted in certain tissues, then the next lower dose level tested of those specific tissues should be examined. Successive examination of the next lower dose level continues until no effects are noted. In addition, all tissues from animals which died prematurely or were sacrificed during the study should be examined microscopically to assess any potential toxic effects.

E. Histopathology of Lymphoid Organs

Histopathological evaluation of the lymphoid organs should be performed as described in the section on immunotoxicity testing (see Chapter V. C. of the 1993 draft "Redbook II") for all animals.

VII. References

1. EPA Health Effects Test Guidelines OPPTS 870.3150, 90-Day Oral Toxicity in Non-Rodents, August 1998
2. OECD Guideline For The Testing Of Chemicals , Repeated Dose 90-day Oral Toxicity Study in Non-Rodents, 409, September 1998
3. Kurt Weingand et al., 1996. "Harmonization of Animal Clinical Pathology Testing in Toxicity and Safety Studies", *Fundamental and Applied Toxicology*, 29, pp 198-201.
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5. Nutrient Requirements of Dogs, Revised, Committee on Animal Nutrition, National Research Council, 1985.
6. Nutrient Requirements of Swine: 10th Revised Edition, Subcommittee on Swine Nutrition, Committee on Animal Nutrition, National Research Council, 1998.
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9. The Application of the Principles of GLP to Computerised Systems, Organisation for Economic Cooperation and Development (OECD), Environmental Monograph No. 116, Paris, 1995.
10. Gaylor, D.W., Suber, R.L., Wolff, G.L. and Crowell, J.A. (1987) Statistical variation of selected clinical pathological and biochemical measurements in rodents (42555) *Proc. Soc. Exp. Biol. Med.* , 185:361-367
11. Ragan, H.A. and Weller, R.E., "Markers of Renal Function and Injury" in *The Clinical Chemistry of Laboratory Animals*, Second Edition 1999, Taylor&Francis, Philadelphia, PA, pp. 520-533

Links on this page:

1. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/default.htm

Redbook 2000: IV.C.5.a Chronic Toxicity Studies with Rodents

July 2007

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter IV.C.5.a. Chronic Toxicity Studies with Rodents

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This guidance represents the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

The FDA has made scientifically justified changes to 1993 "draft" Redbook Chapter IV.C.7., Combined Chronic Toxicity/Carcinogenicity Studies with Rodents, and developed Chapter IV.C.5.a., Chronic Toxicity Studies with Rodents, after consulting other authoritative guidelines[6] [7] [12] and publications[1] (see also relevant sections below). This section of Redbook 2000 supersedes the 1993 "draft" Redbook Chapter IV.C.7.

The FDA acknowledges that it is complicated and difficult to conduct a combined study due to difficulty in setting and administering appropriate dose levels for both types of studies concurrently. In addition, the general objectives of these two types of studies are different. However, when pre-chronic studies provide reasonable estimates of toxicity to predict the information (e.g., treatment doses) to be used in a single bioassay, a chronic toxicity study may be combined with a carcinogenicity study and reveal information about an ingredient's potential to be a carcinogen as well as the maximum dose that produces no adverse effects. On a case-by-case, an *in-utero* exposure phase may also be added to a chronic toxicity study (or combined chronic toxicity/carcinogenicity study) to determine early developmental effects that may increase the incidence of chronic disease outcomes (and/or cancers). Sponsors/submitters of petitions/notifications are encouraged to become familiar with the Carcinogenicity Studies with Rodents (Chapter IV.C.6. [2]) and *In-utero* Exposure Phase for Addition to Carcinogenicity Studies or Chronic Toxicity Studies with Rodents (Chapter IV.C.8. [3]) during the development of a combined study design. The petitioner/notifier should also consult with the FDA before conducting a combined study.

Chronic toxicity studies with rodents should be conducted for a minimum of 12 months (one-year). Results of

these tests can be used, 1) to characterize the toxicity of a food ingredient following prolonged and repeated exposure, and 2) to determine toxicological dose-response relationships needed to establish the maximum dose that produces no adverse effects (i.e., NOEL or NOAEL). The following guidance is written primarily for rats or mice, if other non-rodents are used, modifications to the guidance may be necessary. Sponsors/submitters of petitions/notifications are encouraged to also become familiar with the Guidelines for Reporting Results of Toxicity Studies (Chapter IV.B.2. [4]), Pathology Considerations in Toxicity Studies (Chapter IV.B.3. [5]), Statistical Considerations in Toxicity Studies (Chapter IV.B.4. [6]), during the development of study design.

I. Good Laboratory Practice

Nonclinical laboratory studies discussed in this chapter should be conducted according to U.S. FDA good laboratory practice (GLP) regulations, issued under Part 58 of Title 21 [7] of the Code of Federal Regulations. This document may be obtained from the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C., 20402, (toll free 866-512-1800 or DC area 202-512-1800). Studies performed under other international/national guidelines may be considered equivalent to those conducted under U.S. FDA GLP regulations. Specific area(s) of non-compliance with FDA GLP regulations should be discussed and justified.

II. Test Animals

A. Care, Maintenance and Housing:

Recommendations about the care, maintenance, and housing of animals contained in the National Research Council, Guide for the Care and Use of Laboratory Animals[13] should be followed unless they conflict with specific recommendations in this chapter.

B. Selection of Species and Strains:

Guidance contained within this chapter is for studies with mice and rats; if other rodent species are used, modifications may be necessary. Both male and female test animals, which are healthy and have not been subjected to previous experimental procedures, should be used.

It is important to consider the test animals' general sensitivity and the responsiveness of particular organs and tissues of test animals to toxic chemicals when selecting rodent species, strains, and substrains for toxicity studies. The selection of inbred, out-bred, or hybrid rodent strains for toxicity tests should be based upon the scientific questions to be answered. Additionally, it is important that test animals come from well-characterized and healthy colonies. The petitioners/notifiers should consult with the Agency scientists before toxicity testing has begun if they have questions about the appropriateness of a particular species, strain, or substrain.

C. Age (start of dosing):

Dosing of rodents should begin after weaning, and following a suitable acclimation period of at least 5 days, and before they are approximately 6-8 weeks old.

D. Number and Sex:

Both experimental and control groups should have at least 20 rodents per sex per group. If interim necropsies are planned, the total number of rodents of each sex per group should be increased by the number scheduled to be sacrificed before completion of the study. A minimum of 10 rodents per sex per group should be available for each interim necropsy.

E. Infected Animals:

Generally, it is not possible to treat animals for infection during the course of a study without the possibility of interaction between the therapeutic agent used for treatment and the test substance. This interaction may seriously confound or complicate the interpretation of study results. However, if problems with infection do occur, the sponsor for the study should use their best judgment in proceeding with the study and inform the Agency of their decision. In addition, the FDA requests that they provide a full and detailed description of the justification for study continuation and possible implications of the infection, and if applicable, the justification and possible implications for treatment of the infection.

F. Animal Identification:

Test animals should be characterized by reference to their species, strain (and substrain), sex, age, and weight. Each animal should be assigned a unique identification number (e.g., ear tag, implanted identification

chip, tattoo).

G. Caging:

Animals should be housed one per cage. This recommendation reflects three points of consideration:

- The amount of feed consumed by each animal in the study cannot be determined with sufficient accuracy when more than one animal is housed in each cage. This information is necessary in the determination of feed efficiency (relationship of feed consumed to body weight gained).
- Minimizing the possibility of confounding analyses in determining whether decreases in body weight gain are due to decreased palatability or test substance mediated toxicity.
- Organs and tissues from moribund and dead animals which are single-caged would not be lost due to cannibalism.

H. Diet:

In general, feed and water should be provided *ad libitum*, and the diets should meet the nutritional requirements of the species[14] for normal growth and longevity. Unless special circumstances apply which justify otherwise, care should be taken to ensure that the diets of the test substance treated groups of animals contain the same levels of calories and nutrients (e.g., fiber, micronutrients) as the diets of the control group. Inadequately controlled dietary variables may result in nutritional imbalances or caloric deprivation that could confound interpretation of the toxicity study results (e.g., lifespan, background rates of tumor incidences) and alter the outcome and reproducibility of the studies. However, the Agency is also aware of some beneficial effects on the survivability of certain animal species that have been on calorie-restricted,[4] [5] or low-protein diets.[2] [9] The Agency may accept such study results if the sponsor provides sufficient historical control data on the diet, and the study is well-conducted.

The following issues are important to consider when establishing diets for animals in chronic toxicity studies:

When the test substance has no caloric value and constitutes a substantial amount of the diet (e.g., more than 5%), both caloric and nutrient densities of the high dose diet would be diluted in comparison to the diets of the other groups. As a consequence, some high dose animals may receive higher test substance doses than expected because animals fed such diluted diets *ad libitum* may eat more than animals in other dosed groups to compensate for the differences in energy and nutrient content of the high dose diets. Such circumstances make it especially important that feed consumption of these animals be as accurately and closely monitored as possible in order to determine whether changes observed could be due to overt toxicity of the test substance or to a dietary imbalance. To further aid in this assessment, two control groups can be used; one group would be fed the undiluted control diet and a second group would be fed the control diet supplemented with an inert filler (e.g., methylcellulose) at a percentage equal to the highest percentage of the test substance in the diet.

When the vehicle for the test substance is expected to have caloric and/or nutritional values, which are greater than that of the control ration, an adjustment in the caloric and/or nutritional components may be necessary.

When administration of the test substance is expected to have an effect on feed intake because of its unpleasant taste or texture, other feeding regimens or experimental designs may be necessary. Consultation with the FDA is recommended when alternatives are being considered.

When the test substance interferes with the absorption of nutrients, leading to nutritional deficiencies or changes in nutrient ratios, this can confound assessment of the toxicological endpoints under consideration. For example, fat soluble vitamins may preferentially partition with a mineral oil or fat substitute which is largely unabsorbed, such that a potential deficiency in these vitamins may result. This potential may be eliminated by additional nutrient fortification of the feed for those groups receiving the test substance. Appropriate levels of nutrient fortification should be determined experimentally.

Other related issues (e.g., advantages and disadvantages of using natural ingredient versus purified diets) are discussed in the National Research Council publication on nutrient requirements of laboratory animals.[14]

I. Assignment of Control and Compound Treated Animals:

Animals should be assigned to control and compound treated groups in a stratified random manner. This will help minimize bias and assure comparability of pertinent variables across compound treated and control groups. In general, mean body weights and/or body weight ranges are used as a basis of randomization. If other characteristics are used as the basis for randomization, they should be described and justified.

Animals in all groups should be placed on study on the same day. If this is not possible because of the large number of animals in a study, animals may be placed on study over several days. When the latter recommendation is followed, a preselected portion of the control and experimental animals should be placed on the study each day in order to maintain concurrence.

J. Mortality:

Excessive mortality due to poor animal management is unacceptable and may be a cause to repeat the study.

K. Autolysis:

Adequate animal husbandry practices should result in considerably less than 10 % loss of animals and tissues or organs in a study because of autolysis. Autolysis in excess of this standard may be a cause to repeat the study.

L. Necropsy:

Necropsy should be performed soon after an animal is sacrificed or found dead, so that loss of tissues due to autolysis is minimized. When necropsy cannot be performed immediately, the animal should be refrigerated at a temperature that is low enough to prevent autolysis (i.e., between 4°C and 8°C), but not so low as to cause cell damage. If histopathological examination is to be conducted, tissue specimens should be taken from the animals and placed in appropriate fixatives when the necropsy is performed.

III. Test Substance

The test substance used in chronic toxicity studies should be the same substance that the petitioner/notifier intends to market or, when appropriate, the test substance may be a constituent chemical or an impurity. A single lot of test substance should be used throughout the study. When this is not possible, lots that are as similar as possible in purity and composition should be used. It is the responsibility of the petitioner/notifier to notify the animal test facility of the purity of the test substance, as well as the identity and concentration of any impurities that might be present.

A. Identity:

The identity of the test substance (e.g., either a single component or a mixture of components) should be known. The petitioners/notifiers are encouraged to consult with the Agency regarding the method(s) of determination of the test substance, and should provide all relevant Chemical Abstract Service (CAS) Registry numbers.

B. Composition/Purity:

The composition of the test substance should be known including the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials.

C. Conditions of Storage:

The test samples should be stored under conditions that maintain their stability and purity until the studies are complete.

D. Expiration Date:

The expiration date of the test material should be known and easily available. Test materials should not be used past their expiration date.

IV. Experimental Design

A. Duration of Testing:

The test animal should be exposed to the test substance 7 days per week for at least 12 months (one year).

B. Route of Administration:

The route of administration of the test substance should approximate that of normal human exposure, and if possible, the oral route should be used. A justification should be provided when using other routes. The same method of administration should be used for all test animals throughout the study. The test substance should be administered in one of the following ways:

- **In the diet**, if human exposure to the test substance is likely to be through consumption of solid foods or a combination of solid and liquid foods. If the test substance is added to the diet, animals should not be able to selectively consume either basal diet or test substance in the diet on the basis of color, smell, or particle size. If the test substance is mixed with ground feed and pelleted, nothing in the pelleting process should affect the test substance (for example, heat-labile substances may be destroyed during pellet production by a steam process). When the test substance is administered in the diet, dietary levels should be expressed as mg of the test substance per kg of feed.
- **Dissolved in the drinking water**, if the test substance is likely to be ingested in liquid form by humans (for example, in soft drinks or beer), or if administration in the diet of rodents is inappropriate. The amount of test substance administered in drinking water should be expressed as mg of test substance per ml of water.
- **By encapsulation or oral intubation (gavage)**, if the two previous methods are unsatisfactory or if human exposure is expected to be through daily ingestion of a single, large bolus dose instead of continual ingestion of small doses. If the test substance is administered by gavage, it should be given at approximately the same time each day. The maximum volume of solution that can be given by gavage in one dose depends on the test animal's size; for rodents, the volume ordinarily should not exceed 1 ml/100 g body weight. If the gavage vehicle is oil, then the volume should be no more than 0.4 ml/100 g of body weight, and the use of a low-fat diet should be considered. It is best to adjust the volume every 1-3 days based on the animal's body weight response. If the test substance should be given in divided doses, all doses should be administered within a 6 hour period. Doses of test substance administered by gavage should be expressed as mg of test substance per ml of gavage vehicle. Finally, the petitioner/notifier should provide information that can allow the Agency to conclude that administration of the test substance by encapsulation or gavage is equivalent in toxicologically important respects to administration in the diet or drinking water. Alternatively, metabolic information on both modes of administration should be provided so that appropriate interpretation of data can be accomplished.

C. Dose groups:

Dose selection for chronic toxicity studies should be based on results from subchronic studies and other related information. Appropriate dose selection should enable one to predict the maximum dose that produces no adverse effects, also known as the NOEL or NOAEL. A rationale should be provided for the dose selection. Administration of the test substance to all dose groups should be done concurrently (see section II.I: Assignment of Control and Compound Treated Animals).

1. Controls:

A concurrent control group of test animals fed the basal diet is necessary. A carrier or vehicle for the test substance should be given to control animals at a volume equal to the maximum volume of carrier or vehicle given to any group of treated animals. Sufficient toxicological information should be available on the carrier or vehicle to ensure that its use will not compromise the results of the study. If there is insufficient information about the toxic properties of the carrier or vehicle used to administer the test substance, an additional control group that is not exposed to the carrier or vehicle should be included. In all other respects, animals in the control group should be treated the same as animals in dosed groups. (See also section II.H: Diet.)

2. Selection of Treatment Doses:

We recommend that a minimum of three dose levels of the test substance should be used in chronic toxicity studies. The following is a general consideration in selecting the treatment dose levels: 1) the high dose should be sufficiently high to induce toxic responses in test animals, and should not cause fatalities high enough to prevent meaningful evaluation of the data from the study; 2) the low dose should not induce biologically significant toxic responses in test animals; and 3) the intermediate dose should be sufficiently high

to elicit minimal toxic effects in test animals (such as alterations in enzyme levels or slight decreases in body weight gains).

We do not recommend that petitioner/notifiers use information unrelated to the toxicity of the test substance as a basis for dose selection of chronic toxicity studies. For example, the highest dose in a chronic toxicity study should not be selected so as to provide a pre-determined margin of safety over the maximum expected human exposure to the test substance, assuming that the results of testing at that dose will be negative.

High Dose:

The high dose in a chronic toxicity study should produce toxicity so that a toxicological profile of the test substance can be obtained. When no toxicity is observed in other studies, however, the high dose could be subject to some preset limits such as the highest percent of the test substance in the diet that could be fed without compromising nutritional balance with other nutrients (e.g., about 5%, see also 'section II.H: Diet' for other important dietary issues).

In general, the high dose tested is estimated following a careful analysis of data from appropriate subchronic toxicity tests. As the scientific community's experience with toxicity testing has accumulated, the need to consider a broad range of biological information when selecting the high dose has become increasingly clear. For example, data from a subchronic (90-day) study concerning changes in body and organ weight and clinically significant alterations in neurological, hematological, urinary and clinical chemistry measurements, in combination with more definitive exposure-related toxic, gross or histopathologic endpoints, can be used to estimate the high dose in a chronic toxicity study.

Although the high dose in a chronic toxicity study should be selected to achieve toxic responses in test animals, and should not cause fatalities high enough to prevent meaningful evaluation of the data from the study, the Agency recognizes that this goal may not always be met. In situations such as these, when it is unclear what dose of the test substance is the high dose, the petitioner/notifier should consult with the Agency to determine an appropriate high dose for the chronic toxicity study.

Low Dose:

The low dose level should not interfere with the normal growth, development, and lifespan of test animals, nor should it produce any other biologically significant signs of toxicity (e.g., NOEL or NOAEL).

Intermediate Dose:

The intermediate dose should produce minimal signs of toxicity. The exact dose selected as the intermediate dose may depend on the pharmacokinetic properties of the test substance.

D. Computerized systems

Computerized systems that are used in the generation, measurement, or assessment of data should be developed, validated, operated, and maintained in ways that are consistent with the intention of the Good Laboratory Practice principles.[11] The FDA has endorsed the use of the Standard for Exchange of Nonclinical Data (SEND) format for electronic transmission of animal study data. You are encouraged to contact the Agency for more information on this electronic protocol.

V. Observations and Clinical Tests

A. Observations of Test Animals:

Routine cage-side observations should be made on all animals inside the cage once or twice a day throughout the study for signs of departure from normal activity, morbidity and mortality. The usual interval between multiple periods of observations should be at least 6 hours. Individual records should be maintained for each animal and, as possible, the onset and progression of any effects should be recorded, preferably using a scoring system. If grossly visible or palpable tumors develop, the following parameters should be recorded; time of onset, location, dimensions, appearance and progression.

An expanded set of clinical evaluations performed on animals inside and outside of the cage, should be carried out to enable detection not only of general signs of departure from normal activity, morbidity and mortality but also of neurologic disorders, behavioral changes, autonomic dysfunctions, and other signs of nervous system toxicity. Specific information about the systematic clinical tests/observations is contained in Chapter IV.C.10[8]. This expanded set of clinical examinations, conducted on animals inside and outside the cage, should be age appropriate and performed on all animals at least once prior to initiation of treatment, and periodically during treatment. Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions or other evidence of autonomic activity (e.g.,

lacrimation, piloerection, pupil size, unusual respiratory pattern). Additionally, changes in gait, posture and response to handling, as well as the presence of clonic or tonic seizures, stereotypic (e.g., excessive grooming, repetitive circling) or bizarre behavior (e.g., self-mutilating, walking backwards) should be recorded. During the course of a study, toxic and pharmacologic signs may suggest the need for additional clinical tests or expanded post-mortem examinations.

B. Body Weight and Feed Intake Data:

Accurate individual body weight, feed, and water consumption measurements are critical in the objective evaluation of the effect of a test substance on experimental animals, since changes in these variables are often the first signs of toxicity. Complete records for these parameters are essential in assessing the time-related occurrence of toxicity-induced changes including tumor formation. A discussion of some of the variables that affect feed consumption and weight gain/loss can be found under sections II.H: Diet and IV.B: Route of Administration.

Body weights for all test animals should be recorded weekly for the first 13 weeks, and monthly thereafter for the duration of the study. Feed consumption (or water consumption if the test substance is administered in the drinking water) should be measured at the same interval as body weights; petitioners/notifiers should also attempt to quantify spillage of feed by test animals. When it is suspected that test substance administration may be affected by any of the following conditions; 1) feed palatability issues, 2) marked changes in body weight, or 3) increased numbers of animal deaths, the petitioners/notifiers should measure weights and feed (water) consumption more frequently after the initial 13 week period (e.g., every two weeks). Petitioners/notifiers should also use this accumulated information to calculate intake of the test substance as mg/kg body weight/day.

C. Clinical Testing:

Ophthalmological examination, hematology profiles, clinical chemistry tests, and urinalyses should be performed as described in the following sections:

1. Ophthalmological Examination:

This examination should be performed by a qualified individual on all animals before the study begins and on control and high-dose animals at the end of the study. If the results of examinations at termination indicate that changes in the eyes may be associated with administration of the test substance, ophthalmological examinations should be performed on all animals in the study.

2. Hematology:

Hematological tests should be performed on all animals during the first 2 weeks of study, at 3, 6 and 12 months during the study. The time of the first sampling may be based on test results from short-term studies. If data trends or significant parameter changes (biological or statistical) are observed that are of concern at the 12-month measurement and the study lasts longer than one-year, additional hematological testing should be conducted at the end of the study.

Ideally, the same rodents should be sampled at each collection time point. Blood samples should be analyzed individually, and not pooled. If, due to the large number of animals, it becomes necessary to draw blood samples on more than one consecutive day at each sampling point, the samples should be obtained at approximately the same time each day.

The following determinations are recommended: hematocrit, hemoglobin concentration, erythrocyte count, total and differential leukocyte counts, mean corpuscular hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration, platelet count, and a measure of clotting potential (e.g., clotting time, prothrombin time, activated partial thromboplastin time).

Test compounds may have an effect on the hematopoietic system and therefore appropriate measures should be employed so that evaluations of reticulocyte counts and bone marrow cytology may be performed if warranted. Reticulocyte counts should be obtained for each animal using automated reticulocyte counting capabilities, or from air-dried blood smears. Bone marrow slides for cytological evaluation should be prepared from each animal. These slides only need to be microscopically examined when effects on the hematopoietic system are noted.

3. Clinical Chemistry:

Clinical chemistry tests should be performed on all animals during the first 2 weeks of study, and at 3, 6 and 12 months during the study. The time of the first sampling may be based on test results from short-term

studies. If data trends or significant parameter changes (biological or statistical) are observed that are of concern at the 12-month measurement and the study lasts longer than one-year, additional clinical chemistry testing should be conducted at the end of the study.

Ideally, the same rodents should be sampled at each collection time point. Blood samples should be drawn at the end of the fasting time and before feeding. Fasting duration should be appropriate for the species and the analytical tests to be performed. Blood samples should be analyzed individually, and not pooled. If animals are sampled on more than one day during a study, blood should he drawn at approximately the same time each sampling day.

Clinical chemistry tests that are appropriate for all test substances include measurements of electrolyte balance, nutrients metabolism, and liver and kidney function. Specific determinations should include:

Hepatocellular evaluation (at least 3 of the following 5)

- Alanine aminotransferase (SGPT, ALT)
- Aspartate aminotransferase (SGOT, AST)
- Sorbitol dehydrogenase
- Glutamate dehydrogenase
- Total bile acids

Hepatobiliary evaluation (at least 3 of the following 5)

- Alkaline phosphatase
- Bilirubin (total)
- Gamma-glutamyl transpeptidase (GG transferase)
- 5' nucleotidase
- Total bile acids

Other markers of cell changes or cellular function

- Albumin
- Calcium
- Chloride
- Cholesterol (total)
- Cholinesterase
- Creatinine
- Globulin (calculated)
- Glucose
- Phosphorous
- Potassium
- Protein (total)
- Sodium
- Triglycerides
- Urea nitrogen
- The Agency understands that the specific nature of the test substance may warrant the consideration of alternative tests. Appropriate justification for alternative tests should be presented in study reports.

In spite of standard operating procedures and equipment calibration, it is not unusual to observe considerable variation in the results of clinical chemistry analyses from day to day.[3] Ideally, clinical chemistry analyses for all dose groups should be completed during one day. If this is not possible, analyses should be conducted in such a way as to minimize potential variability.

4. Urinalyses:

The determination of volume of urine collected, urine specific gravity, pH, glucose, and protein, as well as microscopic analysis of urine for sediment and presence of blood and/or blood cells, are recommended[8] before dosing, and at 3, 6 and 12 months during the study. If data trends or significant parameter changes (biological or statistical) are observed that are of concern at the 12-month measurement and the study lasts longer than one-year, additional urinalyses testing should be conducted at the end of the study. These tests should be performed on all animals.

VI. Necropsy and Microscopic Examination

A. Gross Necropsy

All test animals should be subjected to complete gross necropsy, including examination of external surfaces, orifices, cranial, thoracic and abdominal cavities, carcass, and all organs. The gross necropsy should be performed by, or under the direct supervision of, a qualified pathologist, preferably the pathologist who will later perform the microscopic examination.

B. Organ Weight

Organs that should be weighed at minimum include the adrenals, brain, epididymides, heart, kidneys, liver, spleen, testes, prostate, thyroid/parathyroid, thymus if present, ovaries and uterus. Before being weighed, organs should be carefully dissected and trimmed to remove fat and other contiguous tissue. Organs should be weighed immediately after dissection to minimize the effects of drying on organ weight.

C. Preparation of Tissues for Microscopic Examination

Generally, the following tissues should be fixed in 10 % buffered formalin (or another generally recognized fixative) and sections prepared and stained with hematoxylin and eosin (or another appropriate stain) in preparation for microscopic examination. Lungs should be inflated with fixative prior to immersion in fixative.

- Adrenals
- Aorta
- Bone (femur)
- Bone marrow (sternum)
- Brain (at least 3 different levels)
- Cecum
- Colon
- Corpus and cervix uteri
- Duodenum
- Epididymides
- Esophagus
- Eyes
- Gall bladder (if present)
- Harderian gland
- Heart
- Ileum
- Jejunum

- Kidneys
- Liver
- Lung (with main-stem bronchi)
- Lymph nodes (1 related to route of administration and 1 from a distant location)
- Mammary glands
- Nasal turbinates
- Ovaries and fallopian tubes
- Pancreas
- Pituitary
- Prostate
- Rectum
- Salivary gland
- Sciatic nerve
- Seminal vesicle (if present)
- Skeletal muscle
- Skin
- Spinal cord (3 locations: cervical, mid-thoracic, and lumbar)
- Spleen
- Stomach
- Testes
- Thymus (if present)
- Thyroid/parathyroid
- Trachea
- Urinary bladder
- Vagina
- Zymbal's gland
- All tissues showing abnormality

D. Microscopic Evaluation

All gross lesions should be examined microscopically. All tissues from the animals in the control and high dose groups should be examined. If treatment related effects are noted in certain tissues, then those specific tissues in the next lower dose level tested should be examined. Successive examination of the next lower dose level continues until no effects are noted. In addition, all tissues from animals that died prematurely or were sacrificed during the study should be examined microscopically. If there are questions related to the review and interpretation of pathological lesions and statistical results, additional discussion may be found in Chapters IV.B.3 [9]. and IV.B.4 [10]. of the Redbook 2000.

E. Histopathology of Lymphoid Organs

Histological evaluation of the lymphoid organs should be performed as described in the section on immunotoxicity testing (see Chapter V.D. of the 1993 draft Redbook II). A recent publication provides further discussion on this subject.[10]

VII. References

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8. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/ucm078323.htm
9. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/ucm078318.htm
10. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/ucm078320.htm

Redbook 2000: IV.C.5.b One-Year Toxicity Studies with Non-Rodents

November 2003

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter IV.C.5.b. One-Year Toxicity Studies with Non-Rodents

Return to *Redbook 2000* table of contents[1]

This guidance represents the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

Long-term, one-year toxicity tests with non-rodents (usually dogs) should be conducted for a minimum of 12 months (one year). Results of these tests can be used to (1) characterize the toxicity of the test substance in non-rodents and (2) determine the dose of the test substance that produces no observed adverse effects (NOEL or NOAEL) for some toxicological endpoints. One-year toxicity tests are not conducted for the purpose of assessing carcinogenicity, although data from these tests may reveal information about the carcinogenicity of the test substance. The following guideline is written for dogs, if other non-rodents are used, modificaitons to the guidline may be necessary. Sponsors/submitters of petitions/notifications are encouraged to also become familiar with the Guidance for Reporting Results of Toxicity Studies (Chapter IV.B.2.), Pathology Considerations in Toxicity Studies (Chapter IV.B.3.), and Statistical Considerations in Toxicity Studies (Chapter IV.B.4.) during the development of study design.

Scientifically justified changes to the 1993 draft "Redbook II" version of this section have been made following consultation with other authoritative guidelines and publications[1-7].

I. Good Laboratory Practice

Nonclinical laboratory studies must be conducted according to U.S. FDA good laboratory practice (GLP) regulations, issued under Part 58. Title 21. Code of Federal Regulations. This document may be obtained from the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C., 20402, (toll free 866-512-1800).

II. Test Animals

A. Care, Maintenance and Housing:

Recommendations about the care, maintenance, and housing of animals contained in NIH publication 85-23, "Guide for the Care and Use of Laboratory Animals"[7], and the DHEW publication no. 78-23 should be followed unless they conflict with specific recommendations in these guidelines. Additionally, dogs should receive appropriate vaccinations prior to commencement of the study.

B. Selection of Species, Strains and Sex:

These guidelines are for studies with dogs. If other non-rodent species are used, modifications of these guidelines may be necessary. Both male and female test animals, that are healthy and have not been subjected to previous experimental procedures should be used.

It is important to consider the test animals' general sensitivity and the responsiveness of particular organs and tissues of test animals to toxic chemicals when selecting non-rodent species, i.e., dogs, for toxicity studies. Additionally, it is important that test animals come from well-characterized and healthy colonies and should be selected so that they are likely to achieve the recommended duration of the study.

C. Age:

Dogs should be obtained so that, following an appropriate time of acclimation, dosing begins when animals are no older than 4 to 6 months of age.

D. Number and Sex:

Equal numbers of male and female dogs should be used in one-year toxicity studies. Experimental and control groups should have at least 4 dogs per sex per dose group at the beginning of the study. If interim necropsies are planned, the number of dogs per sex per group should be increased by the number scheduled to be sacrificed before completion of the study. These recommendations will help ensure that the number of animals that survive until the end of the study will be sufficient to permit a meaningful evaluation of toxicological effects.

E. Infected Animals:

Generally, it is not possible to treat animals for infection during the course of a study without risking interaction between the compound used for treatment and the test substance. This interaction may confound or complicate the interpretation of study results.

F. Animal Identification:

Test animals should be characterized by reference to their species, strain (and substrain), sex, age, and weight. Each animal must be assigned a unique identification number (e.g., ear tag, implanted identification chip, tatoo).

G. Caging:

Animals should be housed one per cage or run (single-caged). This recommendation reflects three principal concerns:

- The amount of feed consumed by each animal in the study cannot be determined when more than one animal is housed in each cage. This information is necessary in the determination of feed efficiency (relationship of feed consumed to body weight gained).
- Minimizing the possibility of confounding analyses and determining whether decreases in body weight gain are due to decreased palatability or substance mediated toxicity.
- Organs and tissues from moribund and dead animals which are single-caged would not be lost due to cannibalism.

H. Diet:

In general, feed and water should be provided *ad libitum* to animals in toxicity studies, and the diets for these studies should meet the nutritional requirements of the species [3-6] for normal growth and reproduction. Unless special circumstances apply which justify otherwise, care should be taken to ensure that the diets of

the compound treated groups of animals are isocaloric (equivalent in caloric density) with and contain the same levels of nutrients (e.g., fiber, micronutrients) as the diets of the control group. Unrecognized or inadequately controlled dietary variables may result in nutritional imbalances or caloric deprivation that could confound interpretation of the toxicity study results (e.g., lifespan, background rates of tumor incidences) and alter the outcome and reproducibility of the studies.

The following issues are important to consider when establishing diets for animals in toxicity studies:

When the test substance has no caloric value and constitutes a substantial amount of the diet (e.g., more than 5%), both caloric and nutrient densities of the high dose diet would be diluted in comparison to the diets of the other groups. It is important that feed consumption of animals be as closely and accurately monitored as possible in order to determine whether changes observed could be due to overt toxicity of the test substance or to a dietary imbalance. To further aid in this assessment, two control groups can be used; one group would be fed the undiluted control diet and a second group would be fed the control diet supplemented with an inert filler (e.g., methylcellulose) at a percentage equal to the highest percentage of the test substance in the diet.

When the vehicle for the test substance is expected to have caloric and/or nutritional values, which are greater than that of the control ration, an adjustment in the caloric and/or nutritional components may be necessary.

When administration of the test substance is expected to have an effect on feed intake because of its unpleasant taste or texture, paired feeding can be used to eliminate the differences in consumption between control and compound treated groups. When a paired feeding study design is to be employed, pairs of animals of the same sex, age, and approximate size are selected and fed the control or the experimental diet. Animals should be single-caged so that feed consumption can be determined daily, and the control animal is then fed an amount of food equal to that which the paired experimental animal ate on the preceding day. If the test substance is non-nutritive and composes a significant proportion of the diet, the pair-fed control animals should be fed an amount of its feed such that it consumes a nutritionally equivalent amount of diet as the paired experimental animal. Additionally, the study should include a second group of control animals fed the normal ration amount to ensure that the impact on any observed experimental result is due to differences in energy or nutrient intake.

When the test substance interferes with the absorption of nutrients, leading to nutritional deficiencies or changes in nutrient ratios, this can confound assessment of the toxicological endpoints under consideration. For example, fat soluble vitamins may preferentially partition with a mineral oil or fat substitute which is largely unabsorbed, such that a potential deficiency in these vitamins may result. This potential may be eliminated by additional nutrient fortification of the feed for those groups receiving the test substance. Appropriate levels of nutrient fortification should be determined experimentally.

It may be preferable to use a semi-purified diet prepared with known amounts of well-characterized ingredients for short-term and subchronic toxicity studies because of batch to batch variations in diet composition (e.g., fiber, mineral, vitamins, isoflavones) in some of the commonly used laboratory animal chows. The use of these semi-purified diets, however, may not be advisable in long-term and reproductive studies due to inadequate historical data related to their influences on animal survival and toxicological endpoints. For example, loss of necessary but unidentified micronutrients in the semipurified diet may interfere with normal reproduction.

Related issues are discussed in the section on Diets for Toxicity Studies in Chapter IV.B.5. in the 1993 draft "Redbook II"

I. Assignment of Control and Compound Treated Animals:

Animals should be assigned to control and compound treated groups in a stratified random manner; this will help minimize bias and assure comparability of pertinent variables across compound treated and control groups (for example: mean body weights and body weight ranges). If other characteristics are used as the basis for randomization then that characterization should be described and justified.

Animals in all groups should be placed on study on the same day; if this is not possible because of the large number of animals in a study, animals may be placed on study over several days. If the latter recommendation is followed, a preselected portion of the control and experimental animals should be placed on the study each day in order to maintain concurrence.

J. Mortality:

Excessive mortality due to poor animal management is unacceptable and may be cause to repeat the study.

For example, under normal circumstances, mortality in the control group should not exceed 10%.

K. Autolysis:

Adequate animal husbandry practices should result in considerably less than 10% of animals and tissues or organs lost to a study because of autolysis. Autolysis in excess of this standard may be cause to repeat the study.

L. Necropsy:

Necropsy should be performed soon after an animal is sacrificed or found dead, so that loss of tissues due to autolysis is minimized. When necropsy cannot be performed immediately, the animal should be refrigerated at a temperature that is low enough to prevent autolysis but not so low as to cause cell damage. If histopathological examination is to be conducted, tissue specimens should be taken from the animals and placed in appropriate fixatives when the necropsy is performed.

III. Test Substance

The test substance used in toxicity studies should be the same substance that the petitioner/notifier intends to market. A single lot of test substance should be used throughout the study, when possible. Alternatively, lots that are similar in purity and composition should be used.

A. Identity:

The identity of the test substance or mixture of substances to be tested should be known. We urge petitioners/notifiers to consult with the Agency in determination of test compound and to provide a Chemical Abstract Service (CAS) Registry Number or Numbers.

B. Composition/Purity:

The composition of the test substance should be known, including the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials.

C. Conditions of Storage:

The test sample should be stored under conditions that maintain its stability, quality, and purity until the studies are complete.

D. Expiration Date:

The expiration date of the test material should be known and easily available. Test materials should not be used past their expiration date.

IV. Experimental Design

A. Duration of Testing:

Animals should be exposed to the test substance 7 days per week for at least 52 weeks (one year).

B. Route of Administration:

The route of administration of the test substance should approximate that of normal human exposure, if possible. For food ingredients (e.g., food and color additives) the oral route of administration is preferred. A justification should be provided when other routes are used. The same method of administration should be used for all test animals throughout the study.

The test substance should be administered in one of the following ways:

- **In the diet**, if human exposure to the test substance is likely to be through consumption of solid foods or a combination of solid and liquid foods. If the test substance is added to the diet, animals should not be able to selectively consume either basal diet or test substance in the diet on the basis of color, smell, or particle size. If the compound is mixed with ground feed and pelleted, nothing in the pelleting process should affect the test substance (for example, heat-labile substances may be destroyed during pellet production by a steam process). When the test substance is administered in the diet, dietary levels should be expressed as mg of the test substance per kg of feed.

- **Dissolved in the drinking water**, if the test substance is likely to be ingested in liquid form (e.g., in soft drinks, beer), or if administration in the diet is inappropriate for other reasons. The amount of test substance administered in drinking water should be expressed as mg of test substance per ml of water.
- **By encapsulation or oral intubation (gavage)**, if the two previous methods are unsatisfactory or if human exposure is expected to be through daily ingestion of single, large doses instead of continual ingestion of small doses. If the test substance is administered by gavage, it should be given at approximately the same time each day. The maximum volume of solution that can be given by gavage in one dose depends on the test animal's size. Doses of test substance administered by gavage should be expressed as mg of test substance per ml of gavage vehicle. Finally, the petitioner/notifier should provide information that can allow the reviewer to conclude that administration of the test compound by encapsulation or gavage is equivalent in all toxicologically important respects to administration in the diet or drinking water. Alternatively, metabolic information on both modes of administration should be provided so that appropriate interpretation of data can be accomplished.

C. Dose groups:

A minimum of three dose levels of the test substance should be used per sex (one dose level per group). A concurrent control group should be included in the study.

1. Selection of Treatment Doses:

Dose selection for toxicity studies should be based on information related to the toxicity of the test substance. Information from 90-day toxicity studies in non-rodents can help determine appropriate doses for the one-year toxicity study in non-rodents (see Chapter IV.C.4.b.).

A minimum of three dose levels of the test substance and a concurrent control group should be used in toxicity studies. When designing and conducting toxicity studies the following should be considered: 1) the high dose should be sufficiently high to induce toxic responses in test animals; 2) the low dose should not induce toxic responses in test animals; and 3) the intermediate dose should be sufficiently high to elicit minimal toxic effects in test animals (such as alterations in enzyme levels or slight decreases in body weight gains). No dose should cause an incidence of fatalities that prevents meaningful evaluation of the data. Administration of the test substance to all dose groups should be done concurrently.

2. Controls:

A concurrent control group of test animals is required. The control group in dietary studies should be fed the basal diet. Exceptions to this and other related information, including a discussion regarding pair-feeding, was provided above in section "II Test Animals, H. Diet".

A carrier or vehicle for the test substance should be given to control animals at a volume equal to the maximum volume of carrier or vehicle given to any dosed group of animals. Sufficient toxicology information should be available on the carrier or vehicle to ensure that its use will not compromise the results of the study. If there is insufficient information about the toxic properties of the vehicle used to administer the test substance, an additional control group that is not exposed to the carrier or vehicle should be included. In all other respects, animals in the control group should be treated the same as animals in dosed groups. (See additional information in section "II Test Animals, H. Diet" above.)

D. Computerized systems

Computerized systems that are used in the generation, measurement, or assessment of data should be developed, validated, operated, and maintained in ways that are compliant with Good Laboratory Practice principles. [8]

V. Observations and Clinical Tests

A. Observations of Test Animals:

Routine cage-side observations should be made on all animals at least once or twice a day throughout the study for general signs of pharmacologic and toxicologic effects, morbidity and mortality. The usual interval between observations should be at least 6 hours. Individual records should be maintained for each animal and the time of onset and the characteristics and progression of any effects should be recorded, preferably using a scoring system.

An expanded set of clinical evaluations, performed inside and outside of the cage, should be carried out in

short-term and subchronic toxicity studies in rodents and non-rodents, in one-year non-rodent toxicity studies, and reproductive toxicity studies in rodents to enable detection not only of general pharmacologic and toxicologic effects but also of neurologic disorders, behavioral changes, autonomic dysfunctions, and other signs of nervous system toxicity. Specific information about the systematic clinical tests/observations is contained in Chapter IV.C.10. This expanded set of clinical examinations (Chapter IV.C.10), conducted inside and outside the cage, should be age appropriate and performed on all animals at least once prior to initiation of treatment, and periodically during treatment. Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions or other evidence of autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern). Additionally, changes in gait, posture and response to handling, as well as the presence of clonic or tonic seizures, stereotypes (e.g., excessive grooming, repetitive circling) or bizarre behavior (e.g., self-mutilating, walking backwards) should be recorded. Tumor development, particularly in long-term studies, should be followed: the time of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumor should be recorded. During the course of a study, toxic and pharmacologic signs may suggest the need for additional clinical tests or expanded post-mortem examinations.

B. Body Weight and Feed Intake Data:

Test animals should be weighed at least once a week. Feed consumption (or water consumption if the test substance is administered in the drinking water) should be measured every week during the one-year toxicity study. Petitioners should use this information to calculate intake of the test substance during each week of the study. Petitioners should also attempt to quantify spillage of feed by test animals, and to determine if spillage is greater with test diets than with control diets. Appropriate discussions of feed spillage should be included in the study report.

C. Clinical Testing:

Ophthalmological examination, hematology profiles, clinical chemistry tests, and urinalyses should be performed as described in the following sections:

1. **Opthalmological Examination:** This examination should be performed by a qualified individual on all animals before the study begins and on control and high-dose animals at the end of the study. If the results of examinations at termination indicate that changes in the eyes may be associated with administration of the test substance, ophthalmological examinations should be performed on all animals in the study.
2. **Hematology**: Blood samples should be obtained from all animals of all groups at the following times: prior to initiation of treatment, during the first two weeks on study (receiving treatment), at 3-month intervals during the study, and at termination. The determination of the second sampling time point should be based on the expected time of initial toxicological effects on the organ systems. Blood samples should be analyzed individually, and not pooled. Blood should be drawn at approximately the same time each sampling day.
    1. The following determinations are recommended:
        1. hematocrit
        2. hemoglobin concentration
        3. erythrocyte count
        4. total and differential leukocyte counts
        5. mean corpuscular hemoglobin
        6. mean corpuscular volume
        7. mean corpuscular hemoglobin concentration
        8. and a measure of clotting potential (such as clotting time, prothrombin time, thromboplastin time, or platelet count).

    Test compounds may have an effect on the hematopoietic system and therefore appropriate measures should be employed so that evaluations of reticulocyte counts and bone marrow cytology may be performed if warranted. Reticulocyte counts should be obtained for each animal using automated reticulocyte counting capabilities, or from air-dried blood smears. Bone marrow slides should be prepared from each animal for evaluating bone marrow cytology. These slides would only need to be examined microscopically if effects on the hematopoietic system were noted.

Clinical Chemistry: Blood samples should be obtained from all animals of all groups at the following times: prior to initiation of treatment, during the first two weeks on study (receiving treatment), at 3-month intervals during the study, and at termination. The determination of the second sampling time point should be based on the expected time of initial toxicological effects on the organ systems. Dogs should be fasted overnight and blood drawn for clinical chemistry tests before feeding. Blood samples should be analyzed individually, and not pooled. Blood should he drawn at approximately the same time each sampling day.

Clinical chemistry tests that are appropriate for all test substances include measurements of electrolyte balance, carbohydrate metabolism, and liver and kidney function. Specific determinations should include:

1. Hepatocellular evaluation: select at least 3 of the following 5
    1. alanine aminotransferase (SGPT, ALT)
    2. aspartate aminotransferase (SGOT, AST)
    3. sorbitol dehydrogenase
    4. glutamate dehydrogenase
    5. total bile acids
2. Hepatobiliary evaluation: select at least 3 of the following 5
    1. alkaline phosphatase
    2. bilirubin (total)
    3. gamma-glutamyl transpeptidase (GG transferase)
    4. 5' nucleotidase
    5. total bile acids
3. Other markers of cell changes or cellular function
    1. albumin
    2. calcium
    3. chloride
    4. cholestrol(total)
    5. cholinesterase
    6. creatinine
    7. globulin (calculated)
    8. glucose (in fasted animals)
    9. phosphorous
    10. potassium
    11. protein (total)
    12. sodium
    13. triglycerides (fasting)
    14. urea nitrogen

1. However, when adequate volumes of blood cannot be obtained from test animals, the following determinations should generally be given priority. FDA understands that the specific nature of the test compound may warrant the consideration of alternative tests. Appropriate justification for alternative tests should be presented in study reports.

    1. alanine aminotransferase
    2. alkaline phosphatase
    3. chloride
    4. creatinine
    5. gamma-glutamyl transpeptidase (GG transferase)

6. glucose (in fasted animals)
7. potassium
8. protein (total)
9. sodium
10. urea nitrogen

Additional clinical chemistry tests may be recommended to extend the search for toxic effects attributable to a test substance. The selection of specific tests will be influenced by observations on the mechanism of action of the test substance. Clinical chemistry determinations that may be recommended to ensure adequate toxicological evaluation of the test substance include analyses of acid/base balance, hormones, lipids, methemoglobin, and proteins.

In spite of standard operating procedures and equipment calibration, it is not unusual to observe considerable variation in the results of clinical chemistry analyses from day to day[9]. Ideally, clinical chemistry analyses for all dose groups should be completed during one day. If this is not possible, analyses should be conducted in such a way as to minimize potential variability.

4. **Urinalyses**: A timed urine-volume collection should take place prior to dosing, at 3-month intervals, and at the end of the study for all animals in the study. The volume of urine collected, specific gravity, pH, glucose, and protein should be determined as well as conducting a microscopic evaluation of urine for sediment and presence of blood/blood cells[10].
5. **Neurotoxicity Screening/Testing** : Screening for neurotoxic effects should be routinely carried out in dogs and other non-rodents (preferably miniature swine). The neurotoxicity screen should be age appropriate and would typically include: (1) specific histopathological examination of tissue samples representative of major areas of the brain, spinal cord, and peripheral nervous system (see organs and tissues listed below under VI.C. Preparation of Tissues for Microscopic Examination) and (2) a functional battery of quantifiable observations and manipulative tests selected to detect signs of neurological, behavioral, and physiological dysfunctions. This functional battery is also referred to as an expanded set of clinical evaluations and is described more fully in section V.A. Observations of Test Animals in this chapter and in Chapter IV.C.10. Neurotoxicity Studies.

   Reports of one-year toxicity studies should include an assessment of the potential for the test substance to adversely affect the structural or functional integrity of the nervous system. This assessment should evaluate data from the neurotoxicity screen and other toxicity data from the study, as appropriate. Based on this assessment, the petitioner should make an explicit statement about whether or not the test substance presents a potential neurotoxic hazard and if additional neurotoxicity testing is deemed appropriate. FDA recommends that additional neurotoxicity testing not be undertaken without first consulting with the Agency.
6. **Immunotoxicity**: Results from tests that are included in the list of primary indicators for immune toxicity (see Chapter V.C. of the 1993 draft "Redbook II") should also be evaluated as part of an immunotoxicity screen. Reports of one-year toxicity tests should include an assessment of the potential for the test substance to adversely affect the immune system. This assessment should evaluate data from the list of primary indicators included in the immunotoxicity screen and other toxicity data from the study, as appropriate. Based on this assessment, the petitioner/notifier should make an explicit statement about whether or not the test substance presents a potential immunotoxic hazard which requires further immunotoxicity testing. Additional immunotoxicity tests are discussed in Chapter V.C. of the 1993 draft "Redbook II" but should not be undertaken without first consulting with the Agency.

VI. Necropsy and Microscopic Examination

A. Gross Necropsy

All test animals should be subjected to complete gross necropsy, including examination of external surfaces, orifices, cranial, thoracic and abdominal cavities, carcass, and all organs. The gross necropsy should be performed by, or under the direct supervision of, a qualified pathologist, preferably the pathologist who will later perform the microscopic examination (see below).

B. Organ Weight

Organs that should be weighed include the adrenals, brain, epididymides, heart, kidneys, liver, spleen, testes,

thyroid/parathyroid, thymus, ovaries and uterus. Organs should be carefully dissected and trimmed to remove fat and other contiguous tissue and then be weighed immediately to minimize the effects of drying on organ weight.

C. Preparation of Tissues for Microscopic Examination

Generally, the following tissues should be fixed in 10% buffered formalin (or another generally recognized fixative) and sections prepared and stained with hematoxylin and eosin (or another appropriate stain) in preparation for microscopic examination. Lungs should be inflated with fixative prior to immersion in fixative.

1. adrenals
2. aorta
3. bone (femur)
4. bone marrow (sternum)
5. brain (at least 3 different levels)
6. cecum
7. colon
8. corpus and cervix uteri
9. duodenum
10. epididymis
11. esophagus
12. eyes
13. gall bladder (if present)
14. heart
15. ileum
16. jejunum
17. kidneys
18. liver
19. lung (with main-stem bronchi)
20. lymph nodes (1 related to route of administration and 1 from a distant location)
21. mammary glands
22. nasal turbinates
23. ovaries and fallopian tubes
24. pancreas
25. pituitary
26. prostate
27. rectum
28. salivary gland
29. sciatic nerve
30. seminal vesicle (if present)
31. skeletal muscle
32. skin
33. spinal cord (3 locations: cervical, mid-thoracic, lumbar)
34. spleen
35. stomach
36. testes
37. thymus (or thymic region)
38. thyroid/parathyroid
39. trachea
40. urinary bladder

41. vagina
42. all tissues showing abnormality

D. Microscopic Evaluation

All gross lesions should be examined microscopically. All tissues from the animals in the control and high dose groups should be examined. If treatment related effects are noted in certain tissues, then the next lower dose level tested of those specific tissues should be examined. Successive examination of the next lower dose level continues until no effects are noted. In addition, all tissues from animals which died prematurely or were sacrificed during the study should be examined microscopically to assess any potential toxic effects.

E. Histopathology of Lymphoid Organs

Histopathological evaluation of the lymphoid organs should be performed as described in the section on immunotoxicity testing (see Chapter V. C. of the 1993 draft "Redbook II") for all animals.

VII. References

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1. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/default.htm

Redbook 2000: IV.C.6 Carcinogenicity Studies with Rodents

January 2006

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter IV.C.6. Carcinogenicity Studies with Rodents

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This guidance represents the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

This guidance represents the Food and Drug Administration's (FDA's) current thinking on this topic. Scientifically justified changes to the 1993 "draft" Redbook version of this section have been made following consultation with other authoritative guidelines and publications.

Carcinogenicity studies (bioassays) in two rodent species (usually rats and mice) are recommended for food ingredients with the highest levels of concern (*e.g.*,Concern Level III direct food additives, food contact substances with cumulative exposure at or greater than 1 ppm). The carcinogenicity studies (preferably in rats) may be combined with chronic toxicity studies (see Chapter IV.C.7). The Agency recommends that *in utero* exposure be included in carcinogenicity studies due to the fact that exposure to food ingredients occurs during all stages of life (more information is included in Chapter IV.C.8). These studies are designed to determine whether a food ingredient possesses carcinogenic activity when administered to rodents in regularly repeated oral doses for the "lifetime" of the test animal.

I. Good Laboratory Practice

Nonclinical laboratory studies discussed in this chapter should be conducted according to U.S. FDA good laboratory practice (GLP) regulations, issued under Part 58 Title 21 Code of Federal Regulations. This document may be obtained from the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C., 20402, (toll free 866-512-1800). Studies performed under other international/national guidelines may be considered equivalent to those conducted under U.S. FDA GLP regulations. Specific area(s) of non-compliance with FDA GLP regulations should be discussed and justified.

II. Test Animals

A. Care, Maintenance and Housing:

Recommendations about the care, maintenance, and housing of animals contained in the National Research Council, Guide for the Care and Use of Laboratory Animals should be followed unless they conflict with specific recommendations in this chapter.

B. Selection of Species and Strains:

Guidelines contained within this chapter are for studies with mice and rats; if other rodent species are used, modifications may be necessary. Both male and female test animals, which are healthy and have not been subjected to previous experimental procedures, should be used.

It is important to consider the test animals' general sensitivity and the responsiveness of particular organs and tissues of test animals to toxic chemicals when selecting rodent species, strains, and substrains for toxicity studies. The selection of inbred, out-bred, or hybrid rodent strains for toxicity tests should be based upon the scientific questions to be answered. Additionally, it is important that test animals come from well-characterized and healthy colonies. Because recent information suggests survivability problems exist for some strains of rats, test animals should be selected that are likely to survive for the recommended duration of the study (see discussions under sections II.D: Number and Sex and IV.A: Duration of Testing). The Agency encourages petitioners/notifiers to consult with Agency scientists before toxicity testing has begun if they have questions about the appropriateness of a particular species, strain, or substrain.

Another Center within the Agency (Center for Drug Evaluation and Research), as part of a pilot program, accepts safety data from six month studies employing genetically modified mice (i.e., transgenic mice) as a replacement for one of the rodent carcinogenicity studies[5]. The Office of Food Additive Safety will consider this type of information only as supplemental data but does not consider such studies to be substitutes for the two (2-year) rodent carcinogenicity bioassays. Data from transgenic rodent carcinogenesis or mutagenesis assays may be useful in evaluating compound-specific questions relating to mechanism of action or tissue distribution. For the determination of carcinogenic risk of certain kinds of food ingredients (i.e., constituents and/or contaminants), the transgenic mouse model is inappropriate in that it does not provide quantitative dose-response data. It also has not, as of this date, been fully validated or accepted by most national and international validation organizations (e.g., Scientific Advisory Committee on Alternative Toxicological Method of the Interagency Coordinating Committee on the Validation of Alternative Methods[12]) or testing laboratories. At this time, there is no large repository of historical control data to establish baseline parameters. Given the nature of consumption patterns of food ingredients (i.e., chronic, lifetime exposures), it is necessary to require the chronic safety testing of food ingredients that would be representative of lifetime exposure in humans, in addition to requiring that only quantitative data derived from fully validated test systems be used in their safety assessment.

C. Age (start of dosing):

Dosing of rodents should begin after weaning, and following a suitable acclimation period of at least 5 days, and before they are approximately 6-8 weeks old.

D. Number and Sex:

Experimental and control groups should have a sufficient number of animals at the beginning of the study to ensure that at least 25 rodents per sex per group survive to the end of the study. Having sufficient animals survive to the end of the study allows for objective assessment of test substance-related tumor development. Survival can be improved by reducing non-compound related animal pathology, which may occur as a result of excessive weight gain (e.g., obesity-related pituitary changes), or as sequelae to other stressors (e.g., parasitic infection).

The Agency recommends that petitioners/notifiers carefully consider their choice of rat strains for carcinogenicity bioassays, since some strains have more serious problems with survivorship than other strains. It is recommended that carcinogenicity studies begin with at least 50 animals per sex per group. Petitioners/notifiers are encouraged to begin bioassays with more than 50 animals per sex per group if survivorship is expected to be a problem with the rat strain used in the study. If fewer than 25 animals per sex per group are expected to survive to the end of the study (24 months, see section IV.A: Duration of Testing), petitioners/notifiers should take particular care to ensure and document early detection of dead animals through attentive and frequent cage-side observations, thus minimizing the loss of tissues from autolysis. In addition, they should consult with the Agency as soon as a problem with survivorship in a carcinogenicity study becomes apparent.

If interim necropsies are planned, the total number of rodents of each sex per group should be increased by the number scheduled to be sacrificed before completion of the study. A minimum of 10 rodents per sex per group should be available for each interim necropsy.

E. Infected Animals:

Generally, it is not possible to treat animals for infection during the course of a study without the possibility of interaction between the compound used for treatment and the test substance. This interaction may seriously confound or complicate the interpretation of study results. However, if problems with infection do occur, the sponsor for the study should use their best judgment in proceeding with the study and inform the Agency of their decision. In addition, the Agency requests that they provide a full and detailed description of the justification and possible implications of the infection, and if applicable, the justification and possible implications for treatment of the infection.

F. Animal Identification:

Test animals should be characterized by reference to their species, strain (and substrain), sex, age, and weight. Each animal should be assigned a unique identification number (e.g., ear tag, implanted identification chip, tattoo).

G. Caging:

Animals should be housed one per cage. This recommendation reflects three points of consideration:

- The amount of feed consumed by each animal in the study cannot be determined when more than one animal is housed in each cage. This information is necessary in the determination of feed efficiency (relationship of feed consumed to body weight gained).
- Minimizing the possibility of confounding analyses in determining whether decreases in body weight gain are due to decreased palatability or test substance mediated toxicity.
- Organs and tissues from moribund and dead animals which are single-caged would not be lost due to cannibalism.

H. Diet:

In general, feed and water should be provided *ad libitum*, and the diets should meet the nutritional requirements for the species[13] for normal growth and longevity. Unless special circumstances apply which justify otherwise, care should be taken to ensure that the diets of the test substance treated groups of animals contain the same levels of calories and nutrients (e.g., fiber, micronutrients) as the diets of the control group. Inadequately controlled dietary variables may result in nutritional imbalances or caloric deprivation that could confound interpretation of the toxicity study results (e.g., lifespan, background rates of tumor incidences) and alter the outcome and reproducibility of the studies. However, the Agency is also aware of some beneficial effects on the survivability of certain animal species that have been on calorie-restricted[14,24], or low-protein diets[15,16]. The Agency may accept such study results if the sponsor provides sufficient historical control data on the diet, and the study is well-conducted.

The following issues are important to consider when establishing diets for animals in carcinogenicity studies:

When the test substance has no caloric value and constitutes a substantial amount of the diet (e.g., more than 5%), both caloric and nutrient densities of the high dose diet would be diluted in comparison to the diets of the other groups. As a consequence, some high dose animals may receive higher test substance doses than expected because animals fed such diluted diets *ad libitum* may eat more than animals in other dosed groups to compensate for the differences in energy and nutrient content of the high dose diets. Such circumstances make it especially important that feed consumption of these animals be as accurately and closely monitored as possible in order to determine whether changes observed could be due to overt toxicity of the test substance or to a dietary imbalance. To further aid in this assessment, two control groups can be used; one group would be fed the undiluted control diet and a second group would be fed the control diet supplemented with an inert filler (e.g., methylcellulose) at a percentage equal to the highest percentage of the test substance in the diet.

When the vehicle for the test substance is expected to have caloric and/or nutritional values, which are greater than that of the control ration, an adjustment in the caloric and/or nutritional components may be necessary.

When administration of the test substance is expected to have an effect on feed intake because of its unpleasant taste or texture, other feeding regimens or experimental designs may be necessary. Consultation

with the Agency is recommended when alternatives are being considered.

When the test substance interferes with the absorption of nutrients, leading to nutritional deficiencies or changes in nutrient ratios, this can confound assessment of the toxicological endpoints under consideration. For example, fat soluble vitamins may preferentially partition with a mineral oil or fat substitute which is largely unabsorbed, such that a potential deficiency in these vitamins may result. This potential may be eliminated by additional nutrient fortification of the feed for those groups receiving the test substance. Appropriate levels of nutrient fortification should be determined experimentally.

Other related issues (e.g., advantages and disadvantages of using natural ingredient versus purified diets) are discussed in the National Research Council publication on nutrient requirements of laboratory animals[13].

I. Assignment of Control and Compound Treated Animals:

Animals should be assigned to control and compound treated groups in a stratified random manner. This will help minimize bias and assure comparability of pertinent variables across compound treated and control groups. In general, mean body weights and/or body weight ranges are used as a basis of randomization. If other characteristics are used as the basis for randomization, they should be described and justified.

Animals in all groups should be placed on study on the same day. If this is not possible because of the large number of animals in a study, animals may be placed on study over several days. When the latter recommendation is followed, a preselected portion of the control and experimental animals should be placed on the study each day in order to maintain concurrence.

J. Mortality:

Excessive mortality due to poor animal management is unacceptable and may be a cause to repeat the study.

K. Autolysis:

Adequate animal husbandry practices should result in considerably less than 10 % loss of animals and tissues or organs in a study because of autolysis. Autolysis in excess of this standard may be a cause to repeat the study.

L. Necropsy:

Necropsy should be performed soon after an animal is sacrificed or found dead, so that loss of tissues due to autolysis is minimized. When necropsy cannot be performed immediately, the animal should be refrigerated at a temperature that is low enough to prevent autolysis (i.e., between 4^{o}C and 8^{o}C), but not so low as to cause cell damage. If histopathological examination is to be conducted, tissue specimens should be taken from the animals and placed in appropriate fixatives when the necropsy is performed.

III. Test Substance

The test substance used in carcinogenicity studies should be the same substance that the petitioner/notifier intends to market or, when appropriate, the test substance may be a constituent chemical or an impurity. A single lot of test substance should be used throughout the study. When this is not possible, lots that are as similar as possible in purity and composition should be used. It is the responsibility of the petitioner/notifier to notify the animal test facility of the purity of the test substance, as well as the identity and concentration of any impurities that might be present.

A. Identity:

The identity of the test substance or mixture of substances to be tested should be known. We urge the petitioners/notifiers to consult with the Agency regarding the determination of the test compound, and to provide all relevant Chemical Abstract Service (CAS) Registry numbers.

B. Composition/Purity:

The composition of the test substance should be known including the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials.

C. Conditions of Storage:

The test samples should be stored under conditions that maintain their stability and purity until the studies are complete.

D. Expiration Date:

The expiration date of the test material should be known and easily available. Test materials should not be used past their expiration date.

IV. Experimental Design

A. Duration of Testing:

The test animal should be exposed to the test substance 7 days per week for 104 consecutive weeks (two years), or for the life span of the animal. In general, the Agency does not recommend early termination of carcinogenicity studies due to decreased survivorship (see section II.D: Number and Sex). Carcinogenicity bioassays should be conducted for a major portion of the test animal's lifetime. While it is desirable to have an optimum number of animals survive to the end of the study, the Agency believes there is more benefit, as well as added sensitivity, to be gained by conducting carcinogenicity bioassays for as long as possible, or for the full 24 months that is recommended in these guidelines.

B. Route of Administration:

The route of administration of the test substance should approximate that of normal human exposure, and if possible, the oral route should be used. A justification should be provided when using other routes. The same method of administration should be used for all test animals throughout the study. The test substance should be administered in one of the following ways:

- **In the diet**, if human exposure to the test substance is likely to be through consumption of solid foods or a combination of solid and liquid foods. If the test substance is added to the diet, animals should not be able to selectively consume either basal diet or test substance in the diet on the basis of color, smell, or particle size. If the compound is mixed with ground feed and pelleted, nothing in the pelleting process should affect the test substance (for example, heat-labile substances may be destroyed during pellet production by a steam process). When the test substance is administered in the diet, dietary levels should be expressed as mg of the test substance per kg of feed.
- **Dissolved in the drinking water**, if the test substance is likely to be ingested in liquid form by humans (for example, in soft drinks or beer), or if administration in the diet of rodents is inappropriate. The amount of test substance administered in drinking water should be expressed as mg of test substance per ml of water.
- **By encapsulation or oral intubation (gavage)**, if the two previous methods are unsatisfactory or if human exposure is expected to be through daily ingestion of a single, large bolus dose instead of continual ingestion of small doses. If the test substance is administered by gavage, it should be given at approximately the same time each day. The maximum volume of solution that can be given by gavage in one dose depends on the test animal's size; for rodents, the volume ordinarily should not exceed 1 ml/100 g body weight. If the gavage vehicle is oil, then the volume should be no more than 0.4 ml/100 g of body weight, and the use of a low-fat diet should be considered. If the test substance should be given in divided doses, all doses should be administered within a 6 hour period. Doses of test substance administered by gavage should be expressed as mg of test substance per ml of gavage vehicle. Finally, the petitioner/notifier should provide information that can allow the Agency to conclude that administration of the test compound by encapsulation or gavage is equivalent in toxicologically important respects to administration in the diet or drinking water. Alternatively, metabolic information on both modes of administration should be provided so that appropriate interpretation of data can be accomplished.

C. Dose groups:

1. Selection of Treatment Doses:

Dose selection for long-term toxicity studies should be based on results from subchronic studies and other related test substance information.

Three to five dose levels of the test substance and a concurrent control group should be used in carcinogenicity bioassays. When designing and conducting carcinogenicity bioassays the following should be considered: 1) the high dose (maximum tolerated dose) should be sufficiently high to induce toxic responses in test animals, and should not cause fatalities high enough to prevent meaningful evaluation of the data from the study; 2) the low dose should not induce toxic responses in test animals; and 3) the intermediate dose(s) should be sufficiently high to elicit minimal toxic effects in test animals (such as alterations in enzyme levels

or slight decreases in body weight gains. Administration of the test substance to all dose groups should be done concurrently (see Section II.I: Assignment of Control and Compound Treated Animals).

High Dose: The high dose should be the maximum tolerated dose (MTD).

It is not acceptable to select doses for carcinogenicity bioassays based on information unrelated to the toxicity of the test substance. For example, the highest dose in a carcinogenicity study should not be selected so as to provide a pre-determined margin of safety over the maximum expected human exposure to the test substance, assuming that the results of testing at that dose will be negative.

These guidelines recommend that the highest dose in carcinogenicity bioassays should be the MTD. In evaluating the results of carcinogenicity bioassays of a substance in food, Agency scientists will consider the question of whether the substance was tested at the MTD as one of several factors that may affect interpretation of the results of the bioassay. The bioassay should include a description of the process used to select the MTD for the study.

The MTD is defined by the National Toxicology Program (NTP) as "that dose which, when given for the duration of the chronic study as the highest dose, will not shorten the treated animals' longevity from any toxic effects other than the induction of neoplasms"[17] The Office of Science and Technology Policy provides the following advice, "The highest dose should be selected after an adequate prechronic study and after evaluating other relevant information, as necessary, to determine the highest dose consistent with predicted minimal target organ toxicity and normal life span, except as a consequence for the possible induction of cancer."[18] In addition, the NTP cautions that the MTD should not cause morphologic evidence of toxicity of a severity that would interfere with the interpretation of the study results.[17]

In general, the MTD is estimated following a careful analysis of data from appropriate subchronic toxicity tests. As the scientific community's experience with toxicity testing has accumulated, the need to consider a broad range of biological information when selecting the MTD has become increasingly clear. For example, data concerning changes in body and organ weight and clinically significant alterations in hematological, urinary and clinical chemistry measurements, in combination with more definitive toxic, gross or histopathologic endpoints, can be used to estimate the MTD.

Although the high dose in a carcinogenicity study should be selected to achieve the MTD, the Agency recognizes that this goal may not always be met. There are uncertainties in predicting the MTD for long-term bioassays from the results of shorter-term studies. Because working definitions of the MTD require the use of scientific judgment, it is sometimes possible for competent investigators looking at the same set of data to arrive at significantly different estimates of the MTD. Such disagreement may be based on different interpretations of the results of metabolic studies or different conclusions about whether an organ alteration is adaptive or toxicological. In situations such as these, when it is unclear what dose of the test substance is the MTD, the petitioner/notifier should consult with the Agency to determine an appropriate high dose (MTD) for the carcinogenicity bioassay.

The Agency recognizes that use of the MTD in carcinogenicity bioassays has several advantages; these include:

- Compensating for the inherent lack of sensitivity of the bioassay, including the relatively small number of rodents used in the study;
- Providing consistency with other models used in toxicology (*e.g.*, high enough doses should be used in order to elicit evidence of the presumed toxicity or increase probability of detecting rare tumors and identifying weak carcinogens); and
- Permitting comparison of carcinogenic potencies of substances tested at the MTD, even when the data are collected from different studies[19].

The Agency acknowledges that its recommendation to conduct carcinogenicity studies at the MTD may result in the use of doses that are so high as to be unrepresentative of the toxicity of the test substance at lower doses in animals or humans. For example, excessively high doses of a test substance can saturate enzyme systems involved in detoxification of the test substance. Given the above, after thorough internal assessment and in an agreement with other authoritative bodies [9,10], the Agency concludes that the MTD is still the best choice for selecting the high dose for carcinogenicity studies. It should be noted that this is also in line with the conclusions of the International Conference on Harmonization which recommends the use of the MTD in choosing the high dose for drug safety testing [7,8].

Low Dose:

The low dose level should not interfere with the normal growth, development, and lifespan of test animals, nor

should it produce any signs of toxicity.

Intermediate Dose:

The intermediate dose should produce minimal signs of toxicity. The exact dose selected as the intermediate dose may depend on the pharmacokinetic properties of the test substance.

Optional Fourth Dose Level:

If significant differences exist in the pharmacokinetic or metabolic profiles of the test substance administered at high and low doses, an optional (fourth) dose level may be included in the study. This dose level should be the highest dose that produces a pharmacokinetic or metabolic profile similar to profiles obtained at lower doses. The number of test animals in the optional group should be selected to provide approximately the same sensitivity for the detection of the carcinogenic effects of the test substance as the higher dose group provides.

2. Controls:

A concurrent control group of test animals fed the basal diet is required. A carrier or vehicle for the test substance should be given to control animals at a volume equal to the maximum volume of carrier or vehicle given to any dosed group of animals. Sufficient toxicology information should be available on the carrier or vehicle to ensure that its use will not compromise the results of the study. If there is insufficient information about the toxic properties of the vehicle used to administer the test substance, an additional control group that is not exposed to the carrier or vehicle should be included. In all other respects, animals in the control group should be treated the same as animals in dosed groups. (See also section II.H: Diet.)

D. Computerized systems

Computerized systems that are used in the generation, measurement, or assessment of data should be developed, validated, operated, and maintained in ways that are consistent with the intention of the Good Laboratory Practice principles[20]. The FDA has endorsed the use of the Standard for Exchange of Nonclinical Data (SEND) format for electronic transmission of animal study data. Contact the Agency for more information on this electronic protocol.

V. Observations and Clinical Tests

A. Observations of Test Animals:

Routine cage-side observations should be made on all animals inside the cage once or twice a day throughout the study for signs of departure from normal activity, morbidity and mortality. The usual interval between multiple periods of observations should be at least 6 hours. Individual records should be maintained for each animal and, as possible, the onset and progression of any effects should be recorded, preferably using a scoring system. If grossly visible tumors develop, the following parameters should be recorded; time of onset, location, dimensions, appearance and progression.

B. Body Weight and Feed Intake Data:

Accurate individual body weight, feed, and water consumption measurements are critical in the objective evaluation of the effect of a compound on test animals, since changes in these variables are often the first signs of toxicity. Complete records for these parameters are essential in assessing the time-related occurrence of toxicity-induced changes. When these data are not carefully recorded the evaluation of the overall cancer-inducing potential for a test substance may be compromised. A discussion of some of the variables that affect feed consumption and weight gain/loss can be found under sections II.H: Diet and IV.B: Route of Administration.

Body weights for all test animals should be recorded weekly for the first 13 weeks, and monthly thereafter for the duration of the study. Feed consumption (or water consumption if the test substance is administered in the drinking water) should be measured at the same interval as body weights; petitioners/notifiers should also attempt to quantify spillage of feed by test animals. When it is suspected that test compound administration may be affected by any of the following conditions; 1) feed palatability issues, 2) marked changes in body weight, or 3) increased numbers of animal deaths, the petitioners/notifiers should measure weights and feed (water) consumption more frequently after the initial 13 week period (e.g., every two weeks). Petitioners/notifiers should also use this accumulated information to calculate intake of the test substance.

C. Clinical Testing:

Ophthalmological examination, hematology profiles, clinical chemistry tests, and urinalyses should be

performed as described in the following sections:

1. Opthalmological Examination:

This examination should be performed by a qualified individual on all animals before the study begins and on control and high-dose animals at the end of the study. If the results of examinations at termination indicate that changes in the eyes may be associated with administration of the test substance, ophthalmological examinations should be performed on all animals in the study.

2. Hematology:

Hematological tests should be performed on at least 10 animals per sex in each group should be made during the first 2 weeks of study, and at 3, 6 and 12 months during the study. The time of the first sampling may be based on test results from short-term studies. If data trends or significant parameter changes (biological or statistical) that are of concern are observed at the 12-month measurement, then an 18-month measurement should be included. Additional hematology testing should be conducted at the end of the study if data trends or significant parameter changes are observed at 18 months to be consistent with other clinical testing.

Ideally, the same rodents should be sampled at each collection time point. Blood samples should be analyzed individually, and not pooled. If, due to the large number of animals, it becomes necessary to draw blood samples on more than one consecutive day at each sampling point, the samples should be obtained at approximately the same time each day.

The following determinations are recommended: hematocrit, hemoglobin concentration, erythrocyte count, total and differential leukocyte counts, mean corpuscular hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration, platelet count, and a measure of clotting potential (e.g., clotting time, prothrombin time, activated partial thromboplastin time).

Test compounds may have an effect on the hematopoietic system and therefore appropriate measures should be employed so that evaluations of reticulocyte counts and bone marrow cytology may be performed if warranted. Reticulocyte counts should be obtained for each animal using automated reticulocyte counting capabilities, or from air-dried blood smears. Bone marrow slides for cytological evaluation should be prepared from each animal. These slides only need to be microscopically examined when effects on the hematopoietic system are noted.

3. Clinical Chemistry:

Clinical chemistry tests should be performed on at least 10 animals per sex per group during the first 2 weeks of study, and at 3, 6 and 12 months during the study. The time of the first sampling may be based on test results from short-term studies. If data trends or significant parameter changes (biological or statistical) that are of concern are observed at the 12-month measurement, then an 18-month measurement should be included. Additional clinical chemistry testing should be conducted at the end of the study if data trends or significant parameter changes are observed at 18 months.

Ideally, the same rodents should be sampled at each collection time point. Blood samples should be drawn at the end of the fasting time and before feeding. Fasting duration should be appropriate for the species and the analytical tests to be performed. Blood samples should be analyzed individually, and not pooled. If animals are sampled on more than one day during a study, blood should he drawn at approximately the same time each sampling day.

Clinical chemistry tests that are appropriate for all test substances include measurements of electrolyte balance, nutrients metabolism, and liver and kidney function. Specific determinations should include:

Hepatocellular evaluation (at least 3 of the following 5)

- Alanine aminotransferase (SGPT, ALT)
- Aspartate aminotransferase (SGOT, AST)
- Sorbitol dehydrogenase
- Glutamate dehydrogenase
- Total bile acids

Hepatobiliary evaluation (at least 3 of the following 5)

- Alkaline phosphatase
- Bilirubin (total)

- Gamma-glutamyl transpeptidase (GG transferase)
- 5' nucleotidase
- Total bile acids

Other markers of cell changes or cellular function

- Albumin
- Calcium
- Chloride
- Cholesterol (total)
- Cholinesterase
- Creatinine
- Globulin (calculated)
- Glucose (in fasted animals)
- Phosphorous
- Potassium
- Protein (total)
- Sodium
- Triglycerides (fasting)
- Urea nitrogen
- The Agency understands that the specific nature of the test substance may warrant the consideration of alternative tests. Appropriate justification for alternative tests should be presented in study reports.

In spite of standard operating procedures and equipment calibration, it is not unusual to observe considerable variation in the results of clinical chemistry analyses from day to day[21]. Ideally, clinical chemistry analyses for all dose groups should be completed during one day. If this is not possible, analyses should be conducted in such a way as to minimize potential variability.

4. Urinalyses:

The determination of volume of urine collected, urine specific gravity, pH, glucose, and protein, as well as microscopic analysis of urine for sediment and presence of blood and/or blood cells, are recommended before dosing, and at 3, 6 and 12 months during the study[22]. If data trends or significant parameter changes (biological or statistical) that are of concern are observed at the 12-month measurement, then an 18-month measurement should be included. Additional urinalysis testing should be conducted at the end of the study if data trends or significant parameter changes are observed at 18 months. These tests should be performed on at least 10 animals per sex per group.

VI. Necropsy and Microscopic Examination

A. Gross Necropsy

All test animals should be subjected to complete gross necropsy, including examination of external surfaces, orifices, cranial, thoracic and abdominal cavities, carcass, and all organs. The gross necropsy should be performed by, or under the direct supervision of, a qualified pathologist, preferably the pathologist who will later perform the microscopic examination.

B. Organ Weight

Organs that should be weighed include the adrenals, brain, epididymides, heart, kidneys, liver, spleen, testes, thyroid/parathyroid, thymus if present, ovaries and uterus. Before being weighed, organs should be carefully dissected and trimmed to remove fat and other contiguous tissue. Organs should be weighed immediately after dissection to minimize the effects of drying on organ weight.

C. Preparation of Tissues for Microscopic Examination

Generally, the following tissues should be fixed in 10 % buffered formalin (or another generally recognized fixative) and sections prepared and stained with hematoxylin and eosin (or another appropriate stain) in preparation for microscopic examination. Lungs should be inflated with fixative prior to immersion in fixative.

- Adrenals
- Aorta
- Bone (femur)
- Bone marrow (sternum)
- Brain (at least 3 different levels)
- Cecum
- Colon
- Corpus and cervix uteri
- Duodenum
- Epididymides
- Esophagus
- Eyes
- Gall bladder (if present)
- Harderian gland
- Heart
- Ileum
- Jejunum
- Kidneys
- Liver
- Lung (with main-stem bronchi)
- Lymph nodes (1 related to route of administration and 1 from a distant location)
- Mammary glands
- Nasal turbinates
- Ovaries and fallopian tubes
- Pancreas
- Pituitary
- Prostate
- Rectum
- Salivary gland
- Sciatic nerve
- Seminal vesicle (if present)
- Skeletal muscle
- Skin
- Spinal cord (3 locations: cervical, mid-thoracic, and lumbar)
- Spleen
- Stomach
- Testes
- Thymus (if present)
- Thyroid/parathyroid
- Trachea
- Urinary bladder
- Vagina
- Zymbal's gland
- All tissues showing abnormality

D. Microscopic Evaluation

All gross lesions should be examined microscopically. All tissues from the animals in the control and high dose groups should be examined. If treatment related effects are noted in certain tissues, then those specific

tissues in the next lower dose level tested should be examined. Successive examination of the next lower dose level continues until no effects are noted. In addition, all tissues from animals that died prematurely or were sacrificed during the study should be examined microscopically. If there are questions related to the review and interpretation of pathological lesions and statistical results, additional discussion may be found in Chapters IV.B.3 and IV.B.4 of the Redbook 2000.

E. Histopathology of Lymphoid Organs

Histopathology evaluation of the lymphoid organs should be performed as described in the section on immunotoxicity testing (see Chapter V.C. of the 1993 draft Redbook II). A recent publication provides further discussion on this subject[23].

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Redbook 2000: IV.C.7 Combined Chronic Toxicity/Carcinogenicity Studies with Rodents

July 2007

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter IV.C.7. Combined Chronic Toxicity/Carcinogenicity Studies with Rodents

Return to *Redbook 2000* table of contents [1]

This section of Redbook 2000 supersedes the 1993 "draft" Redbook Chapter IV.C.7. The FDA acknowledges that it is complicated and difficult to conduct a combined chronic toxicity/carcinogenicity rodent study particularly with an *in-utero* exposure phase. This is often due to difficulty in setting and administering appropriate dose levels for both types of studies concurrently. Additionally, the general objectives of these two types of studies are different. Nevertheless, when pre-chronic studies provide reasonable estimates of toxicity to predict the information (i.e., treatment doses) to be used in a single bioassay, a chronic toxicity study may be combined with a carcinogenicity study and reveal information about an ingredient's potential to be a carcinogen as well as the maximum dose that produces no adverse effects.

The FDA recommends that the petitioner/notifier consult with the FDA before conducting a combined study. Sponsors/submitters of petitions/notifications are also encouraged to become familiar with the Guidance for Chronic Toxicity Studies with Rodents (Chapter IV.C.5.a. [2]), Carcinogenicity Studies with Rodents (Chapter IV.C.6. [3]), In-Utero Exposure Phase for Addition to Carcinogenicity Studies or Chronic Toxicity Studies with Rodents (Chapter IV.C.8. [4]), Reporting Results of Toxicity Studies (Chapter IV.B.2. [5]), Pathology Considerations in Toxicity Studies (Chapter IV.B.3. [6]), and Statistical Considerations in Toxicity Studies (Chapter IV.B.4. [7]) during the development of study design.

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5. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/ucm078409.htm
6. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/ucm078318.htm
7. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/ucm078320.htm

Redbook 2000: IV.C.8 In-Utero Exposure Phase for Addition to Carcinogenicity Studies or Chronic Toxicity Studies with Rodents

July 2007

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter IV.C.8. *In-Utero* Exposure Phase for Addition to Carcinogenicity Studies or Chronic Toxicity Studies with Rodents

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This guidance represents the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

Scientifically justified changes to 1993 "draft" Redbook version of this section have been made after consulting with other authoritative guidelines[3] [4] [5] [10] [11] [12] [19] [20] [22] [23] and publications (see the relevant sections below).

The FDA recommends including an *in-utero* exposure phase in a carcinogenicity or a chronic toxicity study conducted with rodents for the safety assessment of potential food ingredients with the highest levels of concern (*e.g.*, Concern Level III direct food additives[2], food contact substances[3] with cumulative exposure at or greater than 1 ppm). The animal toxicity studies recommended in this chapter are designed to determine whether a test food ingredient has early developmental effects that may increase the incidence of cancers and/or chronic disease outcomes (e.g., altered glucose tolerance, diabetes mellitus, cardiovascular disorders) when administered in regularly repeated oral doses for the duration of the study in the test animals.

An *in-utero* exposure phase should be added to one of the two recommended rodent carcinogenicity studies (or bioassays; see Chapter IV.C.6[4].). In general, the *in-utero* phase should be added to a bioassay study with rats since the rat is the recommended species for reproduction studies (see Chapter IV.C.9.a[5].) and the FDA has a larger database on carcinogenicity bioassays with *in-utero* exposure in rats than in mice. When chronic toxicity studies are the only long-term studies in support of the safety of a food ingredient, the FDA recommends on a case-by-case basis that an *in-utero* exposure phase be added to at least one of the studies.

The purpose of this chapter is to provide specific guidance for the design and conduct of an *in-utero* exposure phase addition to bioassay or chronic toxicity studies of food ingredients. However, these general procedures

may also be applied to a combined chronic toxicity/carcinogenicity study or shorter-term toxicity studies with modifications (e.g., duration, dose, etc). The FDA encourages petitioners and/or notifiers to consult with the appropriate FDA scientists before toxicity testing has begun if they have questions about the appropriateness of adding an *in-utero* exposure phase to any of these studies. Sponsors/submitters of petitions/notifications are encouraged to also become familiar with the Guidelines for Reporting Results of Toxicity Studies (Chapter IV.B.2 [6].), Pathology Considerations in Toxicity Studies (Chapter IV.B.3 [7].), Statistical Considerations in Toxicity Studies (Chapter IV.B.4 [8].), during the development of study design.

I. Good Laboratory Practice

Nonclinical laboratory studies discussed in this chapter should be conducted according to U.S. FDA good laboratory practice (GLP) regulations, issued under Part 58 of Title 21 [9] of the Code of Federal Regulations. This document may be obtained from the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C., 20402, (toll free 866-512-1800 or DC area 202-512-1800). Studies performed under other international/national guidelines may be considered equivalent to those conducted under U.S. FDA GLP regulations. Specific area(s) of non-compliance with FDA GLP regulations should be discussed and justified.

II. Test Animals

A. Care, Maintenance and Housing:

Recommendations about the care, maintenance, and housing of animals contained in the National Research Council, Guide for the Care and Use of Laboratory Animals [24] should be followed unless they conflict with specific recommendations in this chapter.

B. Selection of Species and Strains:

Guidance contained within this chapter is for studies with mice and rats; if other rodent species are used, modifications may be necessary. Both male and female test animals, which are healthy and have not been subjected to previous experimental procedures, should be used.

It is important to consider the test animals' general sensitivity and the responsiveness of particular organs and tissues of test animals to toxic chemicals when selecting rodent species, strains, and substrains for toxicity studies. The selection of inbred, out-bred, or hybrid rodent strains for toxicity tests should be based upon the scientific questions to be answered. Strains selected should not have low fecundity and should be sensitive to teratogens and embryotoxins. Additionally, it is important that test animals come from well-characterized and healthy colonies. Because recent information suggests survivability problems exist for some strains of rats, test animals should be selected that are likely to survive for the recommended duration of the study (see discussions under sections II.D: Number and Sex and IV.A: Duration of Testing). The FDA encourages petitioners/notifiers to consult with the appropriate FDA scientists before toxicity testing has begun if they have questions about the appropriateness of a particular species, strain, or substrain.

Another Center within the FDA (Center for Drug Evaluation and Research), as part of a pilot program, accepts safety data from six month studies employing genetically modified mice (i.e., transgenic mice) as a replacement for one of the rodent carcinogenicity studies.[20] The Office of Food Additive Safety will consider this type of information only as supplemental data but does not consider such studies to be substitutes for the two (2-year) rodent carcinogenicity bioassays. Data from transgenic rodent carcinogenesis or mutagenesis assays may be useful in evaluating compound-specific questions relating to mechanism of action or tissue distribution. For the determination of carcinogenic risk of certain kinds of test substances (i.e., constituents and/or contaminants in food ingredients), the transgenic mouse model is inappropriate in that it does not provide quantitative dose-response data. It also has not, as of this date, been fully validated or accepted by most national and international validation organizations (e.g., Scientific Advisory Committee on Alternative Toxicological Method of the Interagency Coordinating Committee on the Validation of Alternative Methods [16]) or testing laboratories. At this time, there is no large repository of historical control data to establish baseline parameters. Given the nature of consumption patterns of food ingredients (i.e., chronic, lifetime exposures), it is important to provide the chronic safety testing of food ingredients that would be representative of lifetime exposure in humans, in addition to also providing that only quantitative data derived from fully validated test systems be used in their safety assessment.

C. Age (start of dosing):

Following a suitable acclimation period of at least 5 days, parental animals should receive the test substance. Females should receive the test substance for a minimum of four weeks prior to mating and males should receive the test substance for at least ten weeks prior to exposure to cover the full spermatogenic cycle.

Dosing of all test and control pups (F1) should begin at weaning (see also section IV.A: Duration of testing).

D. Number and Sex:

Experimental and control groups should have a sufficient number of animals at the beginning of the study to ensure that at least 25 rodents per sex per group survive to the end of the study. Having sufficient animals survive to the end of the study allows for objective assessment of test substance-related effects including tumor development. Survival can be improved by reducing non-compound related animal pathology, which may occur as a result of excessive weight gain (e.g., obesity-related pituitary changes), or as sequelae to other stressors (e.g., parasitic infection).

The FDA recommends that petitioners/notifiers carefully consider their choice of rat strains for bioassays or chronic toxicity studies, since some strains have more serious problems with survivorship than other strains. It is recommended that these studies begin with at least 70 animals per sex per group. Petitioners and/or notifiers should begin bioassays with more than 70 animals per sex per group if survivorship is expected to be a problem with the rat strain used in the study. If fewer than 25 animals per sex per group are expected to survive to the end of the study (e.g., 1-year or longer in chronic study and 2-years in bioassay, see section IV.A: Duration of Testing), petitioners/notifiers should take particular care to ensure and document early detection of dead animals through attentive and frequent cage-side observations, thus minimizing the loss of tissues from autolysis. In addition, they should consult with the FDA as soon as a problem with survivorship in a carcinogenicity or chronic study becomes apparent.

One male and one female per litter are preferred; no more than two males and two females per litter should be included in any group. For example, if the petitioner decides that each group should contain 70 animals per sex, at least 70 litters/group should be produced in the *in-utero* phase. Thus, for this example the number of parental animals per sex for the *in-utero* phase should be sufficient to ensure at least 70 litters per group.

If interim necropsies are planned, the total number of rodents of each sex per group should be increased by the number scheduled to be sacrificed before completion of the study. A minimum of 10 rodents per sex per group should be available for each interim necropsy.

E. Mating Procedures:

For each mating, a female should be placed with a single randomly selected male from the same dose group until pregnancy occurs or two to three weeks have elapsed. Animals should be separated as soon as possible after evidence of copulation has been observed. If mating has not occurred after two to three weeks, the animals should be separated without further opportunity for mating. Mating pairs should be clearly identified in the data. Sibling matings should be avoided. Each morning, all females should be examined for the presence of sperm in the vaginal lavage or the presence of a vaginal plug; if sperm and/or a vaginal plug are found, this is considered day zero of gestation. Near parturition, pregnant females should be caged separately in delivery or maternity cages that contain nesting materials. Pregnant females in test and control groups should be allowed to litter naturally.

F. Standardizing the Number of Pups per Litter:

Standardization of the number of pups per litter through culling is optional. Litters may be standardized to 10 or 8 based on historical litter size for the strain. It is recommended that standardization be performed on postnatal day 4 by reducing all litters of more than 10 to 10 (or more than 8 to 8) in a random manner. If possible, the retained litter-mates should consist of equal numbers of males and females; excess males or females should be randomly selected out. Random selection is important to guard against the human tendency to keep the fit animals in the study.

G. Selection of Pups (F1):

One animal per sex per litter, or up to two animals for single sex litters, should be randomly selected.

H. Infected Animals:

Generally, it is not possible to treat animals for infection during the course of a study without the possibility of interaction between the therapeutic agent used for treatment and the test substance. This interaction may seriously confound or complicate the interpretation of study results. However, if problems with infection do occur, the sponsor for the study should use their best judgment in proceeding with the study and inform the FDA of their decision. In addition, the FDA requests that they provide a full and detailed description of the

justification for study continuation and possible implications of the infection, and if applicable, the justification and possible implications for treatment of the infection.

I. Animal Identification:

Test animals should be characterized by reference to their species, strain (and substrain), sex, age, and weight. Each animal should be assigned a unique identification number (e.g., ear tag, implanted identification chip, tattoo).

J. Caging:

Animals should be single-caged during the study, except during mating and lactation. This recommendation reflects three points of consideration:

- The amount of feed consumed by each animal in the study cannot be determined with sufficient accuracy when more than one animal is housed in each cage. This information is necessary in the determination of feed efficiency (relationship of feed consumed to body weight gained).
- Minimizing the possibility of confounding analyses in determining whether decreases in body weight gain are due to decreased palatability or test substance mediated toxicity.
- Organs and tissues from moribund and dead animals which are single-caged would not be lost due to cannibalism.

K. Diet:

In general, feed and water should be provided *ad libitum*, and the diets should meet the nutritional requirements to support pregnancy in the test species as well as of the species for normal growth and longevity.[25] Unless special circumstances apply which justify otherwise, care should be taken to ensure that the diets of the test substance treated groups of animals contain the same levels of calories and nutrients (e.g., fiber, micronutrients) as the diets of the control group. Inadequately controlled dietary variables may result in nutritional imbalances or caloric deprivation that could confound interpretation of the toxicity study results (e.g., lifespan, background rates of tumor incidences) and alter the outcome and reproducibility of the studies. However, the FDA is also aware of some beneficial effects on the survivability of certain animal species that have been on calorie-restricted, [7] [8] or low-protein diets. [1] [15] The FDA may accept such study results if the sponsor provides sufficient historical control data on the diet, and the study is well-conducted.

The following issues are important to consider when establishing diets for animals in toxicity studies:

When the test substance has no caloric value and constitutes a substantial amount of the diet (e.g., more than 5%), both caloric and nutrient densities of the high dose diet would be diluted in comparison to the diets of the other groups. As a consequence, some high dose animals may receive higher test substance doses than expected because animals fed such diluted diets *ad libitum* may eat more than animals in other dosed groups to compensate for the differences in energy and nutrient content of the high dose diets. Such circumstances make it especially important that feed consumption of these animals be as accurately and closely monitored as possible in order to determine whether changes observed could be due to overt toxicity of the test substance or to a dietary imbalance. To further aid in this assessment, two control groups can be used; one group would be fed the undiluted control diet and a second group would be fed the control diet supplemented with an inert filler (e.g., methylcellulose) at a percentage equal to the highest percentage of the test substance in the diet.

When the vehicle for the test substance is expected to have caloric and/or nutritional values, which are greater than that of the control ration, an adjustment in the caloric and/or nutritional components may be necessary.

When administration of the test substance is expected to have an effect on feed intake because of its unpleasant taste or texture, other feeding regimens or experimental designs may be necessary. Consultation with the FDA is recommended when alternatives are being considered.

When the test substance interferes with the absorption of nutrients, leading to nutritional deficiencies or changes in nutrient ratios, this can confound assessment of the toxicological endpoints under consideration. For example, fat soluble vitamins may preferentially partition with a mineral oil or fat substitute which is largely unabsorbed, such that a potential deficiency in these vitamins may result. This potential may be eliminated by additional nutrient fortification of the feed for those groups receiving the test substance.

Appropriate levels of nutrient fortification should be determined experimentally.

Other related issues (e.g., advantages and disadvantages of using natural ingredient versus purified diets) are discussed in the National Research Council publication on nutrient requirements of laboratory animals.[25]

L. Assignment of Control and Compound Treated Animals:

Animals should be assigned to control and compound treated groups in a stratified random manner. This will help minimize bias and assure comparability of pertinent variables across compound treated and control groups. In general, mean body weights and/or body weight ranges are used as a basis of randomization. If other characteristics are used as the basis for randomization, they should be described and justified.

Animals in all groups should be placed on study on the same day. If this is not possible because of the large number of animals in a study, animals may be placed on study over several days. When the latter recommendation is followed, a preselected portion of the control and experimental animals should be placed on the study each day in order to maintain concurrence.

M. Mortality:

Excessive mortality due to poor animal management is unacceptable and may be a cause to repeat the study.

N. Autolysis:

Adequate animal husbandry practices should result in considerably less than 10 % loss of animals and tissues or organs in a study because of autolysis. Autolysis in excess of this standard may be a cause to repeat the study.

O. Necropsy:

Necropsy should be performed soon after an animal is sacrificed or found dead, so that loss of tissues due to autolysis is minimized. When necropsy cannot be performed immediately, the animal should be refrigerated at a temperature that is low enough to prevent autolysis (i.e., between 4^{o}C and 8^{o}C), but not so low as to cause cell damage. If histopathological examination is to be conducted, tissue specimens should be taken from the animals and placed in appropriate fixatives when the necropsy is performed.

III. Test Substance

The test substance used in carcinogenicity or chronic toxicity studies with an *in-utero* exposure phase should be the same substance that the petitioner/notifier intends to market or, when appropriate, the test substance may be a constituent chemical or an impurity. A single lot of test substance should be used throughout the study. When this is not possible, lots that are as similar as possible in purity and composition should be used. It is the responsibility of the petitioner/notifier to notify the animal test facility of the purity of the test substance, as well as the identity and concentration of any impurities that might be present.

A. Identity:

The identity of the test substance (e.g., either a single component or a mixture of components) should be known. The petitioners/notifiers are encouraged to consult with the FDA regarding the method(s) of determination of the test compound, and should provide all relevant Chemical Abstract Service (CAS) Registry numbers.

B. Composition/Purity:

The composition of the test substance should be known including the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials.

C. Conditions of Storage:

The test samples should be stored under conditions that maintain their stability and purity until the studies are complete.

D. Expiration Date:

The expiration date of the test material should be known and easily available. Test materials should not be used past their expiration date.

IV. Experimental Design

A. Duration of Testing:

The parental animals should receive the test substance starting at a minimum four weeks (or ten weeks exposure is preferable for males to cover full spermatogenic cycle) prior to mating. Exposure should be continued throughout pre-mating, mating, gestation, and lactation until the F1 animals have been weaned. Dosing of all test and control F1 animals should begin at weaning, and continue for 7 days per week for the duration of the study (e.g., 1-year or longer in chronic study and 2-years in bioassay).

In general, the FDA does not recommend early termination of carcinogenicity studies due to decreased survivorship (see discussions under section II.D: Number and Sex). Carcinogenicity bioassays should be conducted for a major portion of the test animal's lifetime. While it is desirable to have an optimum number of animals survive to the end of the study, the FDA believes there is more benefit, as well as added sensitivity, to be gained by conducting carcinogenicity bioassays for as long as possible, or for no longer than the full 24 months that is recommended in this guidance.

B. Route of Administration:

The route of administration of the test substance should approximate that of normal human exposure, and if possible, the oral route should be used. A justification should be provided when using other routes. The same method of administration should be used for all test animals throughout the study. The test substance should be administered in one of the following ways:

- **In the diet**, if human exposure to the test substance is likely to be through consumption of solid foods or a combination of solid and liquid foods. If the test substance is added to the diet, animals should not be able to selectively consume either basal diet or test substance in the diet on the basis of color, smell, or particle size. If the test substance is mixed with ground feed and pelleted, nothing in the pelleting process should affect the test substance (for example, heat-labile substances may be destroyed during pellet production by a steam process). When the test substance is administered in the diet, dietary levels should be expressed as mg of the test substance per kg of feed.
- **Dissolved in the drinking water**, if the test substance is likely to be ingested in liquid form by humans (for example, in soft drinks or beer), or if administration in the diet of rodents is inappropriate. The amount of test substance administered in drinking water should be expressed as mg of test substance per ml of water.
- **By encapsulation or oral intubation (gavage)** , if the two previous methods are unsatisfactory or if human exposure is expected to be through daily ingestion of a single, large bolus dose instead of continual ingestion of small doses. If the test substance is administered by gavage, it should be given at approximately the same time each day. The maximum volume of solution that can be given by gavage in one dose depends on the test animal's size; for rodents, the volume ordinarily should not exceed 1 ml/100 g body-weight. If the gavage vehicle is oil, then the volume should be no more than 0.4 ml/100 g of body weight, and the use of a low-fat diet should be considered. It is best to adjust the volume every 1-3 days based on the animal's body weight response. If the test substance should be given in divided doses, all doses should be administered within a 6 hour period. Doses of test substance administered by gavage should be expressed as mg of test substance per ml of gavage vehicle. Finally, the petitioner/notifier should provide information that can allow the FDA to conclude that administration of the test substance by encapsulation or gavage is equivalent in toxicologically important respects to administration in the diet or drinking water. Alternatively, metabolic information on both modes of administration should be provided so that appropriate interpretation of data can be accomplished.

C. Dose Groups:

1. Controls:

A concurrent control group of test animals fed the basal diet is necessary for all studies. A carrier or vehicle for the test substance should be given to control animals at a volume equal to the maximum volume of carrier

or vehicle given to any dosed group of animals. Sufficient toxicological information should be available on the carrier or vehicle to ensure that its use will not compromise the results of the study. If there is insufficient information about the toxic properties of the carrier or vehicle used to administer the test substance, an additional control group that is not exposed to the carrier or vehicle should be included. In all other respects, animals in the control group should be treated the same as animals in dosed groups. (See also section II.K: Diet.)

2. Selection of Treatment Doses for Carcinogenicity Studies with an *in-utero* exposure phase:

It is recommended that a minimum of three dose levels of the test substance be used in carcinogenicity bioassays with an *in-utero* exposure phase. As a result of maternal or fetal toxicity, it may be necessary to use lower doses during the *in-utero* phase of the studies in order to produce sufficient offspring for the post-weaning phase. Data justifying this protocol modification should be provided; it is recommended that pilot studies be performed to select doses. Results from metabolism and pharmacokinetic studies should also provide guidance in selecting an appropriate dosage regimen.

When designing and conducting carcinogenicity bioassays with an *in-utero* exposure phase the following should be considered: 1) the high dose (maximum tolerated dose) should be sufficiently high to induce toxic responses in test animals, and should not cause fatalities high enough to prevent meaningful evaluation of the data from the study; 2) the low dose should not induce toxic responses in test animals; and 3) the intermediate dose(s) should be sufficiently high to elicit minimal toxic effects in test animals (such as alterations in enzyme levels or slight decreases in body weight gains. Administration of the test substance to all dose groups should be done concurrently (see discussions under section II.L: Assignment of Control and Compound Treated Animals).

High Dose: The high dose should be the maximum tolerated dose (MTD).

It is not acceptable to select doses for carcinogenicity bioassays with an *in-utero* exposure phase based on information unrelated to the toxicity of the test substance. For example, the highest dose should not be selected so as to provide a pre-determined margin of safety over the maximum expected human exposure to the test substance, assuming that the results of testing at that dose will be negative.

This guidance recommends that the highest dose in carcinogenicity bioassays with an *in-utero* exposure phase should be the MTD. FDA scientists will consider the question of whether the substance was tested at the MTD as one of several factors that may affect interpretation of the results of the bioassays. The bioassays should include a description of the process used to select the MTD for the study.

The MTD is defined by the National Toxicology Program (NTP) as "that dose which, when given for the duration of the chronic study as the highest dose, will not shorten the treated animals' longevity from any toxic effects other than the induction of neoplasms".[21] The Office of Science and Technology Policy provides the following advice, "The highest dose should be selected after an adequate prechronic study and after evaluating other relevant information, as necessary, to determine the highest dose consistent with predicted minimal target organ toxicity and normal life span, except as a consequence for the possible induction of cancer.".[13] In addition, the NTP cautions that the MTD should not cause morphologic evidence of toxicity of a severity that would interfere with the interpretation of the study results.[21]

In general, the MTD is estimated following a careful analysis of data from appropriate subchronic toxicity tests. As the scientific community's experience with toxicity testing has accumulated, the need to consider a broad range of biological information when selecting the MTD has become increasingly clear. For example, data concerning changes in body and organ weight and clinically significant alterations in neurological, hematological, urinary and clinical chemistry measurements, in combination with more definitive toxic, gross or histopathologic endpoints, can be used to estimate the MTD.

Although the high dose in a carcinogenicity study with an *in-utero* exposure phase should be selected to achieve the MTD, the FDA recognizes that this goal may not always be met. There are uncertainties in predicting the MTD for long-term bioassays from the results of shorter-term studies. Because working definitions of the MTD require the use of scientific judgment, it is sometimes possible for competent investigators looking at the same set of data to arrive at significantly different estimates of the MTD. Such disagreement may be based on different interpretations of the results of metabolic studies or different conclusions about whether an organ alteration is adaptive or toxicological. In situations such as these, when it is unclear what dose of the test substance is the MTD, the petitioner/notifier should consult with the FDA to determine an appropriate high dose (MTD) for the carcinogenicity bioassay with an *in-utero* exposure phase.

The FDA recognizes that use of the MTD in carcinogenicity bioassays with an *in-utero* exposure phase has several advantages; these include:

- Compensating for the inherent lack of sensitivity of the bioassay, including the relatively small number of rodents used in the study;
- Providing consistency with other models used in toxicology (e.g., high enough doses should be used in order to elicit evidence of the presumed toxicity or increase probability of detecting rare tumors and identifying weak carcinogens); and
- Permitting comparison of carcinogenic potencies of substances tested at the MTD, even when the data are collected from different studies. [9]

The FDA acknowledges that its recommendation to conduct carcinogenicity studies with an *in-utero* exposure phase at the MTD may result in the use of doses that are so high as to be unrepresentative of the toxicity of the test substance at lower doses in animals or humans. For example, excessively high doses of a test substance can saturate enzyme systems involved in detoxification of the test substance. Given the above, after thorough internal assessment and in an agreement with other authoritative bodies,[6] [26] the FDA concludes that the MTD is still the best choice for selecting the high dose for carcinogenicity studies even with an *in-utero* exposure phase. It should be noted that this is also in line with the principles discussed by the International Conference on Harmonization which recommends the use of the MTD in choosing the high dose for drug safety testing.[4] [5]

Low Dose:

The low dose level should not interfere with the normal growth, development, and lifespan of test animals, nor should it produce any signs of toxicity.

Intermediate Dose:

The intermediate dose should produce minimal signs of toxicity. The exact dose selected as the intermediate dose may depend on the pharmacokinetic properties of the test substance.

Optional Fourth Dose Level:

If significant differences exist in the pharmacokinetic or metabolic profiles of the test substance administered at high and low doses, an optional (fourth) dose level may be included in the study. This dose level should be the highest dose that produces a pharmacokinetic or metabolic profile similar to profiles obtained at lower doses. The number of test animals in the optional group should be selected to provide approximately the same sensitivity for the detection of the carcinogenic effects of the test substance as the higher dose group provides.

3. Selection of Treatment Doses for Chronic Toxicity Studies with an *in-utero* exposure phase:

It is recommended that a minimum of three dose levels of the test substance be used in chronic toxicity studies with an *in-utero* exposure phase. Dose selection should be based on results from subchronic studies and other related test substance information (i.e., metabolism and pharmacokinetic studies). As a result of maternal or fetal toxicity, it may be necessary to use lower doses during the *in-utero* phase of the chronic-feeding studies in order to produce sufficient offspring for the post-weaning phase. Data justifying this protocol modification should be provided.

The FDA acknowledges that it is complicated and difficult to conduct a combined chronic toxicity/carcinogenicity rodent study with an *in-utero* exposure phase due to difficulty in setting and administering appropriate dose levels for both types of studies concurrently. However, when pre-chronic studies provide reasonable estimates of toxicity to predict the information on treatment doses to be used in a single bioassay, a chronic toxicity study may be combined with a carcinogenicity study with an *in-utero* exposure. It is recommended that the petitioner/notifier consult with the FDA before conducting a combined study.

The following is a general consideration in selecting the treatment dose levels for chronic toxicity studies with an in-utero exposure phase: 1) the high dose should be sufficiently high to induce toxic responses in test animals, and should not cause fatalities high enough to prevent meaningful evaluation of the data from the study; 2) the low dose should not induce biologically significant toxic responses in test animals; and 3) the intermediate dose should be sufficiently high to elicit minimal toxic effects in test animals (such as alterations in enzyme levels or slight decreases in body weight gains).

High Dose:

The high dose in a chronic toxicity study should produce toxicity so that a toxicological profile of the test substance can be obtained. We do not recommend that petitioner/notifiers use information unrelated to the toxicity of the test substance as a basis for dose selection. For example, the highest dose should not be

selected so as to provide a pre-determined margin of safety over the maximum expected human exposure to the test substance, assuming that the results of testing at that dose will be negative. When no toxicity is observed in other studies, however, the high dose could be subject to some preset limits such as the highest percent of the test substance in the diet that could be fed without compromising nutritional balance with other nutrients (e.g., about 5%, see also 'section II.H: Diet' for other important dietary issues).

In general, the high dose tested is estimated following a careful analysis of data from appropriate subchronic toxicity tests. As the scientific community's experience with toxicity testing has accumulated, the need to consider a broad range of biological information when selecting the high dose has become increasingly clear. For example, data from a subchronic (90-day) study concerning changes in body and organ weight and clinically significant alterations in neurological, hematological, urinary and clinical chemistry measurements, in combination with more definitive exposure-related toxic, gross or histopathologic endpoints, can be used to estimate the high dose in a chronic toxicity study.

Although the high dose in a chronic toxicity study should be selected to achieve toxic responses in test animals, and should not cause fatalities high enough to prevent meaningful evaluation of the data from the study, the Agency recognizes that this goal may not always be met. In situations such as these, when it is unclear what dose of the test substance is the high dose, the petitioner/notifier should consult with the Agency to determine an appropriate high dose for the chronic toxicity study.

Low Dose:

The low dose level should not interfere with the normal growth, development, and lifespan of test animals, nor should it produce any other biologically significant signs of toxicity (e.g., NOEL or NOAEL).

Intermediate Dose:

The intermediate dose should produce minimal signs of toxicity. The exact dose selected as the intermediate dose may depend on the pharmacokinetic properties of the test substance.

D. Computerized systems

Computerized systems that are used in the generation, measurement, or assessment of data should be developed, validated, operated, and maintained in ways that are consistent with the intention of the Good Laboratory Practice principles.[18] The FDA has endorsed the use of the Standard for Exchange of Nonclinical Data (SEND) format for electronic transmission of animal study data. You are encouraged to contact the FDA for more information on this electronic protocol.

V. Observations and Clinical Tests

A. Observations of Parental Animals:

Routine cage-side observations of all parental animals should be made for general signs of departure from normal activity, morbidity and mortality once or twice a day until F1 animals are weaned. The usual interval between multiple periods of observations should be at least 6 hours. Individual records should be maintained for each animal and, as possible, the onset and progression of any effects should be recorded, preferably using a scoring system. If grossly visible or palpable tumors develop, the following parameters should be recorded; time of onset, location, dimensions, appearance and progression.

In a chronic toxicity (or combined chronic/carcinogenicity) study with an *in-utero* exposure phase, an expanded set of clinical evaluations should be carried out on animals inside and outside of the cage to enable detection not only of general signs of departure from normal activity, morbidity and mortality but also of neurologic disorders, behavioral changes, autonomic dysfunctions, and other signs of nervous system toxicity. This expanded set of clinical examinations, conducted on animals inside and outside the cage, should be performed on all animals at least once prior to initiation of treatment, and periodically during treatment. Specific information on this type of evaluation is contained in Chapter IV.C.10[10]. (see also the section below).

If reproductive parameters (e.g., fertility index, gestation length, gestation index, live-born index, etc) are collected, they should be included in the study report.

B. Observations of F1 Animals:

These animals should be observed carefully for signs of departure from normal activity, morbidity and mortality at least twice daily throughout the study period. The usual interval between multiple periods of observations should be at least 6 hours. Observations of general appearance and the presence of dead pups should be recorded. The total number of pups per litter and the number of pups per sex should be recorded.

Individual records should be maintained for each animal and, as possible, the onset and progression of any effects should be recorded, preferably using a scoring system. If grossly visible or palpable tumors develop, the following parameters should be recorded; time of onset, location, dimensions, appearance and progression.

In a chronic toxicity (or combined chronic/carcinogenicity) study with an *in-utero* exposure phase, an expanded set of clinical evaluations should be carried out on animals inside and outside of the cage to enable detection not only of general signs of departure from normal activity, morbidity and mortality but also of neurologic disorders, behavioral changes, autonomic dysfunctions, and other signs of nervous system toxicity. Specific information about the systematic clinical tests/observations is contained in Chapter IV.C.10 [11]. This expanded set of clinical examinations, conducted on animals inside and outside the cage, should be age appropriate and performed on all animals periodically during treatment. Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions or other evidence of autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern). Additionally, changes in gait, posture and response to handling, as well as the presence of clonic or tonic seizures, stereotypic (e.g., excessive grooming, repetitive circling) or bizarre behavior (e.g., self-mutilating, walking backwards) should be recorded. During the course of a study, toxic and pharmacologic signs may suggest the need for additional clinical tests or expanded post-mortem examinations.

C. Body Weight and Feed Intake Data:

Accurate individual body weight, feed, and water consumption measurements are critical in the objective evaluation of the effect of a test substance on experimental animals, since changes in these variables are often the first signs of toxicity. Complete records for these parameters are essential in assessing the time-related occurrence of toxicity-induced changes. When these data are not carefully recorded the evaluation of the overall cancer-inducing potential for a test substance may be compromised. A discussion of some of the variables that affect feed consumption and weight gain/loss can be found under sections II.K: Diet and IV.B: Route of Administration.

Parental animals should be weighed immediately before the first dose of the test substance is administered, and weekly throughout gestation and lactation. If the substance is given by gavage, animals should be weighed every 1-3 days. Feed consumption should be measured weekly. Water consumption should be measured weekly if the test substance is administered in the water.

Body weights for all F1 animals should be recorded weekly for the first 13 weeks after weaning, and monthly thereafter for the duration of the study. Feed consumption (or water consumption if the test substance is administered in the drinking water) should be measured at the same interval as body weights.

Petitioners/notifiers should also attempt to quantify spillage of feed by experimental animals. When it is suspected that test compound administration may be affected by any of the following conditions; 1) feed palatability issues, 2) marked changes in body weight, or 3) increased numbers of animal deaths, the petitioners/notifiers should measure weights and feed (water) consumption more frequently after the initial 13 week period (e.g., every two weeks). Petitioners/notifiers should also use this accumulated information to calculate intake of the test substance as mg/kg body weight/day.

D. Clinical Testing:

Ophthalmological examination, hematology profiles, clinical chemistry tests, and urinalyses should be performed in all F1 animals as described in the following sections:

1. Ophthalmological Examination:

This examination should be performed by a qualified individual on all F1 animals during the first 2 weeks of study and on control and high-dose animals at the end of the study. If the results of examinations at termination indicate that changes in the eyes may be associated with administration of the test substance, ophthalmological examinations should be performed on all F1 animals in the study.

2. Hematology:

Hematological tests should be performed on at least ten F1 animals per sex per group during the first 2 weeks of study, and at 3, 6 and 12 months during the study. If data trends or significant parameter changes (biological or statistical) are observed that are of concern at the 12-month measurement and the study lasts longer than one-year, an 18-month measurement should be included.

Ideally, the same rodents should be sampled at each collection time point. Blood samples should be analyzed

individually, and not pooled. If, due to the large number of animals, it becomes necessary to draw blood samples on more than one consecutive day at each sampling point, the samples should be obtained at approximately the same time each day.

The following determinations are recommended: hematocrit, hemoglobin concentration, erythrocyte count, total and differential leukocyte counts, mean corpuscular hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration, platelet count, and a measure of clotting potential (e.g., clotting time, prothrombin time, activated partial thromboplastin time).

Test compounds may have an effect on the hematopoietic system and therefore appropriate measures should be employed so that evaluations of reticulocyte counts and bone marrow cytology may be performed if warranted. Reticulocyte counts should be obtained for each animal using automated reticulocyte counting capabilities, or from air-dried blood smears. Bone marrow slides for cytological evaluation should be prepared from each animal. These slides only need to be microscopically examined when effects on the hematopoietic system are noted.

3. Clinical Chemistry:

Clinical chemistry tests should be performed on at least ten F1 animals per sex per group during the first 2 weeks of study, and at 3, 6 and 12 months during the study. If data trends or significant parameter changes (biological or statistical) are observed that are of concern at the 12-month measurement and the study lasts longer than one-year, an 18-month measurement should be included.

Ideally, the same rodents should be sampled at each collection time point. Blood samples should be drawn at the end of the fasting time and before feeding. Fasting duration should be appropriate for the species and the analytical tests to be performed. Blood samples should be analyzed individually, and not pooled. If animals are sampled on more than one day during a study, blood should be drawn at approximately the same time each sampling day.

Clinical chemistry tests that are appropriate for all test substances include measurements of electrolyte balance, nutrients metabolism, and liver and kidney function. Specific determinations should include:

Hepatocellular evaluation (at least 3 of the following 5)

- Alanine aminotransferase (SGPT, ALT)
- Aspartate aminotransferase (SGOT, AST)
- Sorbitol dehydrogenase
- Glutamate dehydrogenase
- Total bile acids

Hepatobiliary evaluation (at least 3 of the following 5)

- Alkaline phosphatase
- Bilirubin (total)
- Gamma-glutamyl transpeptidase (GG transferase)
- 5' nucleotidase
- Total bile acids

Other markers of cell changes or cellular function

- Albumin
- Calcium
- Chloride
- Cholesterol (total)
- Cholinesterase
- Creatinine
- Globulin (calculated)
- Glucose
- Phosphorous
- Potassium
- Protein (total)

- Sodium
- Triglycerides
- Urea nitrogen
- The FDA understands that the specific nature of the test substance may warrant the consideration of alternative tests. Appropriate justification for alternative tests should be presented in study reports.

In spite of standard operating procedures and equipment calibration, it is not unusual to observe considerable variation in the results of clinical chemistry analyses from day to day. [2] Ideally, clinical chemistry analyses for all dose groups should be completed during one day. If this is not possible, analyses should be conducted in such a way as to minimize potential variability.

4. Urinalyses:

The determination of volume of urine collected, urine specific gravity, pH, glucose, and protein, as well as microscopic analysis of urine for sediment and presence of blood and/or blood cells, are recommended[14] during the first 2 weeks of study, and at 3, 6 and 12 months during the study. If data trends or significant parameter changes (biological or statistical) are observed that are of concern at the 12-month measurement and the study lasts longer than one-year, an 18-month measurement should be included. These tests should be performed on at least ten F1 animals per sex per group.

VI. Necropsy and Microscopic Examination

A. Gross Necropsy

Termination of Parental and F1 Animals not Selected for the Post-Weaning Phase

These animals should be killed after selection of the F1 animals to be continued on studying. If toxic signs or reproductive toxicity are observed, these animals should be subject to a complete gross necropsy.

Termination of F1 Animals Selected for the Post-Weaning Phase

All of these F1 animals should be subjected to complete gross necropsy, including examination of external surfaces, orifices, cranial, thoracic and abdominal cavities, carcass, and all organs. The gross necropsy should be performed by, or under the direct supervision of, a qualified pathologist, preferably the pathologist who will later perform the microscopic examination.

B. Organ Weight

Organs that should be weighed at minimum include the adrenals, brain, epididymides, heart, kidneys, liver, spleen, testes, prostate, thyroid/parathyroid, thymus if present, ovaries and uterus. Before being weighed, organs should be carefully dissected and trimmed to remove fat and other contiguous tissue. Organs should be weighed immediately after dissection to minimize the effects of drying on organ weight.

C. Preparation of Tissues for Microscopic Examination

Generally, the following tissues should be fixed in 10 % buffered formalin (or another generally recognized fixative) and sections prepared and stained with hematoxylin and eosin (or another appropriate stain) in preparation for microscopic examination. Lungs should be inflated with fixative prior to immersion in fixative.

- Adrenals
- Aorta
- Bone (femur)
- Bone marrow (sternum)
- Brain (at least 3 different levels)
- Cecum
- Colon
- Corpus and cervix uteri
- Duodenum
- Epididymides
- Esophagus

- Eyes
- Gall bladder (if present)
- Harderian gland
- Heart
- Ileum
- Jejunum
- Kidneys
- Liver
- Lung (with main-stem bronchi)
- Lymph nodes (1 related to route of administration and 1 from a distant location)
- Mammary glands
- Nasal turbinates
- Ovaries and fallopian tubes
- Pancreas
- Pituitary
- Prostate
- Rectum
- Salivary gland
- Sciatic nerve
- Seminal vesicle (if present)
- Skeletal muscle
- Skin
- Spinal cord (3 locations: cervical, mid-thoracic, and lumbar)
- Spleen
- Stomach
- Testes
- Thymus (if present)
- Thyroid/parathyroid
- Trachea
- Urinary bladder
- Vagina
- Zymbal's gland
- All tissues showing abnormality

D. Microscopic Evaluation

All gross lesions should be examined microscopically. All tissues from the F1 animals in the control and high dose groups should be examined. If treatment related effects are noted in certain tissues, then those specific tissues in the next lower dose level tested should be examined. Successive examination of the next lower dose level continues until no effects are noted. In addition, all tissues from both parental and F1 animals that died prematurely or were sacrificed during the study should be examined microscopically. If there are questions related to the review and interpretation of pathological lesions and statistical results, additional discussion may be found in Chapters IV.B.3. [12] and IV.B.4. [13] of the Redbook 2000.

E. Histopathology of Lymphoid Organs

Histopathology evaluation of the lymphoid organs should be performed as described in the section on immunotoxicity testing (see Chapter V.D. of the 1993 draft Redbook II). A recent publication provides further discussion on this subject.[17]

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Redbook 2000: IV.C.9.a Guidelines for Reproduction Studies

July 2000

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter IV.C.9.a. Guidelines for Reproduction Studies

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I. Abstract

In the U. S., the Food and Drug Administration (FDA) is the agency responsible for ensuring that the are safe for all consumers. In order to determine the safety of these food ingredients for consumption, appropriate information and results from a series of tests must be made available to the agency. In 1982, in an effort to provide guidance to the food industry concerning the appropriate tests for the determination of safety, the FDA issued the *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Foods*, commonly referred to as the *Redbook*.(23) In 1993, based on the expansion of technology and the use of food ingredients, as well as the refinement of the scientific criteria for establishing safety, the FDA updated its guidelines and issued the draft *Redbook II*.(24) Since the draft *Redbook II* was issued, additional refinements have been made in the procedures for the multigeneration reproduction study and for the assessment of effects on male reproduction. The latest proposed guidelines for multigeneration studies are provided here, in Redbook 2000.

II. Introduction

During the past several decades, the technology of food processing has changed dramatically and the use and variety of food ingredients have increased. In the U. S., the Food and Drug Administration (FDA) is the agency responsible for ensuring that food ingredients are safe for all consumers. Safety, as it pertains to food ingredients , is defined in the *Code of Federal Regulations* as a "reasonable certainty ... that the substance is not harmful under the intended conditions of use".(25) In order to obtain a "reasonable certainty" of meeting the regulation, appropriate information and results from a series of tests must be made available to the agency. Just as the technology and use of food ingredients have expanded, so the scientific criteria for establishing safety have also been refined.

In an effort to provide guidance to industry concerning the appropriate tests for the determination of safety, the FDA issued the *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Foods*.(23) The book is commonly referred to as *the Redbook*. In 1993, based on increased knowledge of toxicological processes and procedures as well as changes in the food industry, the FDA updated its guidelines and issued the draft *Redbook II*.(24)

In 1982, a three-generation reproduction study in rats was recommended, with a teratology phase as part of the battery of tests for substances in concern levels two and three. In 1993, a multigeneration reproduction study with a teratology phase for concern levels two and three was still required, but the multigeneration

study was streamlined to two generations with a single litter per generation.

Concern levels, as determined by the agency, are "relative measures of the degree to which the use of an additive may present a hazard to human health".[24] The concern level is based on the extent of human exposure (dose) and the toxicological effects on biological systems. There are three broad bands of concern levels. Concern level three represents the highest probable risk to human health. Concern level one represents the lowest probable risk. Concern level two is intermediate between high and low risk.

Draft *Redbook II* also included general guidelines for assessing effects on male reproductive function and optional neurotoxicity and immunotoxicity screens. Since Draft *Redbook II* was released, additional refinements have been made in the procedures for the multigeneration reproduction study and for the assessment of effects on male reproduction. The latest proposed guidelines for multigeneration studies are provided here.

In a multigeneration reproduction study, the test substance is administered to parental (F_0) males and females prior to and during mating, gestation, and through the weaning of F_1 offspring. The test substance is then given to selected F_1 generation offspring during their growth and development to adulthood, and through the mating period. Pregnant F_1 generation females continue to receive the test substance throughout gestation and until the F_2 generation offspring until the offspring are weaned.

III. Guideline for Reproduction Studies

The guideline for reproduction studies detailed below pertains to substances given orally to rodents. It is designed to evaluate the effects of a test substance on the reproductive systems of both males and females, the postnatal maturation and reproductive capacity of offspring, and possible cumulative effects through several generations. A study can provide information concerning the effects of a substance on gonadal function, estrous cycles, mating behavior, conception, parturition, neonatal morbidity, mortality, lactation, weaning, growth and development of the offspring, and target organs in the offspring. The study may also serve as a guide for subsequent tests. The end points evaluated and the indices calculated must provide sufficient information and statistical power to permit the FDA to determine whether the chemical is associated with changes in reproduction and fertility. Additional and historical information is found in Collins,[4] Francis and Kimmel,[6] and U.S. Environmental Protection Agency.[21]

The minimal reproduction study recommended consists of two generations, with one litter per generation (see Figure 1). If results of developmental and other toxicity tests indicate that a test substance may be associated with developmental toxicity, the minimal reproduction study should be expanded. This guideline contains optional procedures for inclusion of additional litters per generation, additional generations, a test for teratogenic and developmental toxicity effects, optional neurotoxicity screening, and optional immunotoxicity screening.

Figure 1. 2-Generation Reproduction and Teratology Study

A. General Recommendations

The following recommendations are applicable to all FDA toxicity studies:

1. Studies should be conducted according to Good Laboratory Practice Regulations (GLPs).[22]
2. Animals should be cared for, maintained, and housed according to the recommendations contained in the *Guide for the Care and Use of Laboratory Animals*.[10]
3. Healthy animals that have not been subjected to previous experimental procedures should be used. Generally, it is not possible to treat animals for infection during the course of a study without the risk of interaction between the treatment drug and the test substance. The females should not be pregnant and should be nulliparous.
4. Test animals should be characterized by reference to their species, strain, sex, and weight or age.
5. Animals should be assigned to control and experimental groups in a stratified random manner to minimize bias and ensure compatibility across experimental and control groups for statistical purposes. Each animal must be assigned a unique number.

B. Dose Range-Finding Study

A dose range-finding study is recommended to determine the most appropriate doses, unless suitable

pharmacokinetic and metabolic data concerning the test substance are available prior to the start of the study. The dose range-finding study should preferably, but not necessarily, be done in pregnant animals. Comparison of the results from a trial study in non-pregnant animals and a main study in pregnant animals should establish whether the test substance is more or less toxic in pregnant animals than in non-pregnant animals.

C. Main Study

1. Experimental Animals, Species and Strain Selection and Housing

Because of the expense and length of time needed for multigeneration studies, the species selected should be one that will yield the greatest amount of information per unit cost. Rodents such as rats and mice are usually selected for use in multigeneration studies. The rat is the preferred species because of the small size of the animals, ease of breeding in the laboratory, gestation length of approximately three weeks, high fertility rate, and spontaneous ovulation. The litters are large enough to allow for inter- and intra-litter comparisons, and the animals are less susceptible to stress effects than are mice. Strains with low fecundity should not be used. Single housing of the animals is recommended, except during mating. The animals' diet should meet all nutritional requirements to support pregnancy and lactation in the test species.

2. Number, Sex, and Age

Exposure to the test substance typically begins when the rats are five to nine weeks of age. All test and control animals should be acclimated to the study conditions before treatment begins. The acclimation period is usually one week except under unusual conditions. Each test and control group should consist of animals of uniform weight and age and should start with a number of animals sufficient to contain approximately 20 males and 20 pregnant females near term. In order to achieve this number, it is usually necessary to start with 30 animals per sex per group in the first parental group (F_0) and 25 animals per sex per group (at least one male and one female from each litter, with a maximum of two of each sex per litter) in the parents of each consecutive generation.

3. Assignment to Dose Groups

Animals should be assigned to test and control groups in a stratified random manner to minimize intergroup weight differences. Each animal should be uniquely identified and the litter of origin for each F_1 animal should be identified.

4. Dose Selection

A minimum of three doses of the test substance (high, intermediate, and low doses) should be used to facilitate the separation of dose-related responses from experimental variation. The high dose should produce some parental toxicity (such as reduced body weight or weight gain) but not more than 10% parental mortality. The dose should not exceed 5% of the diet for non-nutritive additives. In dietary studies for macronutrient additives, high doses should be based on nutritional effects rather than toxicological end points. The lowest dose should not induce observable adverse parental effects and should be set at a level which is expected to provide a minimal margin of safety. The intermediate dose(s) should be spaced to allow an arithmetic or geometric progression between the high and low doses. The addition of one or more extra groups is preferable to large intervals between doses.

5. Control Group(s)

A concurrent control group is required. Control animals should be fed and handled the same as dosed animals and should be caged in such a way as to preclude airborne or other contamination by the test substance. For dietary studies, the control group should be fed the basal diet. When a carrier vehicle for the test substance is used, the volume of vehicle given to control rats should be equal to the maximal amount of vehicle given to any dosed group. If there are insufficient data on the toxic properties of the vehicle used in administering the test substance, a sham control group could be included. An additional control group that is not exposed to the vehicle should be included in the study. If a test substance causes reduced dietary intake, a pair-fed control group should be considered.

6. Duration of Testing

Animals should be exposed to the test substance during the entire study. Males of the first parental group (F_0) should be dosed for the duration of spermatogenesis and epididymal transit (at least ten weeks) before mating and throughout the mating period, in order to detect adverse effects on spermatogenesis by the test

substance. The first parental females (F_0) should be exposed before mating for the same length of time (at least ten weeks) as the males and then through mating and pregnancy, to the weaning of the F_{1a} litter. Litters (usually F_{1a} and F_{2a}) should be exposed from the prenatal period throughout their entire postnatal lives. If a third generation is planned, these litters also should be exposed from the prenatal period and throughout their entire lives.

7. Substance Administration

The test substance may be administered to rodents in the diet, in drinking water, or by gavage (stomach tube intubation). The same route of administration should be used for all animals throughout the study. If the test substance is given by gavage it is best to adjust the volume daily or every three days based on the animal's body weight.

8. Mating Procedures

For each mating, a female should be placed with a single randomly selected male (one:one mating) from the same dose group until pregnancy occurs or two to three weeks have elapsed. Mating of one male with two female rats is permitted in the event that a male dies.

Animals should be separated as soon as possible after evidence of copulation has been observed. If mating has not occurred after two to three weeks, the animals should be separated without further opportunity for mating. Mating pairs should be clearly identified in the data. Each morning, all females should be examined for the presence of sperm in the vaginal lavage or the presence of a vaginal plug; if sperm and/or a vaginal plug are found, this is considered day zero of gestation. Near parturition, pregnant females should be caged separately in delivery or maternity cages and may be provided with nesting materials. Pregnant females in test and control groups should be allowed to litter normally.

9. Standardizing the Number of Pups per Litter

Standardization of the number of pups per litter through culling is optional. Litters may be standardized to ten (or eight) based on historical litter size for the strain. It is recommended that standardization be performed on postnatal day four by reducing all litters of more than ten (or eight) to ten (or eight) in a random manner. If possible, the retained litter-mates should consist of equal numbers of males and females. Random selection is important to guard against the human tendency to keep the most fit animals in the study.

10. Selection of Parental Animals for Next Generation

At least one male and one female should be randomly selected from each litter for mating with another pup of the same dose level but different litter to produce the next generation. If there are insufficient litters from which to make a selection, then no more than two males and two females per litter should be included in the group. As many litters as possible should be represented. The mating procedures for the F_1 males and females should be carried out in the same manner as the F_0 parental animals. Care should be taken that siblings are not mated on a study. F_1 males and females not selected for mating should be terminated after weaning.

11. Optional Third Generation

If overt reproductive, morphologic, and/or toxic effects of a test substance are observed in offspring during the two-generation reproduction study, the study may be extended to a third generation to determine cumulative effects of the substance. Selection of animals for mating and mating for an additional generation should be carried out by the same procedures as for the first generation. Randomly mated animals from the F_{2a} litter should be mated to produce the third generation. F_{3a} animals should be weaned and either necropsied or used for a longer-term toxicity study.

12. Optional Second Mating

If production of a second litter is necessary, the dams should be mated again approximately one to two weeks after weaning of the F_{1a} or F_{2a} litter.

13. Optional Teratology Phase

The teratology phase should be incorporated into the multigeneration reproduction study unless justification can be provided for conducting a separate developmental study. In a reproduction study, either the F_{2b} or the F_{3b} litter can be used to determine fetotoxic effects of the test substance. If a teratology phase is to be performed, pregnancy should be timed by the presence of sperm in the vaginal lavage or by the presence of a vaginal plug, and this considered as day zero of gestation. Approximately one day before expected parturition, the dams should be euthanized and Cesarean sections performed. The uterus should be opened and examined

for the presence of early and late deaths, and *corpora lutea* should be counted. Each live fetus should be removed from the uterus. The weight and sex of each live fetus should be determined. Each live fetus should be examined for gross malformations and then for skeletal or soft-tissue abnormalities. Additional, detailed procedures are found in the Food and Drug Administration Proposed Testing Guidelines for Developmental Toxicity Studies.(13)

14. Clinical Observations

Each animal should be observed at least twice each day. The first observation should be a thorough clinical examination. The second may involve the observation of the animals through their cages. Observation of the animals through their cages is satisfactory for pregnant animals near term and for animals nursing their litters. Observation times should be selected to permit detection of the onset and progression of all toxic and pharmacologic effects of the test substance and to minimize the loss of animals and organs/tissues. Relevant behavioral changes and all signs of toxicity, including mortality, should be recorded. Estrous cycle length and normality should be evaluated daily by vaginal smears for all F_0 and F_1 females during a minimum of three weeks prior to mating and during cohabitation. The duration of gestation should be calculated from day zero of pregnancy. Care should be taken to prevent the induction of pseudopregnancy.

Individual records should be maintained for all adult animals and offspring selected for the next generation. Toxicological and pharmacological symptoms and signs, including behavioral abnormalities, should be recorded daily; records should include the date of onset, duration, and intensity of symptoms and signs.

Animals should be weighed immediately before the test substance is first administered, once weekly thereafter, and at necropsy. Feed consumption also should be recorded weekly at a minimum. If the substance is given in the diet, weekly body weights are acceptable. If the substance is given by gavage it is best to adjust the volume daily or every three days on the basis of the animal's body weight. Water consumption should be measured if the test substance is administered in the water and may also be measured if it is thought that the substance might influence fluid consumption.

15. Growth of Offspring

Each litter should be examined as soon as possible after delivery for the number of pups, stillbirths, live births, and the presence of gross anomalies. Dead pups should be necropsied and observed for possible gross defects and the cause of death unless excessive autolysis renders specimens useless.

The neonates should be carefully observed, and their sex and weight should be noted on postnatal days zero (day of birth), four, seven, fourteen, and 21. Other appropriate days are acceptable to monitor postnatal growth and other developmental indices.

Anogenital distance should be measured at day zero for all F_2 pups that show treatment-related effects in F_1 sex ratio or sexual maturation. The age and weight of each animal on the day of vaginal opening or balano-preputial separation should be recorded for F_1 weanlings selected for mating.

16. Optional Neurotoxicity Screening

Multigeneration reproduction studies provide an excellent vehicle to screen for potential developmental neurotoxicity. Periodic examination of the developing offspring provides information to help detect treatment-related changes in development, the appearance of neurological disorders, and other signs of nervous system toxicity. The examination of the offspring should be as brief as possible to minimize the period of separation from the dam. During the examination, any abnormalities in the animal's appearance or behavior should be noted as well as markers to gauge age-appropriate physical development (such as eye opening, genital development, and incisor eruption) and functional development (such as righting reflex, startle response, and motility). As an alternative option, satellite groups of litters (using suitable numbers for adequate statistical analysis) may be used to screen for developmental neurotoxicity. The inclusion of other endpoints should be encouraged, such as the assessment of cognitive function development. All data derived from the examination of the experimental offspring, including positive and negative findings, should be documented, statistically analyzed as appropriate, using the litter as the statistical unit, and reported. Additional information is available in Sobotka *et al.*(19)

17. Optional Immunotoxicity Screening

Multigeneration reproduction studies can screen the effects of a test substance on a developing immune system and evaluate the potential for immunotoxicity by the use of non-invasive (Type I) tests and invasive (Type II) tests.(24) Since the effects of *in-utero* exposure are not normally assessed in chronic, acute, and subchronic studies, Type I immunotoxicity testing should be conducted on exposed dams and F_1 male and

female offspring. By careful planning, animals used or produced in the reproduction study may be evaluated for Type I indicators, and possibly Type II. For example, when F_0 parental males have completed the mating cycle, representative animals could be sacrificed or fed longer if data from chronic, acute, and subchronic studies are not available. After weaning, F_0 parental females that are no longer needed are an excellent source of animals for evaluating potential immunotoxicity effects. For evaluating the effects of *in-utero* exposure, neonatal specimens from culled litters could be used for histologic evaluation of the neonatal lymphoid organs. Only a small number of weanlings of each sex are selected for further use in the reproduction study; the remaining animals could be sacrificed and evaluated at three weeks or allowed to mature to six or eight weeks of age. At this time, Type I testing and/or functional Type II testing could be performed. Similar opportunities exist in the F_2 generation. Additional information is available in Hinton *et al.* (9)

18. Gross Necropsy and Microscopic Examination

All adult males and females should be terminated when they are no longer needed for the assessment of reproductive findings. Any dam that shows signs of imminent abortion, premature delivery, or moribund condition should be necropsied on the day such signs are observed. Dead pups (pups that die spontaneously during the postpartum phase) should be necropsied and observed for possible gross defects and the cause of death unless they are excessively autolyzed.

At the time of termination, all parental animals should be examined macroscopically for any structural abnormalities or structural changes. This should include examination of external surfaces, orifices, cranial cavity, carcass, and all organs. Special attention should be paid to the organs of the reproduction system. The uterus should be examined for the presence of implantation sites and resorptions. The uterus may be stained with sodium or ammonium sulfide (16) or other appropriate chemical to help visualize the implantation sites.

a. Necropsy of Weanlings

   At the time of termination, at least two pups per sex per litter from unselected F_1 and F_2 weanlings should be examined macroscopically for any structural abnormalities or structural changes. Special attention should be paid to the organs of the reproduction system.

   Brain, thymus, and spleen should be weighed from the F_1 and F_2 weanlings that are examined macroscopically for structural abnormalities or structural changes. At necropsy, grossly abnormal organs and tissues from pups from all dose groups should be preserved and then examined histopathologically.

b. Necropsy of Parental Animals

   At necropsy, the following organs of all F_0 and F_1 control and treated parental animals should be observed and weighed: brain, pituitary, liver, kidneys, adrenal glands, spleen, known target organs, and reproductive organs. Uterus and ovaries of females should be weighed. For males, both testes, seminal vesicles with coagulating glands, and the prostate should be weighed. In addition, the total epididymal weight should be determined for one epididymis that will be fixed for histopathology, and both total epididymal and cauda epididymal weight should be determined for the epididymides that will be used for observing sperm morphology, numbers, and motility. Seminal vesicles and prostates should be weighed separately. The source of the prostate weight should be identified (*e.g.*, as ventral and/or dorsal and/or dorsolateral prostate). At necropsy, the contralateral testis and epididymis (the non-fixed testis and epididymis) should be utilized for the determination of homogenization resistant spermatid numbers and cauda epididymal sperm reserves, respectively. Additionally, sperm from the cauda epididymis (or proximal vas deferens) should be collected for the evaluation of sperm motility and sperm morphology.

   Since testis weight varies only slightly within a given species, a change in testis weight may indicate that a test substance has had an adverse effect on the testis. Seminal vesicles and prostates are androgen-dependent organs and changes in their weights may indicate a change in the endocrine status of the animal or the ability of the testis to produce androgen.

   Organ weight should be reported as absolute weight and as a relative weight (*e.g.*, organ-to-body or organ-to-brain weight).

   i. Fixation of Tissues and Organs

      At necropsy, the following organs and tissues, or representative samples thereof, from all parental animals, should be fixed and stored in a medium suitable for histopathological examination. For parental females, the vagina, uterus with cervix, ovaries with oviducts, adrenal and pituitary glands, target organs, and grossly abnormal tissue should be preserved. For parental males, one testis, one epididymis, seminal vesicles, coagulating glands, prostate, and adrenal and pituitary

glands, target organs, and grossly abnormal tissue should be preserved. Testicular tissues should be fixed in Bouin's or a comparable fixative and stored in a suitable medium for histopathological examination. Several articles and books have been written recently to describe methods that can be used to preserve testicular tissue and evaluate histopathology.[8],[15] If an immunotoxicity screen is being included in the study, the appropriate procedures and organs mentioned in *Redbook II*[24] should be followed.

ii. General Histopathology

Full histopathological examination of the organs should be performed for ten randomly selected control and high-dose F_0 and F_1 animals per sex. If the high-dose group reveals a treatment-related effect, ten animals from each intermediate dose group should be randomly selected and examined. Tissues and organs preserved from the additional animals in each group may be examined to provide additional data.

In addition, a full histopathological examination should be performed on the reproductive organs of animals suspected of reduced fertility from intermediate dose groups. Signs of reduced fertility include failure to mate, conceive, sire, or deliver healthy offspring; effects on estrous cycle; reduced reproductive organ weight; and reduced testicular spermatid counts or cauda epididymal sperm counts.

iii. Histopathology of Female Reproductive Organs

The post-lactational ovary should contain primordial and growing follicles as well as the large *corpora lutea* of lactation. Histopathological examination should detect qualitative depletion of the primordial follicle population. A quantitative evaluation of primordial follicles should also be conducted. If the high-dose animals reveal a treatment-related effect, all groups should be examined. The following evaluation technique may be used, but others may be used if the number of animals, ovarian section selection, and section sample size are statistically appropriate. Substance-induced depletion of primordial follicles can be identified by removing five sections from the inner third of each ovary. The sections should be at least 0.1 mm (100 µm) thick. Examination should include enumeration of the total number of primordial follicles from these ten sections for comparison with control ovaries. Examination should also confirm the presence or absence of growing follicles and *corpora lutea* in comparison with control ovaries. Additional information can be found in Bolon *et al.*,[1] Bucci *et al.*[2] and Heindel.[7]

iv. Histopathology of Male Reproductive Organs

Histopathological assessment of the epididymis should include an evaluation of the corpus, cauda and caput epididymis. This can be accomplished by examining a longitudinal section through all three regions of the epididymis in order to identify such lesions as sperm granulomas, leukocyte infiltration, aberrant cell types within the lumen, or the absence of clear cells in the cauda epididymal epithelium.[5]

Careful histopathological examination of the testis is recognized as a sensitive method to identify effects on spermatogenesis. Testicular tissue should be examined with a knowledge of testis structure, the process of spermatogenesis, and the classification of spermatogenesis. If an effect is observed, it should be described in detail.[15] If testicular effects are quantitated, the methods used should be described in detail.

A thorough histological evaluation of the testis should include an examination of the interstitial compartment and the seminiferous tubule compartment. A histopathological evaluation of the intertubular cell compartment of the testis should include a general assessment of the Leydig cells, the blood vessels, and the cell types other than the Leydig cells typically found in the intratubular space. The general appearance of the seminiferous tubules should be noted This should be followed by an examination of the seminiferous tubule compartment to detect any disruption in the normal sequence of the events that occurs during the normal process of spermatogenesis. The seminiferous epithelium should then be carefully observed to detect any of the following: presence of multinucleated cells, missing germ cell layers, increased germ-cell degeneration, abnormal development in germ cells, sperm release delay or failure, presence of germ cells in the seminiferous tubule lumen, and any changes in the Sertoli cells (vacuolization, sloughing, or nuclear changes). The general condition of the boundary layer should be noted.

D. End Points of Female Reproductive Toxicity

End points of reproductive toxicity are usually expressed as indices that encompass the animals' responses to the test substance from conception to weaning. The following indices should be calculated for each reproduction study: female fertility, gestation, and live-born indices; weaning index or lactation index; sex ratio; and viability indices at postnatal days four, seven, fourteen, and 21.

1. Female Fertility Index

The female fertility index represents the percent of matings that result in pregnancies. It is calculated as follows: (number of pregnancies/number of matings) X 100. This index reflects the total number of dams that have achieved pregnancy, including those that deliver at term, abort, or have fully resorbed litters. This index depends on male libido and fertility as well as female cyclicity and receptiveness.

2. Gestation Index

The gestation index evaluates the efficiency of pregnancy that results in at least one live offspring. In this index, the litter with one live offspring is counted the same as one with more than one live offspring. The index is calculated as follows: (number of litters with live pups/number of pregnancies) X 100.

3. Live-born Index

Related to the gestation index, the live-born index (number of pups born alive/total number of pups born) X 100 is a measure of the total number of offspring lost, regardless of litter.

4. Weaning Index

The weaning index represents the ability of pups to survive from day four to day 21. It is calculated as follows: (number of pups alive at day 21/number of pups alive and kept on day four) X 100. This index corrects for the reduction of pups on day four. If the pups are not reduced, a related index, the lactation index, is calculated: (number of pups alive on day 21/number of pups alive on day four) X 100. Regardless of the etiology, a decrease in the weaning index indicates adverse reproductive effects.

5. Sex Ratio and Percentage by Sex

Determining the sex of pups at birth and verifying their sex at each weighing permits the relative fitness of each sex to be calculated as the offspring mature. The sex ratio is useful in detecting if the test substance is preferentially affecting one sex. This parameter is usually calculated as follows: (number of males/number of females). The related calculation (number of females or males/total number of animals) X 100 yields the percentage of total animals that are male or female.

6. Viability Indices

The viability indices are measures of the offsprings' ability to survive during specific brief intervals of their lives, from birth (day zero) to day four, day four to day seven, day seven to day fourteen, day fourteen to day 21, or they may reflect longer intervals, such as day zero to day seven, day zero to day 21, *etc.* For example, the day seven viability index is calculated as follows: (number of pups alive on day seven/number of pups alive and kept on day four) X 100. The pups' ability to survive may reflect the adequacy of postnatal nourishment, maternal neglect, and postnatal absorption of a toxic substance that is excreted in the mother's milk. Regardless of etiology, decreases in viability indices indicate adverse reproductive effects. Other appropriate days are acceptable to monitor postnatal growth and other developmental indices.

E. End Points of Male Reproductive Toxicity

The following end points of male reproductive toxicity should also be assessed if there is evidence of male-mediated effects on developing offspring. End points should be measured in all animals in each of the control and high dose groups. If treatment-related effects are observed, then animals from each intermediate dose group should be evaluated.

1. Evaluation of Testicular Spermatid Numbers

Testicular spermatid enumeration is a measure of sperm production from the stem cells and their survival through all phases of spermatogenesis. The enumeration of spermatid numbers should primarily be used in chronic studies where spermatid numbers have stabilized; in short-term studies treatment may not have impacted the late spermatid population. From the number of spermatids per testis, the efficiency of sperm production and daily sperm production rate can be calculated.[(14)]

The second testis (first testis was used for histopathology) from all F_0 and F_1 generation males used for

mating should be collected and stored frozen until testicular spermatid numbers are enumerated. Homogenization-resistant spermatid numbers may be determined by enumerating elongated spermatid nuclei after the testis has been homogenized in a medium containing detergent.[14]

2. Sperm Evaluation for Motility, Morphology and Numbers

Motility is influenced by abstinence, the time between obtaining and evaluating the sample, pH of the medium, sample chamber depth, and temperature. Sperm samples obtained from the cauda epididymis (or proximal vas deferens) should be collected and evaluated for the percent of progressively motile sperm. [17] Care should be taken to avoid artifactual cell death during sample preparation so that the percentage of progressively motile sperm from control animals is consistently high (>70%).[11]

Sperm motility can be assessed by microscopic techniques or with a computer-assisted sperm analysis (CASA) system.[17] For microscopic evaluation, an acceptable counting chamber of sufficient depth is used to combine the assessment of motility with sperm numbers and sperm morphology. When the CASA system is utilized,[3],[18],[20] the derivation of progressive motility relies on user-defined thresholds for average path velocity and straightness or linear index. All samples should be videotaped or otherwise recorded. The video may be retained as raw data. In the event that sperm motility is not videotaped, then a sperm motility assessment from all animals in all dose levels should be performed.

Inasmuch as sperm morphology in rodents is generally stable, characteristic of the animal strain, and exhibits little variability, an increase in the number of morphologically abnormal sperm indicates that the test substance has gained access to the germ cells. This should be considered an adverse reproductive effect. Sperm should be collected from all F_0 and F_1 generation males selected for mating from the control and all dose levels for sperm morphology analysis.

Sperm (minimum 200 per sample) from the cauda epididymis or proximal vas deferens should be examined as a fixed wet preparation [12],[17] and classified as either normal (both head and midpiece appear normal) or abnormal (*i.e.*, fusion, isolated heads, misshapen heads and/or tails).[26]

The total number of sperm in the cauda epididymis should be enumerated.[14] Cauda sperm reserves can be derived from the concentration and volume of sperm in the suspension used to complete the qualitative evaluations, and the number of sperm recovered by subsequent mixing and/or homogenizing the remaining cauda tissue. Sperm in the concentrated suspension can be frozen for subsequent evaluation of cauda epididymal sperm numbers. If sperm counts are reported in relation to the weight of the epididymis, the absolute counts should be reported in order to clarify declines in sperm number.

F. Analysis of Data

Values from control and test groups of animals should be compared statistically. The following techniques may be used, but others may be substituted if they are appropriate. The fertility and gestation indices may be analyzed by a one-tailed Fisher exact test. For the sex ratio index, a two-tailed Fisher exact test may be used. Data for the viability and weaning indices may be transformed by the Freeman-Tukey arc-sine transformation for binomial proportions. The transformed data may then be analyzed by an analysis of variance (ANOVA) followed by a one-tailed protected least significant difference (LSD) test to compare the control with the treated groups if the ANOVA $p<0.10$. The average litter size and the number of viable pups throughout the reproduction phase may be analyzed by ANOVA followed by a protected LSD test (one-tailed). For the growth (weight gain) and organ weight analyses, an analysis of covariance may be used followed by a protected LSD test (two-tailed) to compare the control and treated groups.

G. Reporting the Results of Reproduction Studies

Reports of all reproduction studies should contain the information required by the Good Laboratory Practice Regulations, including a copy of the study protocol and all amendments, absolute values for all parameters, complete data (individual pups) and tables of data summarized and analyzed by litter. Because the maternal animal and not the developing organism is the individual treated during gestation, data generally should be calculated as incidence per litter or as number and percent of litters with particular end points. All major indices and end points discussed in the previous section should be calculated. The dosage rate of test substance (doses) should be reported as mg/kg/day (milligrams of test substance per kilogram of body weight per day).

Problems commonly encountered in the review of multigeneration reproduction studies include: insufficient numbers of pregnant animals per control or treatment group, non-random selection procedures, and statistical analyses of data on a per-pup basis instead of a per-litter basis. Careful consideration of recommended guidelines and the submission of protocols for review by FDA before conducting the studies should help

eliminate such problems.

In addition to the various indices in reproduction studies, data on the average number of pups that survived during a specific interval (*e.g.*, average number of pups that survived from birth to day four, or the average number of pups that were weaned) should be examined. This analysis considers the total effect of the test substance at all stages to that point and is a more sensitive indicator than each index separately.

Relevant historical control data may be used to increase the understanding of the study results. When used, historical data should be compiled and presented with appropriate additional information, such as dates of study, strain of animals, vehicle, and route of administration.

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Redbook 2000: IV.C.9.b Guidelines for Developmental Toxicity Studies

July 2000

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter IV.C.9.b. Guidelines for Developmental Toxicity Studies

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I. Abstract

The Food and Drug Administration (FDA) is the agency responsible for ensuring that food ingredients used in the U.S. are safe for all consumers. In 1982, in an effort to provide guidance concerning appropriate tests, the FDA issued *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food*, commonly known as the *Redbook*.(7) The *Redbook* included detailed guidelines for testing the effects of food ingredients on mothers and their developing fetuses. Based on refinements in safety assessment and risk evaluation as well as expansion of knowledge concerning the metabolism and pharmacokinetics of food ingredients, the need to revise and update the 1982 document became apparent. In 1993, *Redbook II* in draft form(8) was made available for public comment. Since then, test end points and developmental landmarks have been refined. The latest proposed guidelines for developmental toxicity studies are provided here.

II. Introduction

The Food and Drug Administration (FDA) is the agency responsible for ensuring that food ingredients used in the U.S. are safe for all consumers. In 1982, the FDA issued *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food*.(7) Based on the color of its cover, the book quickly became known as *the Redbook*. The *Redbook* included detailed guidelines for testing the effects of food ingredients on mothers and their developing fetuses. The tests included a chapter on teratology/developmental toxicity studies as well as reproduction studies that spanned several generations. Guidelines for teratology/developmental toxicity studies are discussed here; guidelines for multigeneration studies are discussed in Chapter IVC9a [2].

Based on refinements in safety assessment and risk evaluation as well as expansion of knowledge concerning the metabolism and pharmacokinetics of food ingredients, the need to revise and update the 1982 document became apparent. In 1993, *Redbook II* in draft form was made available for public comment.(8) Changes in the chapter on reproduction and teratology/developmental toxicity guidelines were based on extensive literature review and public comments. In late 1996, current drafts of this and several other chapters of *Redbook II* were presented at a *Redbook* Update Symposium and the guidelines were compared with current draft guidelines from other national and international regulatory groups.(2) Since then, test end points and developmental landmarks have been refined. The latest proposed guidelines for developmental toxicity studies are provided here in Redbook 2000.

In a developmental toxicity study, the test substance is administered to pregnant animals at least from the day of implantation to the day prior to the day of expected parturition. A short time before the day of expected parturition, the pregnant females are euthanized, the uterine contents are examined, and the fetuses are removed. The fetuses are observed, preserved, and examined for skeletal and soft-tissue abnormalities.

The purpose of developmental toxicity studies is to evaluate the effects of test substances on developing fetuses that result from exposure of either parent prior to conception or to mothers during prenatal development. The adverse effects are as end points that may be used to evaluate the toxic potential of a test substance. The four major manifestations of an effect on the developing organism are: death, structural anomaly, altered or retarded growth, and functional deficiency. For many substances, these manifestations are related to dosage and to the developmental timing and duration of exposure. While high doses produce death, low doses that permit survival may produce malformed, growth retarded, or functionally deficient offspring.

III. Guideline for Developmental Toxicity Studies

The developmental toxicity test may be done as a stand-alone study, or may be part of a multigeneration reproduction study. If it is combined with a reproduction study, assessment of teratological effects may be performed on either the first or second generation, but it is usually performed on the last litter of the generation to maximize exposure to the test agent. As part of a multigeneration study, the fetuses may be exposed to the test substance from conception. In a stand-alone study, treatment must begin early enough to include organogenesis for the species used and should continue to the day prior to the expected day of parturition. This guideline may be used with substances given orally to the rat, mouse, hamster, and rabbit. If the test substance is believed to have the capacity to alter the rate of its own metabolism through induction of metabolizing enzymes or as a result of damage incurred by the liver, then consideration should be given to evaluating the teratogenic potential of the compound by using a separate study.

A. General Recommendations

The following recommendations are applicable to all FDA toxicity studies:

1. Studies should be conducted according to Good Laboratory Practice Regulations (GLPs).[(6)]
2. Animals should be cared for, maintained, and housed according to the recommendations contained in the *Guide for the Care and Use of Laboratory Animals*.[(3)]
3. Healthy animals that have not been subjected to previous experimental procedures should be used. Generally, it is not possible to treat animals for infection during the course of a study without the risk of interaction between the treatment drug and the test substance. The females should not be pregnant and should be nulliparous.
4. Test animals should be characterized by reference to their species, strain, sex, and weight or age.
5. Animals should be assigned to control and experimental groups in a stratified random manner to minimize bias and ensure compatibility across experimental and control groups for statistical purposes. Each animal must be assigned a unique number.

B. Dose Range-Finding Study

A dose range-finding study is recommended to determine the most appropriate doses, unless suitable pharmacokinetic and metabolic data concerning the test substance are available prior to the start of the study. The dose range-finding study should preferably, but not necessarily, be done in pregnant animals. Comparison of the results from a trial study in non-pregnant animals and a main study in pregnant animals should establish whether the test substance is more or less toxic in pregnant animals than in non-pregnant animals.

C. Main Study

1. Experimental Animals, Species and Strain Selection

When pharmacokinetic and metabolic data or other information on the test substance suggest the most appropriate species for developmental toxicity testing, that species should be used. In the absence of such data, the preferred species are the rat and rabbit. These guidelines include information on the mouse and hamster in addition to the rat and rabbit. These animals are small, easy to care for, and have historically provided consistent results that can be extrapolated to human effects. The strains selected should have high fecundity and should be sensitive to teratogens and embryotoxins. Scaling of doses between species should be

based on pharmacokinetic differences between them, unless precluded by differences in overt toxicity.

2. Animal Husbandry

Single housing of the animals is recommended, except during mating. Food and water should be provided *ad libitum*. The animals' diet should meet all nutritional requirements to support pregnancy in the test species. Special attention should be paid to diet composition when the test material itself is a nutrient, because such material may have to be incorporated into the diet at levels which may interfere with normal nutrition. Under these circumstances, an additional control group fed basal diet may be necessary.

3. Number, Sex, and Age

All test and control animals should be young, mature, primiparous, pregnant females of uniform age and size. A sufficient number of females should be used so that each test and control group consists of approximately 20 pregnant rats, mice, hamsters, or rabbits near term. These are the minimum numbers of pregnant animals for developmental toxicity testing. The objective is to ensure that enough litters are produced to permit effective evaluation of the teratogenic potential of the test substance.

4. Duration of Testing

The test substance should be administered daily throughout the treatment period. The minimum treatment period recommended for developmental toxicity studies is from implantation to Cesarean section one day prior to the expected day of parturition. In rats the approximate timing for this period includes days six through twenty; in mice, days six through eighteen; in hamsters, days four through fifteen; and in rabbits, days six through 29. Alternatively, treatment may be extended to include the entire period of gestation, from fertilization to the day of Cesarean section. If the developmental toxicity test is being conducted as part of a multigeneration reproduction study, the animals are dosed from before conception until they are necropsied. The presence of sperm in the vaginal lavage or the presence of a vaginal plug is considered day zero of gestation.

5. Route of Administration

The test substance or vehicle should be administered by the route that most closely approximates the pattern of human exposure (diet or drinking water). Oral intubation (gavage) may be appropriate in instances where human exposure is *via* a bolus dose or when it is essential for the animal to receive a specified amount of the test substance. Gavage may also be required when analysis of the agent in the diet is not possible, when the agent is not stable in the diet, or when the agent is not palatable. The maximum volume of solution that can be given by gavage in one dose depends on the test animal's size; for rodents, this should not exceed 1 ml/100 g body weight. If the test substance must be given in divided doses, all doses should be administered within a six-hour period, unless there is justification for increasing the duration of dosing. If the test substance is given by gavage, it should be given at approximately the same time each day, and the volume should be adjusted on a daily basis or every three days based on the animal's body weight. In diet and drinking-water studies, the amount consumed depends on each animal.

6. Mating Procedures

In-house mating of the animals is recommended. A sufficient number of males should be mated to ensure a large gene pool. Siblings should not be mated. Each male may be mated to either one or two females. The following morning, each female should be examined for the presence of sperm in the vaginal lavage or the presence of a sperm plug. The presence of sperm in the vaginal lavage or the presence of a vaginal plug is considered day zero of gestation (day zero of gestation in rabbits is the day insemination is performed or observed).

7. Control and Dosed Groups

Healthy animals should be assigned to test and control groups in a stratified random manner to minimize inter-group weight differences and ensure statistical comparability of relevant variables. The animals may also be assigned in a random procedure which results in comparable mean body weight values among all groups. At least three test groups and one control group should be used in the developmental toxicity study. All groups should be concurrent.

When the test substance is administered in a vehicle, the vehicle without the test substance should be administered to the control group at a volume equal to the maximal amount of vehicle given to any dosed group. If a vehicle or other additive is used to facilitate dosing, the effects on absorption, distribution, metabolism, or retention of the test substance should be considered, as well as alterations of toxicity due to

effects on the chemical properties of the test substance. Effects of the vehicle on food consumption, water consumption, or nutritional status of the animals should also be considered.

If there are insufficient data on the toxic properties of the vehicle used in administering the test substance, a sham control group should also be included. If no vehicle is used, then the controls should be sham treated. In all other respects, the control group must be handled and maintained in a manner identical to that used with the groups given the test substance.

Unless limited by the physical or chemical properties of the substance, the high dose should induce some developmental and/or maternal toxicity but not more than approximately ten percent mortality. The high dose should not exceed five percent of the diet for non-nutritive additives. In dietary studies for macronutrient additives, the high dose should be based on nutritional effects rather than toxicological end points.

The low dose should not induce observable effects attributable to the test substance and should be set at a level which is expected to provide a margin of safety. The intermediate doses should be spaced to allow an arithmetic or geometric progression between the low and high doses. The addition of one or more groups is preferable to the use of large intervals between doses.

8. Maternal Toxicity and its Significance

End points which may serve as indicators of maternal toxicity include mortality, body weight, body weight gain, organ weights, feed and water consumption, clinical signs of toxicity, and gross or microscopic lesions. The calculation of a corrected mean maternal weight gain (difference between initial and terminal maternal body weight less the gravid uterus weight) may also be used as an index of maternal toxicity.

Various test substances have selective toxic effects on the male, the female, or the offspring, while other substances exhibit non-specific effects. When mother and offspring are adversely affected by a test substance, it can be difficult to determine if the developmental toxicity is mediated by maternal toxicity or occurs independently of it. Due to differences in metabolism, distribution, and elimination of the test substance, the sensitivity of the maternal system can vary significantly from that of the fetus. The response of the fetus can also differ markedly from that of the mother as a result of the developmental processes taking place that have no counterpart in the adult.

Developmental effects without maternal toxicity are commonly regarded as the most serious manifestations of toxicity, because their occurrence is thought to be the result of greater sensitivity of the developing organism. When developmental effects are found in the presence of maternal toxicity, the primary cause is often left to speculation. Without sufficient evidence to support the premise that developmental toxicity is always a secondary toxic effect in the presence of maternal toxicity, a default is needed. Developmental effects that occur in the presence of minimal maternal toxicity are thus considered to be evidence of developmental toxicity, unless it can be established that the developmental effects are unquestionably secondary to the maternal effects. In situations where developmental effects are observed only at doses where there is a substantial amount of maternal toxicity, then the possible relationship between maternal toxicity and the developmental effects should be evaluated in order to make a proper assessment regarding the toxicity of a test substance.

9. Clinical Observation and Examination of Dams and Fetuses

Throughout the study, each animal should be observed at least twice daily. The first observation should be a thorough clinical examination. The second may involve observing the animals through the cages. Observation times should be selected to permit detection of the onset and progression of all toxic and pharmacologic effects of the test substance and to minimize the loss of animals and organs/tissues. Relevant behavioral changes and all signs of toxicity, morbidity, or mortality should be recorded.

Dams should be weighed immediately before the first dose of the test substance is administered (usually on gestation day six for mice, rats, and rabbits; on gestation day four for hamsters), weekly until necropsy, and at the time of necropsy. If the test substance is given in the diet, weekly body weight is acceptable. If the test substance is given by gavage, body weights should be measured daily or at least every three days. At a minimum, weekly measurements of feed consumption should be made. Fluid consumption should be measured as appropriate. Any dam that shows signs of imminent abortion or premature delivery during the study should be necropsied on the date such signs are observed.

The test should be terminated approximately one day before the expected day of parturition (day 20 or 21 for rats, day 29 for rabbits, day eighteen for mice, and day fifteen for hamsters), when the dams should be subjected to gross pathologic examination. Immediately after the dams are killed, fetuses should be delivered by hysterotomy. Care should be taken to ensure that all fetuses (except those sacrificed before the end of the study) are delivered at approximately the same stage of fetal development. The intact uterus should be

removed and weighed in order to calculate the adjusted body weight gain. The contents of the uterus should then be examined for embryonic or fetal deaths and for the number of live fetuses. For dead fetuses, it is usually possible to estimate and describe the approximate time of death *in utero* (early and late deaths). The number of *corpora lutea* should be determined for all pregnant animals.

The uterus of each dam that does not appear to be pregnant should be stained in a solution of sodium or ammonium sulfide[4] or other appropriate chemical to enhance the visibility of resorption sites. Evaluation of the females during Cesarean sections and subsequent fetal analyses should be conducted blind in order to minimize unconscious bias.

After removal from the uterus, the weight and sex of each fetus should be determined. The fetus should be examined externally, and all deviations from normal should be noted. Additional end points may be measured, such as the crown-to-rump distance of each fetus. The sex of rabbit fetuses should be determined by internal examination. Each fetus should be weighed individually, and the mean fetal weight per sex per group should be calculated.

Fetuses should be evaluated for skeletal and soft-tissue anomalies. For rodents, approximately one-half of the rodent fetuses should be preserved in Bouin's solution and sectioned by the Wilson serial section technique to evaluate alterations of the soft tissues.[9] The remaining fetuses should be prepared and stained for skeletal anomalies (Alizarin red stain for bone and optional Alcian blue stain for cartilage). The assignment to soft-tissue or skeletal examination should be done randomly or alternately. The alternation procedure is sometimes not followed when an abnormality is found which would be better observed by a different technique. For example, a specimen with an obvious skeletal defect would be prepared for skeletal examination. For identification of rodent bones, the atlas of Yasuda and Yuki [10] may be consulted. Alternatively, all rodent fetuses may be freshly dissected [1],[5] to discover soft-tissue abnormalities, then fixed and examined for skeletal anomalies.

Each rabbit fetus should be examined for both soft-tissue and skeletal malformations and variations. The bodies should be evaluated for soft-tissue anomalies by fresh dissection, followed by fixation and an examination for skeletal anomalies. Internal head structures should be evaluated in at least one-half of the fetal heads of rabbit fetuses. This evaluation should include at least the eyes, brain, nasal passages, and tongue.

10. Histopathology

When a developmental toxicity study is performed as a stand-alone study, there is no need to perform histopathology unless abnormalities are noted in the organs at the time of Cesarean section.

11. End Points Measured

Because the maternal animal is treated during gestation rather than the developing organism, data should be calculated as incidence per litter or as the number and percent of litters with particular end points. The degree of maternal toxicity may be useful in assessing the relevance of any embryotoxicity or fetotoxicity observed in the treated groups. Parameters used to measure maternal toxicity include body weight and adjusted body weight, feed and fluid consumption, daily clinical observations, and necropsy data such as organ weights.

If treatment is given throughout gestation, implantation may be affected. If, however, treatment begins after implantation, conception and implantation rates should be the same in control and treated groups. End points to be measured per litter should include the number of implantations, *corpora lutea*, live fetuses (and with separate sexes), dead fetuses, and resorbed fetuses. For litters with live fetuses, mean male and female body weights and the incidence per litter of all divergences from normal fetal development (skeletal and visceral analysis) should also be reported.

12. Analysis of Data

Values from control and test groups of animals should be compared statistically. The following techniques may be used, but others may be substituted if they are appropriate. Maternal body weights may be compared by analysis of co-variance after adjustment for initial body weight, and then analyzed by protected least significant difference tests. Fetal body weights may be evaluated using nested analysis of variance. Anomalies in litters may be compared by Fisher's exact test. Fetal survival and incidence of abnormalities per litter may be compared by analysis of variance after the data have been transformed by use of the Freeman-Tukey arc-sine transformation.

D. Reporting the Results of Developmental Toxicity Studies

Reports of all studies should contain the information required by the Good Laboratory Practice Regulations, including a copy of the study protocol and all amendments, absolute values for all parameters, complete data (individual pups) and tables of data summarized and analyzed by litter. Because the maternal animal is treated during gestation rather than the developing organism, data should be calculated as incidence per litter or as number and percent of litters with particular end points. The dosage rate of the test substance (doses) should be reported as mg/kg/day (milligrams of test substance per kilogram of body weight per day).

Problems commonly encountered in the review of developmental toxicity studies include insufficient numbers of pregnant animals per control or treatment group, non-random selection procedures, and statistical analyses of data on a per-fetus basis instead of a per-litter basis. Careful consideration of recommended guidelines and a review of protocols by the Agency before studies are conducted should help eliminate such problems.

Relevant historical control data may be used to increase the understanding of the study results. When used, historical data should be compiled and presented in an appropriate manner with additional information, such as dates of study, strain of animals, vehicle, and route of administration.

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Redbook 2000: IV.C.10 Neurotoxicity Studies

July 2000

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter IV.C.10. Neurotoxicity Studies

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I. Summary

This chapter defines neurotoxicity and the broad spectrum of adverse effects to the nervous system that may occur in the adult and developing organism. Emphasis is placed on the need to effectively minimize the risk of human neurotoxicity by assessing the neurotoxic potential of food ingredients. The chapter proceeds with explicating the nature and extent of information needed for an assessment of neurotoxic potential and suggests a strategy for obtaining this information as a routine part of the toxicological testing to evaluate the safety of chemicals proposed for use as food ingredients. Consistent with the basic strategy advocated by FDA for toxicological testing, the assessment of neurotoxic potential would be most effectively carried out through a structured process of tiered testing in which chemicals are initially screened for signs of neurotoxicity as part of those toxicity studies recommended for entrance-level testing of proposed food ingredients . Chemicals identified as possible neurotoxicants become candidates for subsequent special neurotoxicity testing designed to confirm and characterize the scope of nervous system involvement and to determine dose-response characteristics, including a quantitative determination of the no-observed-adverse-effect level. The basic elements of a neurotoxicity screen and of special neurotoxicity testing are presented and the principle points

to consider in protocol design are discussed.

II. Background

The nervous system regulates and maintains diverse biological processes that are essential not only for survival but also for maintaining an acceptable quality of life. The proper functioning of the nervous system enables an organism to receive information from its internal and external environments and to orchestrate appropriate adaptive physiological and behavioral responses. An extensive body of data demonstrates that diverse chemical substances can alter the structure and function of the nervous system in a variety of ways with notable human health consequences[1]. Alterations that significantly compromise an organism's ability to function appropriately in its environment are considered *adverse.* **Neurotoxicity** refers to any adverse effects of exposure to chemical, biological or physical agents on the structure or functional integrity of the developing or adult nervous system. Neurotoxic effects may involve a spectrum of biochemical, morphological, behavioral, and physiological abnormalities whose onset can vary from immediate to delayed following exposure to a toxic substance, and whose duration may be transient or persistent. Depending upon their severity, some of these abnormalities may have life-threatening consequences; more commonly, they result in diminished quality of life. Neurotoxicity may result from effects of the toxic substance acting directly on the elements of the nervous system or acting on other biological systems which then adversely affect the nervous system. From a safety standpoint neurotoxic effects resulting from either a direct or indirect action of a chemical on the nervous system are important components of a chemical's toxicological profile. However, in those instances where neurotoxicity occurs secondary to some non-nervous system toxicity, the latter would typically represent the more sensitive endpoint.

In 1982, the FDA issued guidelines for toxicological testing of food ingredients[2]. Although neurotoxicity was neither explicitly discussed nor defined in these guidelines, there were certain elements included in the conventional toxicity studies which have traditionally been used to assess nervous system toxicity. In general, these included a routine pathological evaluation of neuronal tissue and cage-side observations for clinical signs of toxicity. In 1985, FDA commissioned the Federation of American Societies for Experimental Biology (FASEB) to assess the utility of these current FDA guidelines for detecting neurotoxic hazards.[3] One conclusion of the FASEB report was that the current guidelines are too broad and nonspecific with respect to the nature and extent of information which needs to be provided to the FDA for an evaluation of a chemical's neurotoxic potential. The limited information derived from conventional toxicity screening studies, as currently conducted and reported, enables little more than the detection of clearly evident nervous system toxicity associated with general neuropathology and overt neurological dysfunction. Little consistent or systematically documented information is typically available about other possibly less severe, but equally important, types of neurotoxic effects including, for example, behavioral and physiological dysfunction and developmental neurotoxicity. Incomplete documentation about the range of adverse effects to the structural and functional integrity of the nervous system limits the effective evaluation of the full spectrum of neurotoxic hazards.[4] The present FDA guidelines are intended to explicate more clearly the nature and extent of information deemed necessary for the assessment of neurotoxic potential and to suggest a strategy for obtaining this information as part of the safety evaluation process.

Until recently, neurotoxicity was equated with neuropathy involving frank neuropathological lesions or overt neurological dysfunctions, such as seizure, paralysis or tremor. Examples of chemically induced neuropathy in humans (for example, from exposure to domoic acid, lead, organic mercury, hexane, carbon disulfide, and tri-ortho-cresylphosphate) emphasize the need for assessing the neurotoxic potential of chemicals to which humans may be exposed.[5] Although neuropathy is appropriately recognized as a manifestation of neurotoxicity, it is now clear that there are numerous other endpoints which may signal nervous system toxicity.[6] Ongoing research on nervous system toxicity continues to reveal the diversity of biochemical, structural, and functional abnormalities that toxicants can elicit, both directly and indirectly.[7] Neurotoxic chemicals invariably initiate their effects at the molecular level, altering cellular neurochemical processes. The qualitative nature of these alterations or their magnitude may be such as to result in cytoarchitectural changes and neuropathological effects accompanied by nervous system dysfunction expressed as physiological or behavioral abnormalities.[8] Motor incoordination, sensory deficits, learning and memory impairment, changes in emotion, and altered states of arousal in the adult and the developing organism are examples of deficits recognized as functional indices of possible neurotoxicity. Notably, physiological or behavioral dysfunctions may occur prior to, or even in the absence of, evident neuropathology or other signs of toxicity.[9] This is exemplified by the behavioral dysfunctions associated with exposure to such neuroactive chemicals as barbiturates, amphetamines, ethanol, lead, and carbon monoxide at exposure levels that elicit little or no

apparent signs of neuropathy.[10] This dissociation of neuropathology and functional changes may involve a number of factors, including the intrinsic toxicity of a chemical and, particularly, the dose and regimen of exposure. Continued reliance on neuropathy as the primary criterion of neurotoxicity is overly simplistic and does not adequately reflect contemporary concerns about the broader spectrum of potential neurotoxic effects on the adult and developing organism.

Among the various approaches that can be used for assessing neurotoxicity, behavioral testing in conjunction with neuropathological evaluation represent a practical means of obtaining a relatively comprehensive assessment of the functional development and integrity of the nervous system within the context of a standard toxicity study.[11] Behavior is an adaptive response of an organism, orchestrated by the nervous system, to some set of internal and external stimuli. A behavioral response represents the integrated end product of multiple neuronal subsystems including sensory, motor, cognitive, attentional, and integrative components, as well as an array of physiological functions.[12] As such, behavior can serve as a measurable index of the status of multiple functional components of the nervous system. Since behavioral testing is non-invasive, it can be applied repeatedly for longitudinal assessment of the neurotoxicity of a test compound, including persistent or delayed treatment-related effects.[13] Furthermore, since neuronal function can be influenced by the status of other organ systems in the body (*e.g.*, cardiovascular, endocrine, and immunologic systems), certain types of behavioral changes may indirectly reflect significant primary toxicity in other organ systems. For this reason it is important to emphasize that the assessment of neurotoxicity necessitates an integrated interpretation of all toxicologic data.

Behavioral testing has been established as a reliable toxicological index in safety assessment. Considerable progress has been made in the standardization and validation of neurobehavioral and neurodevelopmental testing procedures.[14] As a result, a variety of behavioral methodologies is available for use in determining the potential of chemical substances to affect adversely the functional integrity of the nervous system in adult and developing organisms.[15] Behavioral testing can be readily incorporated into toxicity testing protocols and, together with neuropathological evaluation, can enhance the ability to assess neurotoxic hazard[16].

Because of the impact that nervous system toxicity can have on human health, assessing the neurotoxic potential of a chemical proposed for use as a food ingredient should be an essential element in that chemical's toxicological profile.[17] Current scientific technology provides ample means of effectively assessing neurotoxic potential of chemical substances[18]. To effectively minimize the risk of potential neurotoxicity in humans, it is important that the best available science be used to develop the necessary information. It should be clear that neurotoxic effects identified in experimental animal models may not always compare exactly with what may occur in humans. Nonetheless, these effects are still interpreted as being indicative of treatment related effects on the nervous system and predictive of possible adverse health effects in humans. As advances in the neurosciences continue to evolve, our understanding of the processes underlying neurotoxicity will become increasingly clear. This will enhance our ability to assess neurotoxicity in a manner that is more predictive of potential human risk and to apply the available neurotoxicological information more reliably in support of regulatory decisions.[19]

III. Evaluating Neurotoxicity

The reliability of assessing the full spectrum of neurotoxic potential for a test substance is directly related to the extent to which the detection and evaluation of neurotoxicity is explicitly included as a specific, defined objective of routine toxicity testing.[20] Consistent with the basic strategy advocated by the FDA for toxicological testing and with the recommendations by expert committees, scientific panels and health-related organizations, the assessment of neurotoxic potential is most efficiently carried out through a structured process of tiered testing.[21] Each stage of testing would focus on different aspects of assessment. In the first stage of testing chemicals would be initially *screened* across a range of dose levels for any clinical or pathological signs of toxicity, including those involving the nervous system. Those chemicals showing evidence of adversely affecting the nervous system may be presumptively identified as candidates for subsequent *specific neurotoxicity testing* to confirm and further characterize the scope of nervous system involvement (*i.e.*, characterization of effects) and to determine dose-response kinetics (*i.e.*, dose-response determination), including a quantitative determination of the no-observed-adverse-effect level (NOAEL).

A tiered approach to neurotoxicity testing and evaluation allows for multiple decision points at which scientifically based decisions can be made about the adequacy of available information and the need for additional testing. To facilitate such decisions, specific summary statements regarding the neurotoxic potential

of the test compound should be included in the evaluation of the results of each level of testing. Since the nervous system interacts dynamically with certain other organ systems in the body, adverse effects to the nervous system should be evaluated within the context of a comprehensive assessment of all significant toxic effects for a test compound. In this regard, the neurotoxicity summary statements should reflect an integrated assessment of all relevant toxicology data which are available. This would include information derived not only from tests specifically focused on the detection of nervous system toxicity (*e.g.*, neuropathology, behavioral dysfunctions, neurochemical alterations or physiological changes), but also from more conventional toxicological testing that focuses on other measures of toxicity, for example, general organ pathology and adverse changes in growth, development, food or water intake, or endocrine status.

The neurobiological implications of some conventional endpoints of toxicity are certainly more evident than others. For example, a compound that induces specific teratogenicity of the nervous system, even at high dose levels, would be suspect for adversely affecting the development of nervous system function at lower doses. The neurotoxicological significance of other types of toxicity, however, may be less obvious. For instance, chemicals found to alter hormonal balance might also be suspected of affecting the structural or functional integrity of the nervous system, since endocrine status and the nervous system are interrelated. Altered growth, which is considered an index of general toxicity, may also signal the presence of neurotoxicity. In the developing organism, abnormal growth may reflect a treatment related neurotoxicity of the mother involving poor care of the nursing offspring. In the adult, altered growth stemming from changes in food or water intake may reflect underlying nervous system dysfunction, since both eating and drinking are consummatory behaviors with neuromuscular and physiological components under neuronal control. It should be clear, however, that such generic toxicological endpoints, by themselves, are not to be taken as evidence of neurotoxicity. Rather, when viewed in conjunction with other available data, such effects may serve to indicate the possibility of treatment related effects on the nervous system. Again, it is important to emphasize the need for integrated interpretation of all available toxicological data in the process of assessing neurotoxic potential.

A. Screening

The first stage in assessing neurotoxicity involves a process of screening to identify those chemicals that exhibit any potential for adversely affecting the nervous system. It should be clear that the primary objective of screening is detection. Chemicals identified as exhibiting a significant potential for neurotoxicity would typically be considered as a possible candidate for additional more specific neurotoxicity testing. Under such conditions, the nature and extent of information which is typically developed by screening methods would not provide a sufficient basis for determining the NOAEL for neurotoxicity. Rather, additional more specific neurotoxicity information developed in subsequent stages of testing would be needed to accurately determine the NOAEL. If significant neurotoxic potential is not identified in screening, then there would typically be neither a basis nor a need to define a NOAEL for neurotoxicity.

There are basically three sources of neurotoxicity screening information. One involves the use of structure activity relationships (SAR), the second relies on published literature and other sources of documentation, and the third involves empirical testing. The usefulness and reliability of SAR for identifying potential neurotoxicants is, at the present time, rather limited due to the fact that SAR databases for neurotoxicity are still being developed. The use of published literature or other types of documented information, to the extent that this type of information is available and appropriate for regulatory application, can be of significant value in identifying chemicals that may affect the nervous system. However, this type of information is usually scattered and typically not available for many food ingredients. At the present time, the primary means of obtaining neurotoxicity screening data is through empirical testing. The experimental data needed to screen chemicals for potential neurotoxicity should be routinely obtained as part of those toxicity studies recommended for "entrance-level" testing of proposed food ingredients. Neurotoxicity screening information could be developed most appropriately in short-term (*e.g.*, 14 to 28-day rodent and non-rodent) studies to screen adult animals exposed to the test chemical across a range of relatively high doses for brief periods of time, in subchronic (*e.g.*, 90-day rodent and non-rodent) and long-term (*e.g.*, one-year non-rodent) studies to screen adult animals following more prolonged exposure across a range of relatively lower doses, and reproduction/developmental studies to screen for potential developmental neurotoxicity in perinatally exposed offspring. The development of neurotoxicity screening information in other types of toxicity studies (*e.g.*, chronic studies) would certainly be acceptable and encouraged.

Screening for neurotoxicity involves the use of valid, cost-effective procedures which can be carried out rapidly and routinely on large numbers of chemicals to detect the presence or absence of immediate or

delayed adverse effects on the nervous system.[22] Neurotoxicity can appear as a wide range of morphological and functional abnormalities involving the nervous system at very specific or multiple levels of its organization.[23] Under the previous guidelines for toxicity testing of proposed food ingredients the identification of neurotoxic effects was based on information derived from a general pathological evaluation of a few sections of neuronal tissue and an unstructured casual observation of test animals in their cages for overt signs of toxicity.[24] This approach focused detection on the more severe forms of neurotoxicity. To maximize the scope of detection, screening should be sufficiently comprehensive to enable the detection of a representative variety of pathological changes and functional disorders of the peripheral, central and autonomic segments of the nervous system.[25] In reproduction/developmental studies, age-appropriate neurotoxicity screening should enable the detection of treatment-related effects on the physical and functional development of the offspring.

1. Elements of a Neurotoxicity Screen

The elements of a basic neurotoxicity screen should include a specific histopathological examination, in conjunction with a systematic clinical evaluation.

- Specific histopathological examination

  A specific histopathological examination should be made of tissue samples representative of all major areas and elements of the brain, spinal cord and peripheral nervous system. Emphasis should be placed more on the carefulness of the histopathological examination of the neuronal tissue and the documentation of findings rather than on the numbers of sections used, provided that all major areas and elements of the nervous system are included. For purposes of screening, either immersion fixation or *in situ* perfusion of tissues is acceptable. Typically, the initial examination may be carried out on tissues from the control and the highest dose group. Positive findings would then be followed by examination of tissues from the other dose groups. The concept of age-appropriateness should also be considered in the morphological evaluation of the immature nervous system.[26]

- Systematic clinical evaluation

  A systematic clinical evaluation of experimental animals should be conducted inside and outside of their cages using a clearly defined battery of clinical tests and observations selected to detect signs of significant neurological disorders, behavioral abnormalities, physiological dysfunctions, and any other signs of nervous system toxicity. Typically, in addition to the animal's physical appearance, body posture and weight, the clinical screen should provide sufficient information to assess the incidence and severity of such endpoints as seizure, tremor, paralysis or other signs of neurological disorder; the level of motor activity and alertness; the animals' reactivity to handling or other stimuli; motor coordination and strength; gait; sensorimotor response to primary sensory stimuli; excessive lacrimation or salivation; piloerection; diarrhea; polyuria; ptosis; abnormal consummatory behavior; and any other signs of abnormal behavior or nervous system toxicity. To accommodate age-appropriate testing, screening for potential developmental neurotoxicity could include measures of postnatal development of representative physical landmarks (for example, body weight and development of external genitalia) and functional milestones (for example, righting reflex, startle response, and motor development) in the experimental offspring. In carrying out the functional evaluation screen, animals should be initially observed in their home cages and then removed to an open arena for the completion of the observations and manipulative testing. As appropriate, more sensitive and objective indices of neurotoxicity, such as tests of learning and memory, and quantitative measures of sensory function and motor behavior, could be included as part of the screen.[27] Further, it is important that the neurotoxicity screening information be supplemented with any other relevant toxicological findings.

2. Considerations in Protocol Design for Neurotoxicity Screening

There are a number of available publications to guide in the design and conduct of neurotoxicity screens appropriate for the adult organism[28] and for the developing and adult offspring.[29] The process of protocol design for deriving neurotoxicity screening information should include consideration of the following:

- Each testing laboratory should develop and maintain an historical database demonstrating its continuing competence in the assessment of neurotoxicity. The neurotoxicity screen should consist of valid test

methodology administered by personnel who, in compliance with GLP requirements, are adequately trained to conduct the procedures appropriately. The reliability and sensitivity of the proposed screening to be used for detecting neurotoxic effects should be documented by the availability of historical or concurrent positive control data.

- To help ensure the complete and consistent application of the neurotoxicity screen throughout a particular study, each study protocol should include a detailed description of the particular screen to be used in that study, including its composition, the test procedures to be followed, the time periods at which the screen is to be carried out, the neuronal structures to be examined, the endpoints to be used, and the methods for recording and analyzing the data. During the conduct of the studies, the detailed clinical evaluation should be carried out systematically, using a prepared checklist of tests and observations when appropriate. All experimental procedures should be documented.

- Since neurotoxicity screening is intended to be a routine part of both general and reproductive toxicity studies, the specific composition of the screen and the endpoints to be recorded should be consistent with the particular focus of the study and, specifically, be appropriate for the age (and species) of the animals to be tested. For example, to screen for potential developmental neurotoxicity, it would be appropriate for a systematic evaluation to be carried out on representative male and female offspring from each experimental litter in reproduction studies and to include measures of the ontogenetic development and maturation of representative physical landmarks (for example, body weight and development of external genitalia) and functional milestones (for example, righting reflex, startle response, and motor development) in those offspring. The evaluation of offspring during the preweaning period should be planned so as to maintain the integrity of the primary reproductive study, for example by minimizing the period of pup separation from the dams. The optional inclusion of other, more sensitive, or more objective indices of neurotoxicity, such as tests of learning/memory and quantitative measures of sensory and motor function, to supplement the basic screening of the developing and/or mature offspring would be encouraged in separate or satellite litters. The concept of age-appropriateness should also be considered in the morphological evaluation of the immature nervous system.[30] There are a number of available publications to guide the design and conduct of clinical testing appropriate for neurotoxicity screening of the adult organism[31] and for developing and adult offspring.[32]
- Testing should be carried out at representative intervals throughout the duration of the study (including, when feasible, a pretreatment baseline) to provide information about the consistency of the neurotoxic effect(s), and, as possible, about their onset, duration and reversibility.
- At the discretion of the sponsor or testing laboratory, satellite groups of animals could be used to carry out the neurotoxicity screen testing.
- A sufficient number of male and female animals from each experimental and control group should be used (as recommended in the guidelines for the primary toxicity protocols) to ensure valid statistical analyses giving consideration to the variability of the endpoints being measured. As possible, the selection of tests should afford the best level of detectability with use of the smallest number of animals. In adult studies the individual animal is routinely used as the statistical unit, whereas in developmental studies the litter is typically considered to be the appropriate statistical unit. For screening purposes, the initial histochemical examination could involve tissues from control and high dose animals. If treatment-related effects are found, the subsequent examination of tissues from the lower dose groups would be warranted.
- The experimental design should include measures to minimize inadvertent bias, for example by using random assignment to treatment groups and, as feasible, carrying out testing with the experimenters blind to treatment conditions. Appropriate procedures should be followed to control for potentially confounding variables, such as housing conditions, diet and nutritional status, circadian cycles, test to test interactions, environmental conditions, and handling. For example, in the process of screening for potential developmental neurotoxicity the direct clinical evaluation of the pregnant or lactating dams should be limited to minimize influence of such handling on maternal behavior.
- To take full advantage of the neurotoxicity screening information routinely developed in toxicological testing, experimental data should be accurately recorded, documented and reported to the FDA. Summary tables of all positive effects should be presented. In addition, all data collected (positive and negative) should be submitted to the FDA to enable review personnel the opportunity of examining the actual study results. As appropriate, data should be analyzed using suitable and acceptable statistical procedures. This information, together with any other pertinent toxicity data, should be incorporated into an integrated assessment of the potential for the test chemical to adversely affect the structural or

functional integrity of the nervous system. Based on this assessment, an explicit statement should be made as to whether or not the test chemical represents a potential neurotoxic hazard which may require special neurotoxicity testing. Study protocols for additional neurotoxicity testing should be developed using valid state-of-the-art methodology.

- Throughout the process of protocol design and testing in the assessment of neurotoxic potential, the opportunity for consultation with FDA is available and encouraged.

Increasing attention is being devoted to the development of *in vitro* systems for assessing the neurotoxicological impact of chemical agents.(33) *In vitro* methods would have practical advantages, such as minimizing the use of live animals, but validation studies remain to be done to correlate *in vitro* results with neurotoxicological responses in whole animals. Such systems, once appropriately validated, may have particularly useful application in screening for potential neurotoxicity and in helping to elucidate mode of action or mechanistic information.

The information collected during screening is used to determine whether or not the test chemical represents a potential neurotoxic hazard and whether additional tests to confirm and characterize the neurotoxicity, to define NOAELs, and to develop other necessary information should be recommended. A number of considerations enter into the scientific interpretation of the neurotoxicity screening information when making this evaluation. These include the adequacy and completeness of the screening assessment; the nature and severity of the effects detected; consistency of effects across dose; consistency of effects across testing intervals within a study; replicability of effects across different types of toxicity studies; presence of other toxic effects; and the margin of difference between the doses producing neurotoxicity and those producing other toxic effects. The extent to which screening provides the information to address these issues adds to the level of confidence in identifying a potential neurotoxic hazard and aids in determining the need to proceed from screening to the development of more comprehensive neurotoxicity information. The decision to proceed with such specialized neurotoxicity testing should be made in consultation with the FDA.

B. Special Neurotoxicity Testing

When a chemical is presumptively identified by SAR, empirical screening, or other sources of information as producing neurotoxicity, that chemical becomes a candidate for additional neurotoxicity testing. Chemicals not identified as having neurotoxic effects during screening will generally not be recommended for subsequent neurotoxicity testing, although exceptions may occur. Special neurotoxicity testing focuses on the characterization of the neurotoxic effects and the determination of dose-response relationships:

1. Characterization of Effects

Following the presumptive identification of chemicals that adversely affect the nervous system, the next level of testing focuses attention on determining the nature and extent to which the nervous system is affected by that chemical (characterization). At this level the neurotoxic effects found during screening are further characterized and studies are conducted to determine whether the test chemical has any other, possibly more subtle, effects on the structural and functional integrity of the nervous system in mature and developing organisms. The in-depth assessment of neurotoxicity at this stage of testing should include information about the nature and severity of effects, the temporal pattern of onset of effects (particularly when delayed neurotoxicity occurs), and the duration of effects. To enhance detection of subtle neuropathological findings, tissues should be perfusion-fixed *in situ* and a detailed histopathological examination (more thorough than the histopathology examination performed during screening) should be carried out involving the use of special stains to highlight relevant neural structures.(34)

The neurofunctional assessment at this level should routinely include a core battery of behavioral and physiological tests designed to detect adverse changes to the primary subfunctions (*e.g.*, cognitive, sensory, motor, and autonomic) of the nervous system in the mature and developing nervous system.(35) The need for additional special tests may logically follow from information obtained during screening; for example, if a chemical is observed to induce convulsions during screening, the seizure potential and pro-convulsant properties of that chemical should be more specifically characterized during the second level of testing.

2. Dose-Response Relationships

A critical element used in defining a chemical's neurotoxic hazard is the no-observed-adverse-effect level (NOAEL), typically using the most relevant and sensitive endpoint identified in previous testing. To enable a more quantitative determination of the NOAEL, ample data should be obtained to thoroughly characterize the dose-response and dose-time relationships in repeated exposure studies, *e.g.*, intermittent and continuous exposure regimes, typically using the most relevant and sensitive endpoint.

The protocols for special neurotoxicity testing, which should be designed in consultation with FDA, should take into consideration elements similar to those involved in the development of protocols for neurotoxicity screening, including the appropriateness and reliability of the test procedures, the suitability of the control measures, and the adequacy of the experimental design and schedule of testing (frequency and duration). Consistent with the guidelines for the primary toxicity testing protocols, special neurotoxicity testing would initially be carried out using rodents as the principal species of choice. However, as appropriate and in consultation with FDA, neurotoxicity studies using non-rodent species may be recommended, on a case-by-case basis, to develop information needed for more reliable cross-species extrapolation of data.[36]

At the stage of special neurotoxicity testing, efforts to develop additional relevant information for a more comprehensive assessment of neurotoxic hazard are certainly encouraged. For example, information regarding the occurrence of treatment-related neurochemical changes, the pharmacokinetic properties of the test compound, or the factors that may modulate the sensitivity of the organism to the test compound could contribute to a better understanding of the neurobiological processes underlying the chemically induced neurotoxicity. This mechanistic type of information would enable a more reliable interpretation of the available animal data for predicting neurotoxic risk in humans.

Endnotes

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1. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/Redbook/default.htm

Chapter V

Additional Recommended Studies

A. Introduction

The Agency recognizes that information about metabolism and pharmacokinetics, neurotoxicity, and immunotoxicity are significant endpoints in assessing the safety of direct food additives and color additives used in food. Recommended strategies for improving the ability to determine metabolism and pharmacokinetics and the neurotoxic and immunotoxic potentials of test substances are described in **Chapters V B**, **C**, and **D**, respectively. Because this chapter addresses toxicity studies that are recommended for the first time by FDA for assessing the safety of direct food additives and color additives used in food (see **Figure 4**, **Chapter III C 1**), they are discussed in greater detail than other recommended toxicity studies (see **Chapter IV C**).

1. Metabolism and Pharmacokinetics

FDA believes that data from studies on the adsorption, distribution, metabolism, and excretion of a chemical can provide insight into mechanisms of toxicity and are essential in the design and evaluation of results from other toxicity studies. Such data should be provided for all direct food additives and color additives used in food that are assigned to Concern Levels II or III. Recommendations for obtaining data on the metabolism and pharmacokinetics of these substances are presented in this document. In general, the Agency recommends that this information be obtained before subchronic and chronic toxicity tests are begun.

2. Neurotoxicity

It is recommended that the assessment of neurotoxic potential be carried out according to a process of tiered testing progressing from the identification of chemicals associated with neurotoxic effects (**screening**), through a characterization of the scope of nervous system involvement (**characterization of effects**), to the determination of dose response kinetics which includes the definition of the no-observed adverse effect level (**dose-response**). Screening for neurotoxic effects, which is considered to be one of the most critical steps in this tiered process, should be routinely and systematically carried out in short-term (see **Chapter IV C 3**), subchronic (see **Chapter IV C 4**), and reproductive and developmental toxicity (see **Chapter IV C 9**) studies. The neurotoxicity screen should include a specific histopathological examination of representative tissue samples of all major areas of the brain, spinal cord, and peripheral nervous system in conjunction with a functional evaluation battery of quantifiable observations and manipulative tests selected to detect signs of neurological, behavioral, and physiological dysfunctions. References to published literature that can guide the petitioner in selecting an appropriate neurotoxicity screen are included.

Study reports should include an integrated assessment of the potential for the test chemical to adversely affect the structural or functional integrity of the nervous system. This assessment should include results of the neurotoxicity screen and other toxicology data, as appropriate. Based on the assessment, an explicit statement should be made as to whether or not the test chemical represents a potential neurotoxic hazard which requires special testing. Recommendations about further neurotoxicity testing, if the results of the initial screens indicate the need for such testing, are included. However we urge petitioners to consult with Center scientists before undertaking additional neurotoxicity tests.

3. Immunotoxicity

An immunotoxicity screen should be routinely carried out in short-term (see **Chapter IV C 3**), subchronic (see **Chapter IV C 4**), and reproductive and developmental toxicity studies (see **Chapter IV C 9**). This screen consists of primary indicators of immunotoxicity described in **Chapter V D 3**; these indicators are a set of hematological, serum protein, histopathological, and body and organ weight endpoints that are routinely evaluated in standard toxicity tests.

Study reports should include an integrated assessment of the potential for the test chemical to adversely affect the immune system. This assessment should be based on results of the immunotoxicity screen (primary indicators of immunotoxicity) and other toxicology data, as appropriate. Based on the results of this assessment, an explicit statement should be made as to whether or not the test chemical represents a potential immunotoxic hazard which requires additional immunotoxicity testing (see **Chapter V D 4** and **5**).

If results of the immunotoxicity screen indicate the need for further testing, information that will help the petitioner choose additional immunotoxicity tests is provided. However, we urge petitioners to consult with Center scientists before undertaking additional immunotoxicity tests.

V B. Metabolism and Pharmacokinetic Studies

Results from animal toxicity studies are used by FDA to determine dose-response characteristics for any effects observed in the evaluation of the safety of food and color additives. Since the delivered dose of a substance to any affected tissue or organ is determined by the pharmacokinetics and metabolism of the substance in the test animal, toxicity studies are more easily interpreted, likely to achieve target doses, and avoid excessive toxicity if data from metabolic and pharmacokinetic studies are available during the planning of short-term, subchronic and/or chronic toxicity studies. Early determination of metabolic pathways and the rates of metabolism in different test species may provide explanations for species differences in any effects which are observed, and suggest biochemical or pharmacologic experiments which might be used to test explanations of such phenomena.

The Agency recommends that petitioners submit data that will enable our scientists to evaluate: 1) the extent of absorption, 2) tissue distribution, 3) pathways and rates of metabolism, and 4) rate(s) of elimination of the parent substance and any metabolites formed for all Concern Level II and III substances (see **Figure 3**). The Agency may recommend submission of additional metabolic and pharmacokinetic data based on the extent to which a chemical is metabolized, the potential toxicity of the metabolites, and the extent to which observed toxic effects seem to correspond to the presence of the parent substance or its metabolites.

1. Considerations in the Design of, Analysis of, and Use of Data from Metabolic and Pharmacokinetic Studies

Pharmacokinetic data can be used to predict plasma concentrations, target tissue doses, and the fate of the administered dose. This information can then help the petitioner and/or the Agency: 1) decide which toxicity studies should be conducted, 2) select doses for chronic toxicity and carcinogenicity studies, 3) determine the mechanism of toxicity and assist in the interpretation of toxicity data, and 4) improve the risk assessment process.

a. Design and Analysis of Metabolic and Pharmacokinetic Studies

Pharmacokinetic studies are most useful when they are performed early in the process of evaluating the toxicity of a chemical. However, additional metabolism and pharmacokinetic studies may be recommended after target organs have been identified in toxicity studies.

Whole animal (oral dosing) studies should be performed to determine gastrointestinal absorption and overall elimination rates for a compound. However, it is often most efficient to perform *in vitro* studies of metabolism before whole animal (oral dosing) studies to determine whether enzyme kinetics may explain known dose response curves or predict non-linear dose response curves. The results of early *in vitro* studies also can be used to optimize the choice of doses in whole animal pharmacokinetic studies.

Additional recommendations concerning the design and analysis of metabolism and pharmacokinetic studies are described below.

i. Test Compound

In selecting the dosage form of a test compound to be administered in metabolic and pharmacokinetic studies, the chemical characteristics of the compound and its route of administration should be considered. The formulation of the test substance used for metabolic and pharmacokinetic studies should exhibit similar patterns of disintegration and/or dissolution as formulations used for toxicity studies. Chemical purity of the test compound should be established; impurities that may affect absorption, distribution, metabolism and excretion of the test compound should be identified. Stability of the compound in its carrier (*i.e.*, food, water, or solvent) also should be determined. Chemical characteristics of the compound (*i.e.*, low solubility, volatility) may make certain routes of administration impossible. It is critical that the dose absorbed into tissues be determined especially in studies where the test substance is added to the feed or water and is ingested *ad libitum*.

Use of radioactive substances facilitates mass balance determinations because radio-labels are relatively easy to detect in samples of tissues and body fluids. Determining the disposition pattern of the radio-label may be adequate for predicting doses that should be used in toxicity studies where the results of a test animal's overall exposure to the substance (parent compound and metabolites) is of concern. The radio-label should not be biologically labile; when a radioactive element is present at more than one position of the test compound, the radio-label should be uniformly distributed in the molecule.

The radiochemical purity of the test substance (radioactivity actually associated with the compound being tested) is another important consideration. If the test compound is not radiochemically pure and radio-labeled impurities are not identified, and if only the distribution of the radio-label in tissues and body fluids is determined, interpretation of the results may be difficult. For example, for a compound that is 95-96% radioactively pure and minimally absorbed (*i.e.*, approximately 2% absorbed), it is impossible to unequivocally differentiate between 2% absorption of the test compound and 100% absorption of a radioactive impurity present at 2%.

ii. Animals

Metabolic and pharmacokinetic data from two rodent species (usually the rat and mouse) and a non-rodent species (usually the dog) are recommended. If a dose dependency is observed in metabolic and pharmacokinetic or toxicity studies with one species, the same range of doses should be used in metabolic and pharmacokinetic studies with other species. If human metabolism and pharmacokinetic data also are available, this information should be used to help select test species for the full range of toxicity tests, and may help to justify using data from a particular species as a human surrogate in safety assessment and risk assessment. (Human metabolism studies should be conducted according to the guidelines in **Chapter VI B**.)

Metabolism and pharmacokinetic studies have greater relevance when conducted in both sexes of young adult animals of the same species and strain used for other toxicity tests with the test substance. The number of animals used in metabolism and pharmacokinetic studies should be sufficient to reliably estimate population variability (see **Chapter V B 1 e**). A single set of intravenous and oral dosing results from adult animals, when combined with some *in vitro* kinetic results, may provide an adequate data set for the design and interpretation of short-term, subchronic and chronic toxicity studies.

Studies in multiple species may clarify what appear to be contradictory findings in toxicity studies (*i.e.*, equal mg/kg bw doses having less effect in one species than in another). If disposition and metabolite profiles are found to be similar, then differences in responses among species could more reliably be attributed to factors other than differences in metabolism. Studies of the pharmacokinetics and metabolism of a substance in neonatal and adolescent animals provide information about any changes in metabolism associated with tissue differentiation and development. Animals with fetuses of known gestational age should be used for determining the disposition of the test substance in the fetus.

iii. Route of Administration

The most critical parameters required in assessing human exposure and target tissue dose are the gastrointestinal absorption rate and internal elimination rates (renal and hepatic) for the test compound. Without an intravenous (IV) dosing study, it is very difficult to determine what percentage of a chemical is absorbed, because the material excreted in the feces is composed of unabsorbed dose plus biliary and non-biliary (mucosal) elimination.

An intravenous study can provide accurate rates of metabolism-- without interference from intestinal flora--plus rates of renal and biliary elimination, if urine and bile are collected. This route also avoids the variability in delivered dose associated with oral absorption and ensures that the maximum amount of radiolabel is excreted in the urine or bile for purposes of detection. Once IV data and parameters are available, they can be used with plasma concentrations from limited oral studies to compute intestinal absorption via the ratio of Areas Under the (plasma and or urine) Curves or via simulations of absorption with gastrointestinal absorption models.

In single-dose pharmacokinetic studies of oral absorption, the primary concerns are with the extent of absorption and peak plasma or target tissue concentrations of the test substance. If the test vehicle affects gastric emptying, it may be necessary to use both fasted and non-fasted animals for pharmacokinetic studies.

iv. Dosage Regimen

Selection of the dosing regimen for metabolism and pharmacokinetic studies depends on the type of information that is needed. Metabolic and pharmacokinetic parameters are usually determined following a single administration of the test compound. Comparing parameters obtained from studies in which a range of single doses have been administered can be used to determine the doses at which saturation of absorption, distribution, metabolism or excretion occurs. Multiple dosing studies can be used to determine the potential of a compound to induce or inhibit its absorption, distribution, metabolism or excretion. Identification and quantification of the major metabolites following administration of single and multiple doses may indicate whether saturation or induction of a particular biotransformation pathway can occur.

In vitro experiments may be useful in screening for dose dependencies, and provide more accurate descriptions of the enzyme kinetics or other processes underlying dose dependencies observed in the whole animal. *In vitro* studies usually indicate identical metabolic pathways and metabolism rates comparable to those obtained from whole animal studies but require fewer animals to perform and can be completed in less time with fewer resources. [1-3]

v. Sampling

Blood (RBCs, plasma, and serum), urine, and feces are the most commonly collected samples. In addition, a few representative organ and tissue samples should be taken, such as liver, kidney, fat, and suspected target organs. Sampling times should depend on the substance being tested and the route of administration. In general, an equal number of blood samples should be taken in each phase of the concentration-versus-time curve. Intravenous (IV) studies usually require much shorter, and more frequent, sampling than is required for oral dosing. Time spacing of samples will depend on the rates of uptake and elimination. In a typical IV study, blood and tissue samples are taken in a "powers of 2" series, *i.e.* samples at 2, 4, 8, 16, and 30 (32) minutes, 1, 2, 4, 8, and 16 hours. Similar coverage could be obtained with only 7 time points by using a "powers of 3" series: 3, 9, and 30 (27) minutes, 1, 3, 9, and 24 (27) hours. Oral dosing studies usually extend to at least 72 hours, or 5 plasma half-lives, ensuring the excretion of 95% of the absorbed dose. The sampling schedule for an oral dosing experiment might be: 15 minutes, 30 minutes, 1, 2, 4, 8, 24, 48, and 72 hours. Such a sampling scheme would provide data coverage for evaluation of absorption, elimination, enterohepatic recirculation and excretion processes.

Whole Body Autoradiography (WBA) has been used with increasing frequency as a means of identifying tissues which concentrate test substances. This technique allows a small number of animals (5 - 10) to be used for screening purposes with a minimal investment in manual labor. FDA encourages the use of WBA with IV dosing,

as a means of screening and selecting tissues of greatest relevance for later oral dosing studies. Animals used for WBA should be sacrificed during the elimination phase, between 1 and 5 plasma half-lives, since bioaccumulation at steady-state is the primary consideration in selecting specific tissues.

The number of animals used in metabolism and pharmacokinetic studies should be large enough to reliably estimate population variability. In the case of rats and mice, tissue and/or blood sample size is usually the limiting factor: analysis of the substance may require 1 ml or more blood, but it is difficult to obtain multiple blood samples of this size from one animal. As a consequence, a larger number of animals is required (3 - 4 per time point, 7 - 9 time points) when small rodents are used. Such an approach has the advantage of allowing limited sampling of critical tissues (*e.g.* liver, fat) at each time point, an option which is usually unavailable with large animals. The use of humans and large animals generally permits collection of multiple (serial) blood samples. For outcrossing populations like humans and large animals, individual differences in the rates of biotransformation are likely to be greater than those of inbred rodent populations; under these circumstances, more samples/sex/group may be needed to reliably estimate variability.

Individual metabolism cages are recommended for collecting urine and feces in oral dosing studies. Excreta should be collected for at least 5 elimination half-lives of the test substance. When urine concentrations will be used to determine elimination rates, sampling times should be less than one elimination half-life (taken directly from the bladder in IV studies); otherwise, samples should be taken at equal time intervals.

vi. *In Vitro* studies

In Vitro measurements employing enzymes, subcellular organelles, isolated cells and perfused organs may be used to augment the dose response information available from less extensive metabolic and pharmacokinetic studies. Because *in vitro* systems generally are less complex than whole animals, elucidation of a test compound's metabolic pathways and the pathways' kinetic characteristics may be facilitated. Such systems can be used to measure binding, adduct and conjugate formation, transport across cell membranes, enzyme activity, enzyme substrate specificity, and other singular objectives. Biochemical measurements that can be made using *in vitro* systems include: Intrinsic clearances of enzymes in an organ or tissue, kinetic constants for an enzyme, binding constants, and the affinity of the test compound and its metabolites for the target macromolecules. The activity of a hepatic drug-metabolizing enzyme *in vivo* may be approximated by kinetic constants that are calculated from *in vitro* studies; when a first-order approximation is used, the ratio of V_{max} to K_m is equal to the intrinsic clearance of the drug. [4,5] *In vitro* measurements made using readily accessible tissues and body fluids from animals and man may also be useful in elucidating mechanisms of toxicity.

vii. Analysis of Data

Data from all metabolism and pharmacokinetic studies should be analyzed with the same pharmacokinetic model and results should be expressed in the same units. Concentration units are acceptable if the organ or sample size is reported, but percent of dose/organ is usually a more meaningful unit. In general, all samples should be analyzed for metabolites that cumulatively represent more than 1% of the dose.

A variety of rate constants and other parameters can be obtained from IV and oral dosing data sets, provided that good coverage of the distribution, elimination, and absorption (oral dose) phases is available. Typical parameters calculated to characterize the disposition of a test substance are: half-lives of elimination and absorption; area under the concentration-versus-time curve (AUC) for blood; total body, renal and metabolic clearances (Cl); volume of distribution (V_d); bioavailability (F); and mean residence and absorption times (MAT, MRT). Some of these parameters, such as half-lives and elimination rates, are easily computed from one another; the half-life is more easily visualized than the rate constant. [6,7]

Computation of oral absorption (k_a) and elimination (E) rates is often complicated by the "flip-flop" of the absorption and elimination phases when they differ by less than a factor of 3. [8] Because of these analysis problems, computation of absorption and elimination rates should not be attempted on the basis of oral dosing

results alone.

Blood-tissue uptake rates (k_{j1}) can often be approximated from data at early ($t < 10$ minutes) time points in IV studies, provided that the blood has been washed from the organ (*e.g.* liver) or the contribution from blood to the tissue residue is subtracted (fat). High accuracy is not usually required since these parameters can be optimized to fit the data when they are used in more complex models. Tissue-blood recycling rates (k_{1j}) and residence times can be computed from partition coefficients if estimates of uptake rates are available.

Tissue/blood partition coefficients (R_{j1}) should be determined when steady-state has been achieved. Estimates based on samples obtained during the elimination phase following a single dose of the test substance may lead to underestimates of this ratio in both eliminating and non-eliminating tissues unless its half-life is very long. Correction of these values for elimination has been described by several authors. [9,10]

It may be important to determine the degree of plasma protein and red blood cell binding of the test substance; calculation of blood clearance rates using plasma or serum concentrations of the substance that have not been adjusted for the degree of binding may under- or over-estimate the true rate of clearance of the test substance from the blood. This is usually done through experiments *in vitro*.

Two classical methods used in the analysis of pharmacokinetic data are the fitting of sums of exponential functions (2- and 3-compartment mammillary models) to plasma and/or tissue data, and less frequently, the fitting of arbitrary polynomial functions to the data (non-compartmental analysis).[8,11,12]

Non-compartmental analysis is limited in that it is not descriptive or predictive; concentrations must be interpolated from data. The appeal of non-compartmental analysis is that the shape of the blood concentration-versus-time curve is not assumed to be represented by an exponential function and, therefore, estimates of metabolic and pharmacokinetic parameters are not biased by this assumption. In order to minimize errors in parameter estimates that are introduced by interpolation, a large number of data points that adequately define the concentration-versus-time curve are needed.

Analysis of data using simple mammillary, compartmental models allows the estimation of all of the basic parameters mentioned above, if data for individual tissues are analyzed with 1 or 2 compartment models, and combined with results from 2 - 3 compartment analyses of blood data. "Curve Stripping" analysis can be applied to such simple models through the use of common spreadsheet programs (*i.e.* LOTUS 1-2-3), as long as a linear regression function is provided in the program. Optimization of the coefficients and exponents estimated may require the use of more sophisticated software: a number of scientific data analysis packages such as RS/1 and SigmaPlot have the necessary capabilities. Specialized programs such as NONLIN [13], CONSAM,[14] or SIMUSOLV [15] will be needed when more complex models must be analyzed. Coefficients and exponents from mammillary models can be used to calculate other parameters; however, they should not be taken too literally, since mammillary models assume that all inputs are to a central pool (blood), which communicates without limitation into other compartments.[16,17] This approach does not include details such as blood flow limitations, anatomical volumes or other physiological limits in the animal.

Physiologically based pharmacokinetic models (PB-PK) were developed to overcome the limitations of simple mammillary models. Physiologically based models describe the disposition of test substances via compartmental models which incorporate anatomical, biochemical and physiological features of specific tissues in the whole animal. The types of information added include organ-specific blood flows, volumes, growth models and metabolism rates. Metabolic parameters often are obtained from *in vitro* studies (*i.e.*, enzyme reaction rates in cultured hepatocytes, plasma protein binding, *etc.*), while other parameters are becoming available as standard parameters in the literature. Parameters from mammillary models can be used to compute the value of parameters used in physiological pharmacokinetic models, using tissue-specific blood flows, anatomical volumes, and other information (literature values). Estimation of parameters for a simple mammillary model is often the first data reduction step in creating a physiological model.[5,18]

Because PB-PK models are based on physiological and anatomical measurements and all mammals are inherently similar, they provide a rational basis for relating data obtained from animals to humans. Estimates of

predicted disposition patterns for test substances in humans may be obtained by adjusting biochemical parameters in models validated for animals; adjustments are based on experimental results of animal and human *in vitro* tests and by substituting appropriate human tissue sizes and blood flows. Development of these models requires special software capable of simultaneously solving multiple (often very complex) differential equations, some of which were mentioned above. Several detailed descriptions of data analysis have been reported.[7,19]

b. Use of Data from Metabolism and Pharmacokinetic Studies

Information from metabolism and pharmacokinetic studies can be used in the design and analysis of data from other toxicity studies. Some examples are described below.

Design of Toxicity Studies: The concentration-versus-time curve, peak, and steady-state concentrations of the test substance in blood or plasma provide information on the distribution and persistence of the substance in the animal which may suggest essential elements in the design of toxicity studies. For example, when metabolic and pharmacokinetic studies indicate that the test compound accumulates in the bone marrow, long-term toxicity tests should include evaluation of the test compound's effect on hematopoietic function and morphology. If a test compound is found to accumulate in milk, an investigator should plan to perform reproductive toxicity studies with *in utero* exposure and a nursing phase (cross-fostering study; see **Chapter IV C 8**). In addition, information from metabolic and pharmacokinetic studies can be used to predict the amount of test compound that enters biological compartments (tissues, organs, *etc.*) that may not suffer a toxic insult but may serve as depots for indirect or secondary exposure.

Setting Dose Levels: There is considerable debate about the use of metabolic and pharmacokinetic data in setting doses to be used in toxicity studies, particularly chronic toxicity and carcinogenicity studies. Current NTP policy for selecting the highest dose in carcinogenicity bioassays is described in **Chapter IV C 6 b**. In 1984, the NTP Ad Hoc Panel on Chemical Carcinogenesis Testing and Evaluation also recommended that pharmacokinetic data be considered along with subchronic toxicity data in setting all dose levels except the maximum-tolerated dose (MTD) in the carcinogenesis bioassay of chemicals.[20] FDA agrees with these statements and recommends that pharmacokinetic data be used in conjunction with the results of short-term and subchronic toxicity studies to set appropriate dose levels for chronic toxicity, reproduction and teratology studies, and for setting dose levels below the MTD (highest dose) in carcinogenicity studies.

Determining Mechanisms of Toxicity: Information from metabolic and pharmacokinetic studies can be used to supplement conventional toxicology data in elucidating mechanisms of toxicity. Metabolites identified by a pharmacokinetic study can suggest mechanisms underlying a toxic response. Biologically reactive intermediates are often implicated in a toxic response; however, such metabolites are usually short-lived, reacting in the vicinity of their formation. The presence of potentially reactive intermediates can be deduced indirectly by measuring the formation of characteristic macromolecular (DNA, RNA, protein) adducts and metabolic conjugates. Measurement of metabolic conjugate vs adduct formation and the affinity of a compound and/or its metabolites for the target molecule may help identify mechanisms of toxicity and effective routes of detoxification.

Information from *in vitro* test systems concerning the formation of critically reactive metabolites may be used to establish the relationship between the formation of the reactive metabolite *in vivo* and duration of exposure to the test compound. This relationship is important in circumstances where critically reactive metabolites are only formed when the capacities of normal metabolic and other defensive or adaptive mechanisms are exceeded. Determining the concentrations of the test substance at which saturation of binding occurs may indicate at what concentration a compound is likely to deplete detoxifying conjugation pools and become available to react with target macromolecules.

Improving the Risk Assessment Process: Information from metabolic and pharmacokinetic studies increasingly is being incorporated into risk assessments. Conventional risk assessments typically involve

linear extrapolation of external dose and an inter-species scale factor based on body weight or body surface area. Risks calculated by this approach may be under- or over-estimated. Many of the biological processes involved in the absorption, distribution, metabolism and excretion of a compound are dose dependent and, therefore, the toxicity observed may not be a simple function of administered dose. Development of appropriate pharmacokinetic models may enhance our ability to use metabolic and pharmacokinetic information in risk assessment.

2. Recommended Metabolism and Pharmacokinetic Studies

FDA believes that data from studies on the absorption, distribution, metabolism, and excretion of a chemical can provide insight into mechanisms of toxicity of chemicals and are essential in the design and evaluation of results from other toxicity studies. FDA believes that a set of basic pharmacokinetic and metabolism studies should be performed for all Concern Level II and III substances, but that additional studies may be recommended for a particular additive. Recommended studies should be performed with two rodent species (usually the rat and mouse) and one non-rodent species (usually the dog). In general, what constitutes an appropriate set of metabolism and pharmacokinetic studies will depend on the anticipated degree and type of toxic response to a test compound and by the estimated magnitude of human exposure to the compound. The recommended set of basic studies are:

- Intravenous studies using a tracer level dose should be conducted in adult male and female animals of species in which toxicity studies have already been conducted or in which chronic toxicity studies are contemplated. Blood, liver, and fat samples should be taken at all time points. The size and timing of urine and bile samples will depend on the dose of tracer and rate of excretion by each of these routes. Samples taken over periods of 30 min to 2 hours, at 2 or 3 time points, should be sufficient for computation of the cumulative excretion by these routes. Plasma, urine and bile should be analyzed for metabolites of the test substance that cumulatively represent more than 1% of the dose. Estimates of uptake and elimination rates should be made for each tissue sampled, using 2-compartment models.

- Studies of the rate of metabolism (of the parent compound) as a function of dose (or concentration) should be conducted *in vivo* or *in vitro*, guided by results of metabolite analyses from the intravenous studies and available toxicology information. Hepatocytes or perfused livers will normally be used for such studies, but an examination of the distribution of metabolites between the plasma, bile and urine after IV dosing may indicate that the kidney is important in the metabolism of some chemicals. Enzyme kinetic parameters resulting from *in vitro* studies may be scaled up to whole organ rates and used to predict rates of metabolism in the whole animal as a function of dose.

- Oral dosing studies should be conducted in *ad libitum* fed animals, to determine the rate and cumulative absorption of the substance. Dosage and sampling times should be selected on the basis of results from toxicity tests, metabolic dose response data (**ii**, above), and elimination rates determined from IV dosing studies. Bioaccumulative tissues should be sampled in addition to blood, urine and feces. A tissue that does not accumulate the substance should also be included for reference purposes. Whole Body Autoradiographic studies are recommended as a method for identifying bioaccumulative tissues prior to the initiation of oral dosing studies.

3. Additional Studies

Studies of enzyme induction and potential pharmacological adaptation should be conducted whenever chronic studies are recommended. The resulting information can be incorporated into multiple or continuous dosing models to simulate the plasma and tissue levels of test substance expected for a variety of doses in chronic studies being planned.

In cases where reproductive studies are recommended, pharmacokinetic experiments evaluating the

distribution of the substance in the fetus, mother's milk, and neonates should be performed as an aid in selecting doses and designing reproductive toxicity studies. If the metabolic potential of the fetal and/or neonatal liver can be assessed in a preliminary *in vitro* study, this step is highly recommended.

Assuming that IV and oral dosing studies have already been completed for both male and female adult animals prior to the reproductive pharmacokinetic studies, sampling can be more limited, ie. excretion studies combined with limited sampling of maternal blood, fetuses, milk, and neonatal tissues may be sufficient for characterization of the metabolic and pharmacokinetic processes of interest in pregnancy.

Depending on the types of toxic effects observed and the importance of understanding the mechanisms of these effects to the safety assessment of a direct food or color additive used in food, additional biochemical or *in vitro* experiments may be submitted by the petitioner in support of any mechanism proposed. Such studies should be substance-specific, and should be based on consultation with CFSAN, as appropriate.

Chapter V B: Metabolism and Pharmacokinetic Studies

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V D. Immunotoxicity Studies

Exposure to various chemicals has been associated with toxicity of the immune system in animals; these include environmental contaminants, chemicals in the occupational environment, and direct and indirect food additives. Regulatory agencies, including FDA, [1] have recognized the importance of these types of effects for assessing the safety of chemicals to which humans may be exposed. Because of the rapid emergence of the field of immunotoxicology during the past two decades and the abundance of information that has accumulated with regard to the immune system as a target organ, various federal agencies and international organizations are preparing guidelines for the conduct of immunotoxicity studies. [2-6] In addition, various testing approaches have been proposed by researchers in the field. [7-13]

1. Immunity: A Brief Review

The immune system has been described in detail in a number of excellent reviews. [14-18] Thus, only those aspects of immunity which are particularly relevant to immunotoxicity testing will be reviewed in this section. Immunological function encompasses a complex array of participating cell types and organ systems. Immunity may be defined in relation to the function of the various cellular components.

a. Humoral Immunity

Humoral immunity is defined in terms of the B-lymphocytes (B-cells), the antibody producing cells of the immune system. The B-cells, named because of their functional similarity to antibody-producing cells derived from the Bursa of Fabricius in birds, are found primarily in the spleen, lymph nodes, Peyer's patches in the gut, peripheral blood and bone marrow. The bone marrow is also the site of origination of B-cell precursors, the stem progenitor cells.

Immunoglobulins (Igs), the class of proteins that is comprised of the antibodies, are further classified with regard to particular peptide regions found on the light and heavy chains. At least five major classes of immunoglobulins have been defined for man and animals: IgA, IgD, IgE, IgG, and IgM. Antibodies function in concert with complement proteins that are produced in the liver and by macrophages to provide protection against bacterial and viral infections. Antibodies also help protect man and animals from agents that cause tumors and from some spontaneously occurring tumor cells.

Humoral immunity can be further classified with regard to the dependence of antibody production on T-lymphocyte help: T-cell dependent and T-cell independent immunities.

b. Cell-Mediated Immunity (CMI)

CMI derives its name from classical studies that demonstrated adaptive cell transfer of immunological function, graft v. host reactivity, etc. CMI is associated with the T-lymphocytes or T-cells (thymus-derived). Various classes of T-cells have been described, such as suppressors, helpers, inducers, and cytotoxic cells. Some of these T-cell types are involved in B-cell immunoregulation. T-cells secrete various peptide factors, referred to as lymphokines or cytokines, that modulate the activity of B- and T-cells. Cytotoxic T-cells participate in direct killing of invading microorganisms and tumor cells. T-cells are now commonly defined in terms of various membrane "antigens", such as T-4 (or CD4) for helper/cytotoxic cells and T-8 (or CD8) for suppressor/cytotoxic cells.

c. Non-Specific Immunity

Non-specific immunity is derived from other cell types that participate in the immune process. Natural killer (NK) cells are a group of cells that share certain properties with T-cells, but probably arise from different stem progenitor cells. [19] These cells are known to play an important role in immune surveillance against spontaneous tumor formation. They also serve as a first line of defense, in cooperation with other phagocytic leukocytes (phagocytes or granulocytes), in the destruction of invading viruses and bacteria. Macrophages (activated monocytes) play a key role in antigen processing and presentation to lymphocytes; they interact with the T- and B-cells to facilitate antibody production. These cells also secrete cytokines, such as interleukin-1, which modulate certain T-cell functions.

Modulation of host resistance to infectious organisms can be the result of either direct or indirect effects on various cell components. Reduction in host resistance is referred to as immunosuppression. Severe or prolonged immunosuppression, as manifested in acquired immunodeficiency syndrome (AIDS), can result in an overwhelming number of infections, tumor formation, and death. Immune enhancement or hyperactivity of the immune system can result in hypersensitivities, such as allergic disorders and autoimmune diseases. The mechanisms of these disorders and diseases are complex and are dependent on factors such as genetic predisposition, age, medical condition, and environment. The development of autoimmunity, which has been associated with the use of various drugs, [20] can have a pronounced toxic effect on a number of organ systems.

True allergic reactions, which are mediated mainly by IgE in man and certain animals, can result in a life-threatening condition known as anaphylactic shock. Certain food additives, such as sulfites, have been restricted in use because of their high sensitizing potential. [21] Other food chemicals have been associated with hypersensitivity-like conditions such as the toxic oil syndrome [22] and tryptophan-induced eosinophilia myalgia.[23]

2. Key Concepts in Immunotoxicity Testing

These guidelines relate to the safety assessment of direct food additives and color additives used in food; such assessments are done on a case-by-case basis. The recommendations for immunotoxicity testing of food and color additives used in food presented in this section may or may not be relevant to those of other agencies and organizations. However, certain concepts from which these recommendations derive are shared by various others[10,11,12,24] including the World Health Organization. [6] Other concepts may be unique to FDA, since these guidelines have been developed within the toxicity testing framework set forth in this book. These concepts are:

- Two types of immunotoxicity tests/procedures are defined: Type 1 Tests are those that do not require any perturbation of the test animal, such as immunization and challenge with an infectious agent.

  i) Primary indicators of immune toxicity are derived from Basic Type 1 Tests, such as hematology and serum chemistry profiles, routine histopathology examinations, and organ and body weight measurements from standard toxicity studies described in other sections of this book. Additional procedures, such as measurements of thymus weights and performance of more definitive histopathological evaluations of immune-associated organs and tissues, have been added.

  ii) Indicators of immune toxicity can also come from Expanded Type 1 Tests. These tests are logical extensions of Basic Type 1 tests; for example, Expanded Type 1 tests may extend the hematology, serum chemistry, and histopathology evaluations of standard toxicity studies. Many of these expanded tests can be performed with the same blood and tissue samples collected for the Basic Type 1 tests; in addition, many of the expanded tests can be performed retrospectively.

- Type 2 Tests include injections or exposure to test antigens, vaccines, infectious agents or tumor cells. If Type 2 tests are to be performed concurrently with a standard toxicity study, a satellite group of animals should be added to the recommended number of test animals in the study. Protocol designs for standard toxicity studies that include a satellite group of animals for Type 2 immunotoxicity tests will be

recommended when available information indicates that a test compound may present an immunotoxic risk.

- Sets of Basic and Expanded Type 1 Tests are defined as Level I Immunotoxicity Tests. Some Level I tests screen for immunotoxic effects in test animals; others focus on defining an immunotoxic effect more specifically, such as determining the mechanism or cell types involved. Analogously, sets of Type 2 tests are defined as Level II Immunotoxicity Tests; Level II tests also can be used to screen for, or more specifically define, immunotoxic effects of food and color additives used in food.

3. Indicators of Possible Immune Toxicity

Basic Type 1 Tests: Primary Indicators

The primary indicators of possible immune toxicity are derived from routine measurements and examinations performed in toxicity studies recommended in other sections of this publication (Basic Type 1 tests). Indicators derived from short-term and subchronic toxicity studies, and developmental toxicity studies with rodents are listed below. If a substance produces one or more of these primary indicators of immune toxicity, more definitive immunotoxicity tests (Expanded Type 1 tests or Type 2 tests) may be recommended; such decisions will be made on a case-by-case basis.

a. Indicators from Short-Term and Subchronic Toxicity Studies

- Hematology Indicators: Elevation or depression in white blood cell (WBC) counts; altered differential WBC counts; lymphocytosis and lymphopenia; and eosinophilia.

- Clinical Chemistry Indicators: Elevated or reduced total serum protein in combination with an abnormal albumin-to-globulin (A/G) ratio. Other indicators often associated with immunologic dysfunction include abnormal levels of liver proteins and enzymes, such as albumin and the transaminases.

- Histopathology Indicators: Abnormalities found during gross and routine histological evaluation of the lymphoid tissues, *e.g.* spleen, lymph nodes, thymus, gut-associated lymphoid tissue (GALT, in particular Peyer's patches), and bone marrow. Morphologic abnormalities such as scattered, focal mononuclear cell infiltrates in non-lymphoid organs (*e.g.* kidney and liver) may be relevant to autoimmune disease. If differences are seen in any lymphoid tissue, attention should be given to "cellularity" and prevalence of activated macrophages. The description could include *in situ* descriptions of the types of cells, density of the cell populations, lymphocyte distribution relative to distinguishing structures or defined areas of the organ. (In these instances, the effect does not need to be defined rigorously for each animal. The number of animals observed, however, should be a statistically significant sample size.) The histopathological analysis of routinely stained (hematoxylin and eosin) samples of the spleen should include descriptions of lymphocyte distribution and proliferation in known T- and B-cell areas, such as the germinal centers (for B-cells) and the periarteriolar lymphocyte sheath (PALS) for T-cells if abnormalities are observed. The histopathologic analysis of the lymph nodes and Peyer's patches should include a description of the immune activation (*i.e.* the relative number of follicles and germinal centers) when abnormalities or lesions are observed in these organs. When abnormalities of the thymus are observed, histopathologic analysis should be descriptive and quantitative as possible with regard to atrophy and necrosis and other observations. If the test compound is shown to either stimulate cell proliferation, or to cause atrophy and cell depletion in any lymphoid organ, the effect is likely to be viewed as a potentially immunotoxic effect requiring more definitive testing.

- Organ and Body Weight Indicators: Elevated or depressed spleen and thymus weights; elevated or depressed organ-to-body-weight ratios for the spleen and thymus (statistical treatment of the organ-to-body-weight ratios should include an analysis of co-variance, with body weight as the co-variant).

Elevated or depressed body weights, although primarily an indicator of endocrine function, may also indicate indirect immunotoxic effects, since endocrine function can significantly effect the immune system.

b. Indicators from Developmental Toxicity Studies

◙ Morbidity and Mortality Indicators: Unusual incidence of maternal infections.

◙ Histopathology Indicators: Abnormalities found during gross evaluation of the fetal liver, spleen, and thymus.

◙ For animals in the F_1 and F_2 generations:

i) Hematology Indicators: Elevation or depression in white blood cell (WBC) counts; altered differential WBC counts; lymphopenia and lymphocytosis; and eosinophilia.

ii) Clinical Chemistry Indicators: Elevated or reduced total serum protein in combination with an abnormal albumin-to-globulin (A/G) ratio.

iii) Histopathology Indicators: Abnormalities found during gross and routine histological evaluation of the lymphoid tissues, *e.g.* spleen, lymph nodes, thymus, gut-associated lymphoid tissue (GALT, in particular Peyer's patches), and bone marrow. Morphologic abnormalities such as scattered, focal mono-nuclear cell infiltrates in non-lymphoid organs (*e.g.* kidney and liver) may be relevant to autoimmune disease. If differences are seen in any lymphoid tissue, attention should be given to "cellularity" and prevalence of activated macrophages. The description could include *in situ* descriptions of the types of cells, density of the cell populations, lymphocyte distribution relative to distinguishing structures or defined areas of the organ. (In these instances, the effect does not need to be defined rigorously for each animal. The number of animals observed, however, should be a statistically significant sample size.) The histopathological analysis of routinely stained (hematoxylin and eosin) samples of the spleen should include descriptions of lymphocyte distribution and proliferation in known T- and B-cell areas, such as the germinal centers (for B-cells) and the periarteriolar lymphocyte sheath (PALS) for T-cells if abnormalities are observed. The histopathologic analysis of the lymph nodes and Peyer's patches should include a description of the immune activation (*i.e.* the relative number of follicles and germinal centers) when abnormalities or lesions are observed in these organs. When abnormalities of the thymus are observed, histopathologic analysis should be descriptive and quantitative as possible with regard to atrophy and necrosis and other observations. If the test compound is shown to either stimulate cell proliferation, or to cause atrophy and cell depletion in any lymphoid organ, the effect is likely to be viewed as a potentially immunotoxic effect requiring more definitive testing.

iv) Organ and Body Weight Indicators: Elevated or depressed spleen and thymus weights; elevated or depressed organ-to-body-weight ratios for the spleen and thymus (statistical treatment of the organ-to-body-weight ratios should include an analysis of co-variance, with body weight as the co-variant). Elevated or depressed body weights, although primarily an indicator of endocrine function, may also indicate indirect immunotoxic effects, since endocrine function can significantly effect the immune system.

4. Expanded Type 1 Immunotoxicity Tests

Assessing the safety of food and color additives used in food usually requires the completion of various

toxicity studies. In addition to the screen of primary indicators of possible immune toxicity provided by these toxicity studies and summarized above, additional tests for further evaluation of the immunotoxic potential of a test substance may be recommended by the Agency. The additional tests can be Expanded Type 1 Tests, discussed in this section, or Type 2 Tests, discussed in the next section. The Agency's recommendation that specific immunotoxicity tests be performed on test substances will be made on a case-by-case basis. Expanded Type 1 immunotoxicity tests include:

- Hematology Tests: Flow cytometric analysis of B-lymphocytes, T-lymphocytes, and T-lymphocyte subsets (TH + TS or CD4 and CD8); immunostaining (immunoperoxidase or immunofluorescence) of B-lymphocytes, T-lymphocytes and T-lymphocyte subsets from peripheral blood or single cell suspensions from the spleen. [25-29]

  i) Hematology Indicators: Decreased or elevated percentages of any of the various lymphocytes relative to controls and abnormalities in the B-cell/T-cell and the TH/TS (CD4/CD8) cell ratios; these should be determined from differential counts of the immunostained preparations or from cytometric analysis.

- Serum Chemistry Tests: Electrophoretic analysis of serum proteins to permit separation and quantification of the relative percentages of albumin and the α-, β-, and τ-globulin fractions; quantification of τ-globulin fractions (IgG, IgM, IgA, and IgE); analysis of total serum complement and components of complement (such as C3) from CH-50 determinations; immunochemical assay of serum cytokines, such as IL-2, IL-1, and τ-interferon; quantification of serum auto-antibodies, such as anti-nuclear, anti-mitochondrial, and anti-parietal cell antibodies.

  i) Serum Chemistry Indicators: Statistically significant variations between experimental and control groups of animals for any of the parameters listed above.

- Histopathology Tests: Immunostaining of B-lymphocytes in the spleen and lymph nodes, using polyclonal antibodies to IgG of the test animals; [30,31] immunostaining of T-lymphocytes and T-lymphocyte subsets in the spleen, using monoclonal or polyclonal antibodies to various cell markers; micro-metric measurements of germinal centers and PALS of the spleen and the follicles and germinal centers of lymph nodes; morphometric analysis of the relative areas of the cortex and medulla of the thymus, using routinely stained histopathology sections.

  i) Histopathology Indicators: Statistically significant variations between experimental and control groups of animals for any of the parameters listed above, using both analysis of variance (ANOVA) and a multiple comparison T-test, such as Dunnett's.[32]

- Tests for *In Vitro* Analysis of the Functional Capacity of Specific Cell Types:

  i) Activity of Natural Killer (NK) Cells: The functional capacity of NK cells can be measured using the classical ^{51}Cr chromium release assay; [19] this assay is well standardized and has been used successfully with both mice and rats in various immunotoxicity studies. [33-35] Of particular concern is reduced NK cell activity, which may be correlated with increased tumorigenesis and infectivity.

  ii) Mitogenic Stimulation Assays for B- and T-Lymphocytes: Certain plant lectins stimulate blastogenesis and DNA synthesis of T- and B-lymphocytes: concanavalin-A (Con-A) and phytohemagglutinin (PHA) are known to preferentially stimulate T-lymphocytes, and an extract from pokeweed (PWM) as well as certain bacterial lipopolysaccharides (LPS) and protein extracts are known to preferentially stimulate B-lymphocytes *in vitro*. Since these assays are carried out *ex vivo*, they can be performed on preparations of peripheral blood. The assays are well characterized for use in various animals species (including man[36]), can be performed on either peripheral blood or spleen-cell suspensions, and have been used in a number of immunotoxicity studies. [2,8,9,10,12,13,35,37] Both reduced and elevated levels of blastogenesis or ^{3}H

incorporation into DNA are of interest in the evaluation of the immunotoxic potential of food and color additives used in food.

iii) Phagocytotic Index of the Macrophage: Various assays to determine the phagocytotic ability of macrophages have been described. [24,38,39] These assays measure the ability of a macrophage to ingest particulate substances, such as plastic beads or iron filings, and can be performed on peripheral blood or single cell suspensions of lymphoid organs, such as the spleen. Other assays measure the capacity of the macrophage to destroy live bacteria through lysosomal enzyme activity. [40]

iv) Stem Cell Assays: Bone marrow preparations can be used to investigate the pluripotent population or specific progenitor populations. [41] Although these assays have not been used extensively in immunotoxicity evaluations, they may be recommended when histopathological evaluation indicates that the test substance may have caused changes in bone marrow.

5. Type 2 Immunotoxicity Tests

Evaluating the functional capacity of the immune system requires injecting a substance that elicits immunological reactivity in a test animal. Various antigens provide information about the types of immunity or cells that may be involved in an immune response. For example, protein antigens usually elicit T-dependent immune responses with subsequent production of antibodies to the protein. Polysaccharides elicit T-independent immune responses. Some antigens elicit cell-mediated immune responses, while immunogens such as complex bacteria and viruses may elicit humoral and cell-mediated responses. All of the antigens listed below have been tested in rodents; when an antigen has been used preferentially with a particular rodent species, this is noted.

- T-Dependent Test Antigens: One of the most widely used antigens for rodents [2,7,8,9,10,24,30,42,43] and non-rodents is sheep red blood cells (SRBC). [1,4-9,30,34,35,42,44] For example, SRBCs have been widely used in mice in the Plaque-Forming Cell Assay: [45,46] antibody-producing spleen cell suspensions are mixed with SRBCs, placed on covered slides, and incubated; each antibody-producing cell causes a small, clear area (plaque) to form on the slide; the plaques are then counted. Other T-dependent test antigens that have been widely used include keyhole limpet hemocyanin [10] and bovine serum albumin.

- T-Independent Test Antigens: Ficoll, a branched chain polysaccharide, haptenated ficoll, polyvinylpyrrolidone, and bacterial lipopolysaccharides have been used as T-independent test antigens with mice and rats. [47]

- Human Vaccines: Human T-dependent vaccines, such as tetanus toxoid, and the T-independent vaccine containing pneumococcal polysaccharide antigens have been used in both rats and mice. [48-50] It is possible to compare responses of the test species to the vaccines with human responses, because standard human sera are available from FDA's Center for Biologics. [51]

- Test Antigens for Cell-Mediated Immune (CMI) Reactivity: Contact sensitizers such as dinitrochlorobenzene (DNCB) have been used to elicit delayed hypersensitivity (DTH) responses as a measure of CMI in animals. These assays can be performed in rodent [52] as well as non-rodent species. The DTH assays are economical and correlate well with decreased CMI and host resistance to infectious agents in humans, [53] as well as animals. [54] The mixed-lymphocyte response (MLR) assay, which uses lymphocytes from a different strain, has been successfully used to evaluate CMI in mice. [2]

- Host Resistance Assays with Infectious Agents: A number of bacterial strains have been used to measure host resistance, including *Listeria monocytogenes*, various strains of *Streptococcus*, and *Escherichia coli*. [54] Useful viral models [55,56] include influenza, herpes, and cytomegalovirus. [57] A yeast infectivity model using *Candida albicans* has been described, as well as parasitic infectivity models using *Trichinella spiralis* and *Plasmodium yoelli*.[55,58]

■ Host Resistance Assays Using Syngeneic Tumor Cells: Various assays of host resistance have been described using a number of cultured tumor cell lines. [58,59] These assays, unlike those involving the infectious agents discussed above, do not require special barrier facilities to prevent infections from spreading throughout an animal colony. Two mouse assays have been validated: the PYB6 sarcoma assay and the B16F10 melanoma assay.[60] An assay using a lung tumor model and the MADB106 tumor cell line also has been validated for use in immunotoxicity studies. [61]

6. Relevance of Primary Indicators of Immune Toxicity to Health

a. Hematological Indicators

Hematologic screens recommended for toxicity studies are basically the same as those performed clinically as human health screens. Depressed or elevated WBC counts may be indicative of direct or indirect effects of the test substance on cellular proliferation and distribution. Total WBC counts are used clinically as a presumptive test for infection; they are also used to evaluate the severity of an inflammatory or allergic process. Routine differential WBC counts are used to differentiate among some types of infections and inflammatory responses; they also are used as a screen for toxicologic or pharmacologic effects: for example, immunosuppressive drugs may cause lymphopenia.

Altered lymphocyte counts may be relevant to immunodeficiency. Increased numbers of polymorphonuclear leukocytes can result from pathogenic infections and from pyrogenic and inflammatory processes. Eosinophilia is often associated with allergenic processes. It may also indicate an infectious, reactive, or neoplastic process. Altered red blood cell counts and platelet counts can be associated with autoimmune processes.

b. Serum Protein Indicators

Estimates of total serum proteins and the albumin/globulin ratio may give useful information about liver and lymphocyte function. The α- and β-globulins (*i.e.* α- and β-G) are primarily produced in the liver; τ-G are a product of the B-lymphocytes. Depressed β-G levels could lead to decreases in complement proteins that are required for phagocytosis; this could produce decreased resistance to bacterial infections. Reduced levels of τ-G also could mean reduced levels of antibodies necessary for humoral immunity to infectious agents. Altered levels of τ-G may indicate an effect on B-lymphocytes, T-lymphocytes, or simultaneous effects on both types of cells.

However, total globulin levels do not give specific information about which immunoglobulin classes are affected. Thus, when globulin levels are reduced, specific quantitative assays for the τ-G subclasses may be recommended. Electrophoretic and immunoelectrophoretic analyses of the serum τ-G subclasses or quantitative assays such as Enzyme-Linked Immunosorbent Assay (ELISA), Radioimmunoassay (RIA), or radial immunodiffusion may be recommended. This information may be important because reductions in τ-G and τ-M may be relevant to infection by opportunistic and pathogenic organisms, and changes in τ-A may indicate effects of the test substance on secretory immunity, such as gut-mediated immunity.

c. Histopathology Indicators

Abnormal results from gross and histological evaluation of the lymphoid organs (usually the spleen, thymus, and lymph nodes) are important indicators of various immunotoxic effects; histological evaluation of Peyer's patches and bone marrow also is recommended in screening for effects of a test substance on the immune system. Atrophy of the thymus gland with associated depletion of cortical thymocytes could be an indication of immunosuppression. Concomitantly, a similar effect on the lymphocytes in the periarterial lymphocyte sheath of the spleen (PALS) would indicate an effect of the test substance on T-cells: both cell-mediated and humoral

immunity can be affected. In the spleen and lymph nodes, defined regions are more densely populated with B-lymphocytes, with activated, antibody-producing B-cells, or with plasma cells. Effects on B-cell regions of these organs could be an indication of immunosuppression or immunoenhancement, depending on the result obtained.

d. Body and Organ Weights

Body and organ weights are generally recorded during toxicity studies. Spleen weights are usually recorded in all toxicity studies, but thymus weights may not be recorded in long-term studies. The thymus gland grows rapidly in young animals but begins to involute as the animals reach sexual maturity. In old animals, the thymus may be difficult to detect and measure because of the degree of involution.

Organ weights by themselves or in relation to body weights can be sensitive measures of organ atrophy or hypertrophy, but yield little information about immunotoxic effects. Reduced organ weights can result from direct effects on lymphocyte proliferation and differentiation and may be relevant for assessing immunosuppression. Hypertrophy of the lymphoid organs is usually associated with increased proliferation of cells (hyperplasia). Increased proliferation of lymphocytes can result from infections, stimulation by xenobiotics, altered metabolic processes, and certain forms of trauma, reactive, or autoimmune processes. In practice, however, changes in organ weights or organ-to-body-weight ratios are more relevant to immune toxicity when they are associated with appropriate histopathology findings.

7. Adequacy and Reliability of Primary Indicators of Immune Toxicity

If all primary indicators of possible immunotoxicity from toxicity studies are negative for a test substance, would this effectively rule out the possibility that the test substance produces significant immunotoxic effects? The answer to this question is complex; some of this complexity derives from the fact that the primary indicators of possible immune toxicity listed above are not sufficiently specific or sensitive to provide unambiguous answers. For example, it is not possible to differentiate B-lymphocytes from T-lymphocytes in routinely stained sections of lymphoid tissues, and standard hematology tests cannot distinguish among subcategories of T-lymphocytes. Special immunochemical stains, however, permit B- and T-cells to be visualized in tissue sections and blood smears, making available more information about the immunotoxic effects of the test substance.

If only short-term toxicity studies are performed on a particular test substance, concern about the adequacy and reliability of the immunotoxic indicators from these studies may be high. Subtle immunotoxic effects or immune toxicities that develop only after prolonged administration of the test substance may not be detected in short-term toxicity studies. Conditions of the longer-term toxicity studies, however, may make it difficult to detect some immune toxicities: the use of barrier facilities is common in carcinogenicity studies; because barrier facilities limit exposure of test animals to exogenous infectious microorganisms, detecting possible immunotoxic effects of a test substance in carcinogenicity studies may be compromised because spontaneous infection rates and mortality are evaluated as primary indicators of possible immunotoxicity in such studies.

Even with this disadvantage, many investigators and regulatory authorities recommend specific tests to identify and characterize immune system toxicities only when screening tests or indicators are positive. [6,11] Additional rationale for this approach comes from the fact that most short-term toxicity studies incorporate at least one dose in the potentially highly toxic dose range. Additional tests for immunotoxicity should be performed to verify positive immunotoxic effects noted during screening studies or to determine if the positive result obtained for a primary indicator was a false positive indication of immunotoxicity. For example, certain test substances may cause increased or decreased food intake; nutritional deprivation from significantly decreased food intake has been shown to cause thymic and splenic atrophy. [62] Effects on the endocrine system, such as stimulation of the production of growth hormone [63] or prolactin [64] and decreased levels of adrenocorticosteroids, [65] can stimulate

growth of the thymus. In response to such stimuli, involution of the thymus may proceed at a different rate in animals exposed to the test substance than in control animals. Therefore, measuring thymic weights at one specified time in a short-term toxicity study could give false positive or false negative indications of the test compound's immunotoxic potential. For this reason, the Agency recommends that a study of the effects of a test substance on thymic growth and involution be conducted at two or more time points during the study (such as midterm and final sacrifice). Because sex differences have been demonstrated for various immunologic studies,[30,66] both sexes should be included in immunotoxicity evaluations.

There are data which suggest that the primary indicators do not evaluate toxic effects on all types of immune-related cells. Recent studies have shown that NK cell function may be affected without concomitant effects on either B- or T-lymphocytes.[61] Other studies have shown that functional defects of specific lymphocytes can occur without apparent changes in the proliferation or morphology of the cells as observed in standard histopathology preparations:[11,67,68] the morphology of the cells is normal and a false negative result would be obtained in these instances.

8. Recommendations for Further Immunotoxicity Testing when Primary Indicators of Immunotoxicity are Positive

Assessing the safety of food and color additives used in food usually requires the completion of various toxicity studies. In addition to the screen of primary indicators of possible immunotoxicity provided by these toxicity studies and summarized above, additional tests for further evaluating the immunotoxic potential of a test substance may be recommended by the Agency. In the sections that follow, the adequacy of primary indicators of immunotoxicity for test substances that have been assigned to each Concern Level will be discussed. The Agency's recommendation that specific immunotoxicity tests be performed on test substances that have been assigned to Concern Levels I, II, and III will be made on a case-by-case basis.

a. Immunotoxicity Studies for Compounds that have been Assigned to Concern Level III

Test compounds that have been classified as Concern Level III substances present the highest level of concern about their safe use as direct food additives and color additives used in food. When these substances undergo toxicity testing, primary immunotoxicity indicators may be negative, marginal, or positive. Immunotoxicity tests suitable for each of these situations will be discussed below.

i. Immunological Tests when Primary Indicators of Immunotoxicity are Negative or Marginal

If the primary immunotoxicity indicators from recommended toxicity tests are not positive, then no additional tests for the immunotoxic potential of the Concern Level III test compound would be recommended unless there were special circumstances. Such circumstances may include: 1) the rodent strains employed in toxicity testing were highly inbred and are know to be resistant to immunotoxic effects; 2) barrier or other facilities were used for long-term and short-term toxicity studies, which may have precluded exposure of the test animals to normal infectious agents present in the environment; and 3) omissions from the recommended guideline for standard toxicity tests, such as not measuring thymus weights during the growth phases of the test animals or omitting histopathological analysis of certain lymphoid organs. In these situations, some Type 1 immunotoxicity tests and a Type 2 immunotoxicity study of host resistance may be recommended, particularly if specific tests for immune toxicity had not been incorporated into subchronic toxicity studies.

ii. Immunological Tests when Primary Indicators of Immunotoxicity are Positive

When any of the primary indicators suggests that a Concern Level III test substance has an immunotoxic effect, additional testing will be recommended in order to assess the extent of risk to the immune system. In addition, positive effects on other target organs may indicate the need to assess the autoimmune potential of the

compound.

Certain indicators may derive from effects on either B-cells or T-cells, or may be derived from effects on both types of cells. However, most of the primary indicators of immune toxicity are nondiscriminating with respect to specific lymphocytes involved and specific immune functions affected. Standard histopathology evaluation may provide some clues if there is an effect on the thymus or if areas in the spleen or lymph nodes are associated with specific types of lymphocytes. The objectives of expanded Levels I and II immunotoxicity tests are to delineate the specific cells type(s) which are affected, to evaluate the extent to which specific immune functions are impaired, and to relate these effects to risks such as infection, hypersensitivity, and carcinogenicity.

The immunotoxicity tests described in the following sections are for use with rats, and all tests should be conducted on each test animal. However, tests have been, or can be, adapted for use with mice or non-rodent species. When mice are used as test animals, serum from animals in each experimental group may need to be pooled if there is an insufficient quantity of serum from each animal to perform recommended hematology tests.

a) Retrospective Level I Tests: No additional animals are needed for Retrospective Level I immunotoxicity tests when at least 10 animals of each sex are used in a standard toxicity study and appropriate samples of blood and tissues are properly treated and preserved. After removing blood cells, serum samples should be prepared by high-speed centrifugation, sterilized by filtration, and stored at 4-5^{o}C in sealed containers. At least half of each lymphoid tissue/organ should be fixed briefly in Bouin's fixative (or other fixative shown to be appropriate) and stored in alcohol; sections from the tissue/organ can be processed for histopathological analysis by routine staining or by immunostaining.

If the standard toxicity study was a subchronic or chronic study (with exposure to the test substance for 90 to 120 days), and primary indicators suggested that the test material may be immunotoxic, the following Retrospective Level I tests should be performed on serum samples from the study:

i) Electrophoresis of serum proteins.

ii) Quantification of serum immunoglobulins (IgG, IgM, IgA, IgE).

iii) Immunostaining for B- and T-lymphocytes in spleen and lymph nodes and micrometric analysis of the number of stained cells in specific regions of these organs.

iv) Screening for serum autoantibodies to DNA, mitochondria, and other cell components in one or more tissues, such as liver and smooth muscle.[69] These tests should be performed when there is an indication that the test substance may affect B- or T-lymphocytes.

v) Immunostaining for bound IgG may be recommended to determine if non-lymphoid organ toxicities noted during the standard toxicity study (particularly a long-term toxicity study) are due to an autoimmune reaction.

If the results of these Retrospective Level 1 tests demonstrate that the primary indicators of immune toxicity were false positives, then no further immunotoxicity testing would be recommended. However, if the results of these tests are inconclusive or confirm an immunotoxic effect of the test substance, additional testing would be recommended. The additional testing may include Type 1 and Type 2 immunotoxicity tests.

b) Additional Level 1 Tests: Additional Level 1 immunotoxicity tests cannot be performed retrospectively, but must be incorporated into the protocol of standard toxicity studies. However, all of the tests described in this section can be performed on the same animals that are used in the standard toxicity study, provided that samples are processed appropriately. For example, half of the spleen can be used to make a cell suspension for cellular analysis immediately following sacrifice of the test animal; the remaining half can be processed for histopathology evaluation. Additional (non-retrospective) Level 1 tests that may be recommended include:

i) Quantitative analysis of the B-cell to T-cell ratio (B/T) using either whole blood cells and spleen preparations or spleen preparations only.

ii) Determination of spleen cellularity (the total number of white blood cells and lymphocytes per gram of wet tissue) and the total number of viable cells per gram of wet tissue or per million white blood cells.

iii) Assay of mitogenic stimulation for B- and T-lymphocytes:

iv) Analysis of NK cells using a suspension of spleen cells:

v) Determination of the phagocytotic index of macrophages:

vi) Electrophoresis of serum proteins: Although this test can be performed retrospectively, it is listed here because it is particularly useful for evaluating toxic immune effects on liver, macrophages, and lymphocytes.

c) Level II Tests: If primary indicators of immunotoxicity from standard toxicity studies suggest that a test compound may be immunotoxic, Level II tests may be recommended to identify specific functional immune defects. These tests may be performed on satellite groups of test animals in conjunction with a standard toxicity study or they may be performed on test animals in a separate immunotoxicity study. In the latter case, Level II tests should be performed with the same species, strain and age of test animals and the same doses of test substance used in the standard toxicity study of comparable duration. In addition, separate Level II immunotoxicity studies should be 3 to 6 weeks in duration so that test animals will be exposed long enough to enable primary and secondary immune effects to be identified. An additional period of time at the end of the study during which the test substance is not administered would permit evaluation of the reversibility of observed immune effects.

The following Level II tests may be recommended:

i) Kinetic evaluation of primary and secondary immune responses of test animals to a T-dependent antigen, such as SRBC, tetanus toxoid, or KLH; serum antibody titers should be measured following initial and secondary injections of the antigen.

ii) Evaluation of the primary humoral response to a T-independent antigen, such as pneumococcal polysaccharides; choice of the optimum challenge dose should be justified.

iii) Evaluation of the delayed hypersensitivity response to a contact sensitizer during the second half of the study. Alternatively, evaluation of the mixed lymphocyte response can substitute for measurement of the DTH response as long as the assay has been validated with the particular rat strain used.

d) Enhanced Level II Tests: These tests are designed to determine if a test substance that produces immune toxicity in Level I or Level II tests also affects host resistance to challenge with infectious agents or tumor cells. Enhanced Level II tests may be performed with either rats or mice, because many host resistance tests have been validated in mice. These tests would be recommended in a variety of circumstances; for example:

i) If primary indicators of immunotoxicity from standard long-term toxicity studies showed increased mortality associated with administration of the test substance and effects on humoral immunity were identified from Level I and Level II tests, then bacterial (*e.g. Listeria monocytogenes*)[58] or viral (*e.g. Influenza*)[55] challenge tests associated with humoral immune protection would be recommended for evaluation of host resistance.

ii) If there are indications that consumption of the test substance is associated with increased tumorigenesis and effects on phagocytosis, tumor challenge tests with PYB6 sarcoma, which tests cytolytic activity of T-cells and NK cells in mice,[60] would be appropriate; a similar test for rats uses the MADB106 tumor line.[61]

iii) Finally, for test materials that have demonstrated T-cell or cell-mediated immune effects, challenge tests that use certain strains of *Streptococcus* [57] or *Plasmodium yoelli* [58] would be appropriate.

b. Immunotoxicity Studies for Compounds that have been Assigned to Concern Level II

Specific immunotoxicity tests generally are not recommended for test compounds that have been assigned to Concern Level II. However, if primary indicators of possible immunotoxicity from toxicity studies conducted on Concern Level II test substances are positive, additional Level I and Level II immunotoxicity tests may be recommended; such recommendations will be made on a case-by-case basis.

c. Immunotoxicity Studies for Compounds that have been Assigned to Concern Level I

Usually, short-term acute exposure studies (up to 30 days) are performed to assess the safety of Concern Level I compounds. Although guidelines for these studies (see **Chapter IV C 3**) do not recommend specific immunotoxicity tests, if primary indicators of possible immunotoxicity from short-term toxicity studies are positive, additional Level I and Level II immunotoxicity tests may be recommended. Such recommendations will be made on a case-by-case basis. One immunotoxicity test which measures the primary humoral response to the T-dependent antigen SRBC has been described for use with both rats [34] and mice [38] and has been recommended for use in short-term screening studies.[2]

9. Animal Models for Immunotoxicity Tests

a. Rodent Models

These guidelines have focused on tests designed to assess immune toxicity in the rat. Specific strains have been used and validated by the Agency, including Sprague-Dawley, Spartan,[30] and Osborne Mendel; [69] the Fisher 344 rat has been recommended by others [61] for studies with environmental compounds. Other strains of rat, such as the Buffalo strain, have been used in special studies to evaluate autoimmune disease potentiation.[70-72] In addition, several mouse strains (mainly inbred strains) have been used to assess immune toxicity.

b. Non-rodent Models

Use of the dog for various immunopharmacologic studies has been described in the scientific literature.[73] Level I immunotoxicity tests described in these guidelines can be performed on most large animal species; Level II immunotoxicity tests in other non-rodent models also may be acceptable, if validated: use of primates has been described. [74] Also, miniature swine have been shown to be an excellent non-rodent species for evaluation of various immune functions. [75-79] The Agency has validated a number of immune function assays for use with this

model.

Immunomodulation of porcine as well as other food animals have been reviewed.[75] Other perspectives on animals selection have been reviewed. [76]

10. Recommended Strategy for Assessing the Immunotoxic Potential of Direct Food Additives and Color Additives Used in Food

- Primary indicators of immunotoxicity should be evaluated for short-term (28-day) toxicity studies, subchronic (90-day) toxicity studies, and developmental toxicity studies. Results of these evaluations should be incorporated into an integrated assessment of the potential for a test chemical to adversely affect the immune system. Based on this assessment, an explicit statement should be made as to whether or not the test chemical represents a potential immunotoxic hazard which requires further testing.

- Additional studies to assess the immune toxicity of food and color additives used in food will depend on the results of the evaluation of primary indicators of immune toxicity, the Concern Level to which the additive has been assigned, and other information available concerning the immunotoxicity of the additive.

11. Conclusion

The hierarchical grouping of recommended immunotoxicity tests by specificity and mechanics (*e.g.* tests that use injectable substances) can facilitate including immunotoxicity testing in standard toxicity studies. Expanded testing on existing samples is possible, and allows for a more definitive identification of potential immunotoxic effects. Such expanded testing may be necessary when additional information about a possible immunotoxic effect is important for the safety assessment of a direct food additive or color additive used in food. Immunotoxicity tests recommended in this section are summarized in **Table 1** below.

Table 1

Summary of Immunotoxicity Testing Recommendations for Direct Food Additives

Basic Testing (Rat Model)

- CBC, WBC differential
- Total serum protein, albumin-to-globulin ratio (A/G)
- Histopathology, gross and microscopic (spleen, thymus, lymph nodes, Peyer's patches, and bone marrow)
- Lymphoid organ and body weights

Retrospective Level I Testing: Included as a Possible Requirement in Standard Toxicity Study

- Electrophoretic analysis of serum proteins* (when positive or marginal effect noted in basic testing)
- Immunostaining of spleen and lymph nodes for B and T cells* (quantification of total Ig)
- Serum autoantibody screen and deposition of Ig (micrometry for semi-quantitation of the proliferative response)

Enhanced Level I Testing: Included as a Possible Requirement for More Complete Screening in the Standard Toxicity Study Core Group, with a Satellite Animal Group, or in a Follow-Up Study

- Cellularity of spleen (lymph nodes, thymus when indicated)
  - Quantification of total B and T cells (blood and/or spleen)
  - Mitogen stimulation assays for B and T cells (spleen)
  - NK functional analysis (spleen)
  - Macrophage quantification and functional analysis (spleen)
  - IL-2 functional analysis (spleen)
- When indicated or for more complete analysis, other endpoints such as total hemolytic complement activity or CH-50 assay with serum

Level II Testing: Includes a Satellite Group or Follow-Up Study for Screening of Functional Immune Effects

- Kinetic evaluation of the humoral response to a T-dependent AG (primary and secondary responses with either SRBC, TT, or other)
- Kinetic evaluation of the primary humoral response to a T-independent AG such as Pvax, TNP-LPS, or other recognized AG
- DTH response to known sensitizer of known T-cell affecter
- Reversibility evaluation

Enhanced Level II Testing: Includes a Satellite Group or Follow-Up Study For Evaluation of Potential Immunotoxic Risk

- Tumor challenge (MADB106 or other with the rat; PYB6 sarcoma with a mouse model)
- Infectivity challenge (*Trichinella*, *Candida* or other with the rat; *Listeria* or other with the mouse)

Abbreviations: CBC = complete blood count; WBC = white blood count; Ig = immunoglobulin; NK = natural killer; IL-2 = interleukin-2; SRBC = sheep red blood cells; and TNP-LPS = trinitrophenol lipopolysaccharide.

* Recommended for inclusion in basic testing.

Chapter V D: Immunotoxicity Studies - References

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Chapter VI

Human Studies:

This chapter presents general guidelines for the conduct of human clinical studies on foods and food ingredients. It also describes the types of human epidemiology data that may be useful to the Agency in assessing the safety of direct food additives and color additives used in food. Because human clinical studies were not included in the 1982 guidelines for direct food additives and color additives used in food, important issues related to these studies are discussed at length in this chapter.

The Agency does not require petitioners to conduct human clinical studies to support the safety of direct food additives and color additives used in food. However, petitioners may elect to perform such studies in certain circumstances, such as when the proposed additive will be consumed by humans at relatively high levels (see **Chapter VII B**). When petitioners conduct human clinical studies on substances intended for use as direct food additives and color additives used in food, however, the Agency recommends that the studies conform to the guidelines presented in this section. As usual, the Agency strongly recommends that petitioners planning to conduct human studies in support of the safety of direct food additives and color additives used in food consult with the Agency before the studies begin.

VI A. Clinical Evaluation of Foods and Food Additives

A major objective in the clinical testing of food and food additives is to assess aspects of safety that cannot be addressed adequately by non-human studies or by existing data on population exposure. For example, the Agency is now confronted with petitions for direct food additives that are intended to substitute for major nutrients such as fat and sugar. Because segments of our population may be exposed to large quantities of these additives for long periods of time, traditional methods of evaluating the safety of these substances may not be adequate. Testing these substances in animals at doses that greatly exaggerate their anticipated human exposures may not be possible. For these substances, human clinical studies may provided additional confidence in the safety of the food or food additive.

A food or food additive generally will be considered suitable for clinical testing if the substance is unlikely to produce significant toxic effects at the levels to which the subjects of the clinical study will be exposed. This usually is determined from the results of toxicity studies in animals or by examining existing data on population exposure. However, in cases where the type of toxic response associated with the consumption of a food or food additive by experimental animals is judged to be severe, exposure of subjects in clinical studies to the additive may need to be significantly below the level found to produce no toxic effects in an appropriate species.

Unlike patients participating in clinical trials of new drugs, no health benefit is anticipated for most test subjects in clinical studies of foods and food additives. Thus, the nature and weight of evidence required to establish the safety of these products for humans before clinical studies can begin may differ from that required to support testing under guidelines for investigational new drugs. Clinical studies of foods and food additives will focus on demonstrating safety; for example, the safety of an additive that may interfere with absorption of nutrients, whose status in the population is uncertain, may need to be evaluated in a clinical study.

1. General Considerations for Clinical Studies of Foods and Food Additives

Principles for the conduct of clinical trials are contained in the May 8, 1979 Federal Register: "*Statement concerning adequate and well-controlled clinical investigations.*" [1] The following guidelines identify general considerations for clinical studies of foods and food additives. Each consideration should be explicitly addressed in the clinical study's protocol.

- Before undertaking costly and time-consuming clinical studies as part of the safety assessment of a food or food additive, the investigator needs to formulate a defensible rationale for conducting human clinical studies and a clear set of objectives.

- Adequate preclinical investigations (including toxicity tests in animals) must have been completed. Results of these tests must establish that there is no expected toxicity to man at doses to be used in clinical studies. A clear, concise description of the design of pre-clinical studies and their results should be presented to FDA. Information about the history of use of the food or food additive outside the United States and documentation of the results of foreign clinical studies involving the food or food additive should also be presented for review.

- In designing protocols for clinical studies, the following should be considered: 1) the results of pre-clinical studies (including toxicity tests in animals) and foreign clinical studies; 2) the chemical nature of the proposed additive; and 3) all organs and organ systems that may be affected in man by consumption of the food or food additive under investigation.

- The sequence of clinical tests should be designed to maximize the safety of the research subjects.

- Guidelines for clinical trials of investigational new drugs should be followed in evaluating the qualifications of the principal investigator and investigating institution. In particular, careful consideration must be given to the qualifications of the investigator and the suitability of the investigating institution's facilities for conducting short- and long-term clinical trials.

FDA recognizes the need for the investigator to exercise sound clinical judgement based on his/her experience in an appropriate field of study. Studies involving healthy volunteers should be performed by investigators skilled in the evaluation of the safety of a variety of compounds. When subjects of a clinical study have a specific disease, as may be the case for clinical evaluation of foods for special dietary uses or special medical purposes, the investigators should be clinicians expert in the disease and disease process.

- The investigator should have high regard for the rights and safety of the test subject(s). The investigator is responsible for the administration of the food additive; thus, he/she must bear the ultimate responsibility for the welfare of the test subjects. All aspects of a clinical study generally are described in the study's protocol; however, because actions that have been identified as being in the best interests of the subjects at the beginning of a clinical study may change during the study, all aspects of the study must remain flexible and subject to modification. Aspects of the clinical study protocol subject to such modification include: 1) The nature and frequency of laboratory tests, 2) the duration of consumption of the food or food additive, and 3) the interval between test subjects' visits to the investigator.

Institutional review of research involving human subjects and the requirement for informed consent will provide additional safeguards for test subjects. Principles of institutional review and informed consent were set forth in the March 13, 1975 Federal Register: "*Technical Amendments Concerning Protection of Human Subjects*;" [2] these are summarized in **Appendix A** (see section VI A 5 below).

- There is some finite risk associated with the administration of every unapproved food and food additive to subjects of a clinical study; despite strict adherence to guidelines, the safety of subjects in the study cannot be guaranteed. Before beginning a clinical study, the investigator should consider what procedures will be used to detect adverse reactions to the test substance during the study. The

investigator should establish criteria that will be used to decide when to discontinue the clinical study; these criteria may be changed during the study if the change is required to support the safety of the subjects.

To further protect the safety of subjects of a clinical study, the sponsor of the study should provide appropriate follow-up after the study has ended. Such follow-up should be conducted or supervised by the investigator of the clinical study.

- Before a clinical study begins, the investigator should consider ways in which quality control of the study will be documented. Effective documentation of quality control will facilitate Agency review of the completed clinical study.

- FDA recommends that investigators use statistical expertise in the planning, design, execution, and analysis of pre-clinical and clinical studies. Such expertise will help ensure that the planned studies will provide the necessary information while minimizing the number of subjects (sample size estimation) and will strengthen the validity of estimates of safety obtained from the studies.

2. Specific Considerations for Clinical Studies of Foods and Food Additives

This section describes specific considerations concerning the protocol design, definition of study population, and statistical analysis of the results of human clinical studies with foods and food additives. These considerations should be explicitly addressed in the clinical study protocol.

a. Protocol Design

Protocols for clinical studies of foods and food additives should be described clearly and in sufficient detail to permit effective review and evaluation by CFSAN. In general, the protocol should be strictly adhered to throughout the clinical study; if the protocol is not adhered to, documentation of necessary modifications should be made (see item 7 in section 1 above). While it is rational and desirable to design studies to obtain specific information about the test substance, the generation of data justifying conclusions other than those originally anticipated can be a valuable result of clinical investigation.

The following are additional recommendations for the design of clinical study protocols for foods and food additives:

- A clear statement of objectives should be provided for each protocol. Good planning usually produces research questions that can be answered by direct inference from the study data. Since studies are frequently designed to answer more than one question, it is useful to list the questions to be answered in order of their priority.

- The rationale for conducting a clinical study should be presented. In addition, pre-clinical and clinical data relevant to the compound being studied and to the proposed protocol should be reviewed.

- A statement explaining the reasons for deciding on a particular length for the clinical study should be included in the protocol. In general, a clinical study should be of sufficient length to permit the demonstration of the safety (or lack of safety) of a food or food additive.

- A statement explaining the reasons for selecting particular dietary levels (dosages) of the food or food additive being tested should be included.

- Experimental design should include appropriate controls. When feasible, studies should be performed blind to avoid selection bias and bias in patient and physician responses.

- Investigators should describe proposed methods of randomization and should present analyses that demonstrate the effectiveness of these methods.

- Objective observation methods should be used when possible and appropriate, observational endpoints should be rigorously defined, and methodology that will be used to quantify endpoints should be described. A statement describing quality control and frequency of data collection (endpoint monitoring) also should be included.

- Limitations that may be imposed on the clinical study because of protocol design or the failure of subjects to comply with the written protocol (such as withdrawals from the study, failure to randomize subjects effectively, technological limits of observations, *etc.*) and the possible effects these limitations may have on the outcome of the study should be addressed.

b. The Study Population

Clinical studies identify physiological responses to test substances in well-defined, small populations. These results are used to make inferences about responses to the test substance in larger, target populations. Study protocols should specify how subjects will be selected, their assignment to alternative test regimens, the specific conditions under which the trial will be conducted, and the nature of the target population to which the subjects' responses will be extrapolated. The following are additional recommendations for defining and selecting subjects for the clinical study:

- Each study protocol must be reviewed and approved by the appropriate Institutional Review Board; written, informed consent must be obtained for each subject in the study (see **Appendix A** in section VI A 5 below).

- Protocols should clearly define the selection criteria for subjects, including diagnostic criteria and reasons for exclusion from the study, and should compare and contrast the study population with the larger population likely to consume the food or food additive.

- Criteria for discontinuing the study should be stated clearly.

- Doses of the test substance should be selected so that a range of subject responses to the substance can be observed and the highest safe dose of the proposed additive can be determined. When individual subjects' responses are expected to be quite variable, testing at multiple doses in a double-blind, placebo-controlled study is recommended.

- A serious problem in clinical studies is determining the degree of subject adherence to the assigned protocol. Careful attention to subject compliance with the protocol is particularly important in outpatient studies. Protocols should state clearly how subjects' compliance will be monitored and should indicate when noncompliance will result in discontinuing the subject in the study. In general, data on subject compliance and noncompliance enhance the credibility of a study.

If it becomes apparent during the study that subjects are not complying with the study protocol, reasons for their noncompliance should be determined. All subjects initially included in a study must be reported on in the study's results, regardless of the degree of their compliance. Some noncompliance may necessitate identifying subgroups for evaluation, such as subjects who fail to consume foods containing the additive and subjects who report excessive use of alcohol or medication.

- The number of subjects to be included in the study should be sufficient to be able to determine the safety of the test substance. Statistical estimates of the required number of subjects will depend upon: 1) The desired limit of detection of subjects' responses to the test substance; 2) the desired assurance against a false positive result; and 3) the acceptable risk of a false negative result.

- While it is desirable that placebo groups be included in early clinical studies of proposed foods and food additives (see page 17), this is not a requirement. Goals of early clinical studies may be 1) to gradually increase the dose of the test substance until physiological effects are observed or 2) to determine absorption and metabolism in humans in an effort to assess the adequacy of animal models used in safety assessments of the test compound. Therefore, subjects must be under careful observation during these studies.

The goals of early clinical studies often can be achieved effectively with an open (non-blind) study protocol. When clinical studies using blind comparisons of the test substance and a placebo or positive control substance should begin varies with the nature of the test material. During all phases of clinical investigation, the objective in using a placebo is to provide an adequate control for the compound under study. However, other methods of adequately controlling clinical studies exist. For example, the use of an active control compound or demonstration of a positive dose response to the food or food additive may constitute adequate control in some studies. For situations in which the natural course of a disease or condition is predictable and for which objective measurements of therapeutic or prophylactic response to the test compound can be made, results of carefully executed, open (non-blind) studies may be compared to historical data.

- Food additives should be studied in all age groups that may be significantly exposed, including, as appropriate, children, women of childbearing potential, older populations, and populations with specific disease conditions. The latter category includes populations that may be particularly exposed to, positively affected by, or at risk from a particular food or food additive.

Pregnancy tests should be administered to women of childbearing potential before the introduction of the test substance and the subject should be advised about suitable contraceptive measures. In general, women of childbearing potential should be excluded from the earliest clinical studies of a test substance. Once an adequate baseline of clinical information about the safety of a food or food additive has been obtained, however, women of childbearing potential may be included in clinical studies. For example, women of childbearing potential may participate in clinical studies when the teratogenic potential of the test substance has been determined to be negative in animals.

Follow-up to detect possible effects of the test substance on the fetus should be provided to women who become pregnant while on the study. Under these circumstances, transplacental passage of the substance and its secretion in milk should be assumed until proven otherwise.

- If the proposed food or food additive has a significant potential for use in children, its safety should be evaluated in children. Usually, studies in children are not attempted until there has been considerable clinical experience with the additive in adults. For certain proposed food additives, however, early clinical study in children may be warranted; in such cases, it is preferable to begin with older children, followed by younger children, infants, and premature infants. Detailed comments on pediatric studies are contained in "*General Considerations for the Clinical Evaluation of Drugs in Infants and Children*."[3] Additional examples of guidelines concerning the clinical testing of foods or food additives in children are provided by the American Academy of Pediatrics.[4,5]

- Generally, physical examinations and laboratory tests should be performed to screen individuals with medically significant abnormalities from the clinical study. Laboratory tests should include the following: 1) Electrocardiograph; 2) urinalysis; 3) various tests on blood samples (for example, complete blood counts including platelet estimates, blood urea nitrogen, serum creatinine, tests of liver function, fasting blood sugar or 2-hour postprandial blood sugar, electrolytes, protein, and albumin); and 4) other tests that may be indicated by the nature of the test compound or from the results of previous animal and human clinical studies (for example, tests of vitamin status, prothrombin time, and blood lipid profiles).

- In early clinical studies, when feasible, all subjects should refrain from taking medication (including over-the-counter drugs) for at least two (and preferably four) weeks before the study begins, unless interactions of the test substance with medication are the focus of the study. In some cases, a longer "washout" period will be required for return to a normal physiologic state before the clinical study begins.

In later clinical studies, it may be desirable to examine the safety of combinations of the test substance and medication(s).

◙ Post-study physical examinations for subjects of clinical studies often are necessary to ensure the subjects' safety. The results of these examinations should be fully documented.

c. Statistical Analyses

The following are general recommendations for statistical analyses in clinical studies of foods and food additives. Additional recommendations are contained in **Chapter IV B 4**.

◙ Investigators are encouraged to seek expert biostatistical assistance prior to formulating the study design.

◙ *A priori* description of the statistical methods to be used in analyzing data from a clinical study should be provided in the study's protocol.

◙ Estimates of statistical power should be used to help determine the optimal number of subjects for a clinical study.

3. Sequence of Clinical Studies for Foods and Food Additives

The rationale behind serially conducted studies is that results of each study may influence the plan of succeeding studies. Investigators are encouraged to discuss data from animal studies and early clinical studies with CFSAN before conducting additional clinical studies.

a. Early Clinical Studies

The purpose of these studies is to determine the metabolism and the level of the food or food additive that gives an adverse or toxic response in man. Physiologic processes that are of primary interest in early clinical studies include: 1) Disposition (absorption, biotransformation, and excretion) of the food or food additive and its metabolites; 2) the potential of the food or food additive to induce enzyme levels or increase activity; 3) interactions between the food or food additive and nutrients that may necessitate balance studies; and 4) interactions between the food or food additive and medications that may necessitate drug bioavailability or drug metabolism studies. Information about the potential use of the test substance and all preclinical information about the test substance should factor into decisions about the appropriate sequence of early clinical studies.

For both ethical and scientific reasons, the initial introduction of a food or food additive into humans should be done with carefully selected subjects. Subjects for early clinical studies should be "normal" volunteers. "Normal" generally means volunteers who are free from health problems that would complicate the interpretation of the study or increase the sensitivity of the subject to the toxic potential of the food or food additive. Children, pregnant women, and women of childbearing potential usually should be excluded from early clinical studies.

Within the limitations described in the preceding paragraph, subjects of early clinical studies should be selected to accurately reflect the general population. Thus, individuals with mild but stable illnesses such as uncomplicated hypertension or arthritis may be considered for inclusion in initial clinical studies on a food or food additive. It also may be permissible--and even desirable--to include subjects with abnormalities for which consumption of the food or food additive may be particularly beneficial. For example, subjects with hyperlipoproteinemia may be included in an early clinical study on a food or food additive that functions as a non-caloric fat substitute. Additional examples include: (a) A food or food additive that will be used in the dietary management of organ failure should be tested in a population with failure of the organ under study; (b) a food or food additive designed to be deficient in a particular nutrient should be tested in a population that is unable

to metabolize the nutrient in question (in fact, such a food or food additive may be harmful to a population with normal metabolism).

Most early clinical studies are sub-chronic (relatively short-term) and are generally less than 4 weeks in duration. These studies vary from single exposure to multiple exposures and examine a range of levels (doses) of the food or food additive. When several doses are being tested in a study, no research subject should be given the next-higher dose until sufficient exposure has occurred with the immediately preceding dose to be certain that serious adverse effects have not occurred.

For each food and food additive subjected to clinical investigation, it is also important to consider the appropriate frequency of laboratory tests and, when indicated by the results of previous studies, tests for specific organ or organ system effects. Independent of the outcome of clinical studies, thorough physical examinations and blood screening should be part of the follow-up for all subjects.

When unanticipated side effects occur in clinical studies, the investigator should determine the time required for elimination of the compound from the subject's system and reversal of the effects.

b. Further Clinical Studies

Additional clinical studies may be designed to determine the safety of the proposed food additive during chronic intake (relatively long-term) and to gather more information about the food additive's adverse effects in humans. These studies should be performed after the general safety of the food or food additive in humans has been established in early, short-term clinical studies. The duration of exposure to the food or food additive in these studies will vary with the nature of the additive. Chronic administration in humans usually means continuous consumption for at least 8 to 12 weeks, unless contraindicated by adverse side-effects.

Relatively long-term clinical studies of food and food additives may emphasize the physiologic processes of enzyme induction or interaction of the additive with other substances (such as nutrients, medications, and other food additives). In addition, when designing studies to determine the safety of chronically consumed food additives, investigators should consider conducting nutrient balance studies; these studies help determine end-organ (or end-organ system) responses to the additive, including neurobehavioral changes.

Finally, clinical studies may be performed to obtain information about adverse effects of the food or food additive on specific subpopulations. For these studies, appropriate subpopulations may include children, pregnant women, women of childbearing potential, and older subjects. These studies may also include subjects with concomitant diseases who are undergoing therapy for the disease, particularly if such subjects represent segments of the population who are likely to consume the food or food additive after it has been approved.

Relatively long-term clinical studies should include a limited number of closely monitored subjects (rarely exceeding several hundred). In the clinical studies described above, the frequency of physical examinations and laboratory tests for subjects will depend upon the nature and relative safety of the food additive. For some subjects, daily supervision may be necessary. Early periods during a study will typically involve more frequent supervision of subjects than later periods. An example of a graded supervision plan would be one in which a test subject is seen by the investigator at least once a week for 2 to 4 weeks, once every other week for 6 to 8 weeks, at monthly intervals for 2 to 3 months, and bimonthly until the end of the follow-up period. Routine laboratory tests should be performed at frequent intervals; frequency and type of special laboratory tests should be determined by the nature of the food or food additive and its intended use.

In both early and chronic clinical studies of food additives, it is particularly important that a single formulation of the test substances be used throughout the study; in addition, investigators should test the compounds that will be marketed. Consideration should be given to relative exposures for particular food uses when such uses may alter the structure or effects of the test substance. A significant change in the formulation or manufacture of the food or food additive during chronic clinical studies may indicate the need for bioavailability studies on the (presumably changed) food or food additive. Results of these studies will enable meaningful comparisons to be made among clinical studies performed with different formulations of the test substance. When

the petitioner intends to market a family of formulations and only a limited number of the formulations will be tested in clinical studies, petitioners should be prepared to demonstrate that the test compounds are fully representative of the family of formulations intended for marketing, particularly with respect to questions of safety.

4. Submitting Reports of Clinical Studies on Foods and Food Additives to CFSAN

In submitting reports of clinical studies to CFSAN, particular emphasis should be placed on clear and concise: 1) statement of study objectives, 2) description of protocols, and 3) presentation of significant findings. Presentation of the results of a series of clinical studies on an proposed food additive should be scientifically logical and should specify the order in which the studies were conducted.

Early, relatively short-term clinical studies include tolerance studies. In reporting the results of tolerance studies, information on dose schedules and range of doses should be included. For relatively short-term clinical studies, the following questions should be answered in determining the safety of the proposed additive:

- What are the absorption, metabolism, tissue deposition, and major routes of excretion of the food or food additive?

- What is the half-life of the food or food additive in the human body? (Analysis of turnover and of other pharmacokinetic parameters of the test substance or its metabolites in various physiological compartments may aid in the interpretation of the results of toxicity studies.) (see **Chapter V B**);

- How may interactions between the food or food additive and nutrients or medications compromise the availability of any of these substances?

- How does the food or food additive affect the function of human organs and organ systems?

- What are the possible adverse reactions to the food or food additive in the general population of individuals who are likely to use the substance and in special (more sensitive) populations?

Reports on relatively long-term clinical studies should emphasize specific organ or organ system responses to the food or food additive and nutrient imbalances that occur with chronic use of the food or food additive.

Finally, the safety of a food or food additive may continue to be monitored after the substance has been approved. This can be accomplished by further clinical testing or by establishing a surveillance system and documenting adverse reactions to the food additive. The need for such a system is expected to vary with the nature and use of the approved food additive. Clinical testing and surveillance also may be useful in establishing the safety of expanded uses of the food or food additive or the safety of an altered food or food additive; these changes may occur as the result of changes in patterns of food consumption or food processing.

5. Appendix A

The following principles are general guidelines for institutional review of, and conformed consent of subjects for, clinical studies. Additional information can be found in the references for this chapter.

a. Principles of Institutional Review

- An Institutional Review Board must be composed of no fewer than 5 persons from various backgrounds to assure complete and adequate review of clinical research activities commonly conducted by the institution. In addition to possessing the scientific competence necessary to review such institutional activities, the Board must be able to evaluate research applications and proposals in terms of

institutional commitments and regulations, applicable law, standards of professional conduct and practice, and community attitudes.

- No member of a Board shall be involved in the initial or continuing review of an activity in which he has a conflicting interest, except to provide information requested by the Board.

- No Board shall consist entirely of persons who are officers, employees, or agents of, or are otherwise associated with the institution, apart from their membership on the Board.

b. Principles of Informed Consent

All subjects in a clinical evaluation are entitled to:

- a fair explanation of the procedures to be followed and the purposes of the procedures, including identification of any procedures that are experimental;

- a description of attendant discomforts and risks that may be reasonably expected;

- a description of benefits they may reasonably be expected;

- disclosure of appropriate alternative procedures that may be advantageous to the subject;

- an offer to answer any inquiries concerning the procedure; and

- instruction that the subject is free to withdraw his consent and discontinue participation in the project at any time, without prejudice to the subject.

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Redbook 2000: VI.B Epidemiology

October 2001

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter VI.B. Epidemiology

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This guidance represents the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

Epidemiology is the study of the distribution and determinants of health-related states and events in specified populations, and the application of this study to the control of health problems.[5] The goal of all epidemiology studies is to uncover relationships between exposure to a specific agent and changes in health status.

Epidemiologic data are important to CFSAN in assessing safety and have been used by the Agency as indicators where avenues of research and further human studies would be most productive. Guidelines for the proper conduct and documentation of epidemiology studies, such as selection of the study population, selection of appropriate controls, exposure assessment, methods used to adjust or control for confounding variables, and statistical analyses will not be discussed here. Appropriate guidelines have been published elsewhere,[1] and should be consulted by the petitioner before submitting epidemiology data for consideration by the Agency.

There are two main categories of epidemiology studies, descriptive and analytic. Descriptive studies are concerned with the existing distribution of variables; they do not test hypotheses or make inferences concerning causality. Analytic studies are designed to examine associations, particularly hypothesized causal relationships, and focus on identifying or measuring the effects of specific risk factors.

1. Descriptive Epidemiology Studies

Descriptive epidemiology studies are relatively inexpensive to conduct and are usually of short duration. However, such studies are limited in their usefulness since no inferences can be made concerning causality. Generally, descriptive epidemiology studies are sentinel devices used to generate hypotheses or to provide evidence that indicates whether there is sufficient cause for conducting a lengthier and costlier analytic study.

a. Correlational Studies

Correlational studies, also called ecological studies, use grouped population data to relate exposure patterns of whole populations to disease incidence or mortality rates for whole populations. Because these studies do not examine the relationship between exposure and disease among individuals, the studies have been traditionally regarded as useful for generating, rather than definitively testing, a scientific hypothesis. Thus, the results of correlational studies would be insufficient to demonstrate a relationship without other types of data to support them.

b. Case Reports

Case reports are a type of descriptive epidemiology study frequently evaluated by CFSAN. Strongly suggestive anecdotal or clinical observations may indicate a possible causal relationship. Analytic epidemiology studies can then be designed to verify and quantify the risks, and to determine the role of confounding factors.

There are two principal avenues through which case reports come to the attention of CFSAN: first, reports published in the peer-reviewed medical literature, and second, reports captured in one or more of CFSAN's ongoing voluntary (also called "passive") adverse event monitoring systems, which include:

The Adverse Reaction Monitoring System (ARMS) - collects spontaneous reports from consumers and health professionals regarding alleged adverse effects from food products.

The Cosmetic Adverse Reaction Monitoring System (CARMS)- collects spontaneous reports from consumers and health professionals regarding alleged adverse effects from cosmetic products.

In addition, CFSAN receives adverse event reports linked to the products it regulates through FDA's MedWatch[2] program.[(4)]

2. Analytic Epidemiology Studies

Although analytic epidemiology studies are more informative than descriptive studies, they are expensive and time-consuming to conduct. The types of analytic epidemiology studies commonly considered by CFSAN in safety evaluations include cross-sectional, prospective, and retrospective studies. Results from such studies, when available, are used in the overall safety evaluation of regulated products. In addition, analytic epidemiology studies constitute the scientific base for the Agency's regulation of health claims on food and food labeling authorized by the Nutrition Labeling and Education Act of 1990.

a. Cross-Sectional Studies

Cross-sectional studies are those in which individuals are observed at only one point in time; such studies are commonly known as surveys. The presence or absence of disease and the presence or absence of suspected etiologic factors are determined in each member of the study population or in a representative sample at one particular time. The advantages of cross-sectional studies are that they are relatively inexpensive to conduct, and can be completed relatively quickly. However, cross-sectional studies reveal nothing about the temporal sequence of exposure and disease, and necessarily use current exposure as a surrogate for past exposure. Also, cross-sectional studies can only measure disease prevalence rather than incidence.

b. Prospective Studies

In prospective studies, also called cohort or follow-up studies, the investigator selects a study population of exposed and non-exposed individuals and follows both groups to determine the incidence of disease. The group can be characterized by factors thought to influence the development or course of the disease and by the presence or absence of risk factors (*e.g.*, exposure or nonexposure to some agent). Prospective studies generally imply study of a large population, study for a prolonged period of years, or both. This type of study design is effective when there is good evidence of an association of the disease with a certain exposure (from clinical observations or from descriptive epidemiology studies), when exposure is rare, but incidence of disease among the exposed is high, and when the time between the exposure and disease is short. The major advantage of prospective studies is that the incidence rates of the disease under study can be measured directly; therefore, absolute and relative risks also can be measured directly. In addition, it is possible to analyze the association of a particular exposure with several diseases, and a temporal relationship between exposure and disease can be established.

There are a number of disadvantages to prospective studies, including: 1) The difficulty and expense of conducting the studies, since both large study populations and long periods of observation are required for definite results; 2) bias may be introduced if every member of the cohort is not followed; 3) the length of the study may be less than the latency period of the disease; for example, if the study is stopped before old age, many important diseases such as cancer may be missed; and, most importantly, 4) prospective studies are very inefficient for studying rare diseases.

Results of prospective studies have been used at CFSAN in assessing the potential carcinogenic risk of some compounds; for example, occupational cohort studies and studies of human populations accidentally exposed to a carcinogen have been used in safety assessments of benzene, dioxin, and methylene chloride. FDA has also provided financial support for prospective studies on accidental exposure to PBB's in a Michigan cohort, and exposure to methylmercury in fish in a cohort of pregnant women (and their offspring) in the Seychelles Islands.

c. Retrospective Studies

In retrospective studies, also known as case-control studies, the investigator selects cases with a specific disease, and appropriate controls without the disease, and obtains data regarding past exposure to possible etiologic factors in both groups. The rates of exposure of the two groups are then compared. A case-control approach is preferred when studying rare diseases, such as most cancers, because a very large number of individuals would be needed in order to draw conclusions in a prospective study. Although it is possible to detect the association of multiple exposures or factors with a particular disease, retrospective studies are generally used to study diseases that have some unique and specific cause, such as infectious agents, in order to avoid the problem of confounding etiologic factors.

Case-control studies can not determine directly absolute risk or relative risk because the incidence of disease is not known in either the exposed or unexposed population as a whole. However, the relative risk can be estimated in retrospective studies by the odds ratio, which is the ratio of the odds of exposure among cases divided by the odds of exposure among controls. The odds ratio is a good approximation of the relative risk when the subject cases are representative of all cases with regard to exposure, the controls are representative of all controls with regard to exposure, and the disease being studied is rare.

Retrospective studies are much less expensive and less time consuming to conduct than are prospective studies; usually, a relatively small population is needed for the study. Also, since the study selects only cases of the disease of interest, there is no bias incurred in determining the endpoint. However, bias is frequently incurred during detection and selection of cases, and during assessment of exposure. Controls should be identical to the exposed cases except for the factor under investigation, a requirement which is often difficult to achieve in practice. As with prospective studies, problems are frequently encountered in attempting to control for competing risk factors and confounders. The investigators can adjust for known confounders either by matching when selecting controls, statistically by stratification, or by use of regression models.

Results of case-control studies have been frequently used in safety evaluations at FDA, primarily to add further information to the overall assessment of safety. In the past, FDA has supported case-control studies on compounds of interest, such as the National Bladder Cancer Study and the use of artificial sweeteners. In addition, FDA often looks carefully at the results of case-control studies in the setting of outbreaks of food-borne disease to identify the food vehicle that was most likely responsible for transmitting the infectious agent. The results then can be used to help target specific food vehicles for microbiologic testing as a means of recovering the pathogen from the implicated food.

d. Meta-Analyses

Meta-analysis has been defined as "the statistical analysis of a large collection of analysis results from individual studies for the purpose of integrating the findings".[3] The results of a well-done meta-analysis may be accepted as a way to present the results of disparate studies on a common scale; however, caution should be exercised before attempting to reduce the results to a single value as this may lead to flawed conclusions.[2]

Several publications in the peer-reviewed literature serve as guidelines for the appropriate conduct of meta-analysis,[6],[7] the principal components of which include:

- Identifying criteria for the inclusion and exclusion of studies and avoiding biases in this process;
- Deciding whether the characteristics of study subjects, their interventions, and outcomes in each study are comparable;
- Using well-defined methods for extracting data from the studies;
- Expressing the results of multiple studies in a consistent fashion;
- Using appropriate statistical methods to assess the data.

Where FDA evaluates a meta-analysis, the Agency considers such an analysis primarily as supporting evidence, rather than as primary evidence, that can confirm the validity of data concerning a hypothesis. The Agency must carefully scrutinize each meta-analysis to assess the soundness of its design and the quality of the data from individual studies to determine the significance of the data. Such scrutiny requires review of the original studies used for the meta-analysis.

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Redbook 2000: VII Glossary: Acronyms and Definitions

April 2004

Toxicological Principles for the Safety Assessment of Food Ingredients
Redbook 2000
Chapter VII. Glossary: Acronyms and Definitions

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Acronym	Definition
Act	"The Act", i.e., Federal Food, Drug, and Cosmetic Act
ABS	chromosome aberration(s)
ADI	acceptable daily intake
A/G	albumin-to-globulin
ANOVA	analysis of variance
B-cells	B lymphocytes
B/T	ratio of B to T lymphocytes
CAC	Cancer Assessment Committee
CAS	Chemical Abstract Service
CCFAC	Codex Committee for Food Additives and Contaminants
CFR	Code of Federal Regulations
CFSAN	Center for Food Safety and Applied Nutrition
CHO	Chinese hamster ovary [cell(s)]
CMI	cell mediated immunity
CSO	consumer safety officer
DNA	deoxyribonucleic acid
DTH	delayed type hypersensitivity
EAFUS	Everything Added to Food in the United States (database)
ECVAM	European Centre for the Validation of Alternative Methods
EDI	estimated daily intake

ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency (United States)
FAP	food additive petition
FASEB	Federation of American Societies for Experimental Biology
FASP	food additive safety profile
FCS	food contact substance
FCN	Food Contact (Substance) Notification
FDA	Food and Drug Administration
GLP	good laboratory practices
GMPs	good manufacturing practices
GRAS	generally recognized as safe
HGPRT	hypoxanthineguanine phosphoribosyl transferase activity
HTD	highest treatment dose
IARC	International Agency for Research on Cancer
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
Ig	immunoglobulin
JECFA	Joint (FAO/WHO) Expert Committee on Food Additives
LOEL	lowest observed effect level
LPS	lipopolysaccharide
LSD	least significant difference (refers to statistical test)
MFO	mixed function oxidase
ML	L5178Y mouse lymphoma cell
MLA	mouse lymphoma assay
MLR	mixed lymphocyte response
MTD	maximum tolerated dose
OECD	Organization for Economic Cooperation and Development
OFAS	Office of Food Additive Safety (CFSAN)
PAFA	Priority-Based Assessment of Food Additives (database)
PALS	periarteriolar lymphocyte sheath
PB-PK	physiologically based pharmacokinetic model
PHA	phytohemagglutinin
PWM	pokeweed mitogen
QAU	Quality Assurance Unit
QRAC	Quantitative Risk Assessment Committee
QRAs	quantitative risk assessments
RBC	red blood cells
Redbook I	*Toxicological Principles for the Safety Assessment of Direct Food Food Additives and Color Additives Used in Food (1982)*
Redbook II	draft *Toxicological Principles for the Safety Assessment of Direct Food Food Additives and Color Additives Used in Food (1993)*
Redbook	*Toxicological Principles for the Safety Assessment of Food Ingredients (2000, additions and*

2000	*updates in 2001, 2003, 2004)*
RIA	radio immunoassay
RNA	ribonucleic acid
SAR	structure activity relationship
SCE	sister chromatid exchange
SHE	Syrian hamster embryo cell
SOP	standard operating procedure
SRBC	sheep red blood cells
T-cells	T-lymphocytes, or thymus derived cells
TK	thymidine kinase
UDS	unscheduled DNA synthesis
WBC	white blood cells
WBA	whole body autoradiography

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Food

1993 Draft Redbook II

Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food *Redbook II* Draft Guidance

This draft guidance represents the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if such an approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach contact the FDA staff responsible for implementing this guidance (Office of Food Additive Safety, 301-436-1200).

Draft Redbook II was distributed in 1993 by the Office of Premarket Approval. Effective June 18, 2001 the Office of Premarket Approval is now the Office of Food Additive Safety. Based on comments, experience, and other information, FDA staff has revised and issued several sections of the draft as final guidance in Redbook 2000[1]. Those sections of the 1993 Draft Redbook II not yet available in Redbook 2000 are being placed here for your reference.

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- 1993 Draft "Redbook II" Chapter II Agency Review of Toxicology Information in Petitions for Direct Food Additives and Color Additives Used in Food (PDF - 86KB)[36]
- 1993 Draft "Redbook II" Chapter IV B 5: Diets for Toxicity Studies (PDF - 73KB)[37]
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Chapter VII

Emerging Issues in Safety Assessment of Food Additives and Color Additives Used in Food

A. Introduction

This section discusses approaches to testing that may be useful in assessing the safety of macro-additives (see **Chapter VI B**), bioengineered additives (see **Chapter VII C**), additives that are enzymes (see **Chapter VII D**), and microbially-derived additives (see **Chapter VI E**). This section also discusses the use of alternatives to whole (vertebrate) animal testing in safety evaluation (see **Chapter VII F**) and FDA's recognition of the potential for direct food additives and color additives used in food to cause both heritable and somatic genetic toxicity (see **Chapter VII G**).

Because the Agency's approaches to determining the safety of these additives will continue to evolve for some time, it is not yet appropriate to provide separate guidelines for acquiring toxicology information on the types of additives in this document. In general, the Agency recommends that petitioners follow guidelines for toxicity tests presented in other sections of this publication. In addition, this section suggests some important issues to consider when planning a program of toxicity testing designed to demonstrate the safety of unique additives. As always, we strongly recommend that petitioners discuss planned testing programs and protocols for toxicity tests with Center scientists before tests begin.

B. Macro-Additives

Macro-additives are a class of food additives that are intended to be replacements for conventional macro-nutrients such as fats, proteins, and carbohydrates and are intended for use at relatively high levels in food. Macro-additives may be nutritive or non-nutritive; they may be reasonably pure, well characterized chemicals or they may be complex mixtures whose complete characterization is not feasible; they may be well absorbed from the gastrointestinal tract or poorly absorbed; they may be manufactured from unusual or novel food sources or obtained by chemical synthesis.

The common characteristic of macro-additives is that they will be consumed in large quantities compared to conventional food additives and, as a consequence, they will present testing problems that require "customized" approaches. For example, it may not be feasible to calculate safety factors in the conventional way, that is, as a fraction of the highest oral dose that has no adverse effects in animals. Other means of providing margins of safety for macro-additives will have to be used; these may include information derived from metabolic, pharmacokinetic, and human clinical studies.

1. Nutritional Concerns in Animal Toxicological Tests

Because of the expected high level of human consumption of these additives, animal test doses that are orders of magnitude greater than the Expected Daily Intake (EDI) for humans will often not be feasible. Attempts to achieve very high doses in the animal studies might result in nutritional imbalances or caloric deprivation that could confound interpretation of the toxicity studies. In order to test the highest dose feasible and yet avoid nutritional problems, it may be necessary for toxicity testing to be preceded by nutritional studies to determine adequate test diets and appropriate control diets for animals in toxicity studies.

If appropriate dietary controls include nutrient enhancement, care should be taken to avoid over-enriching the diet or changing nutrient ratios that would mask toxicological endpoints under consideration. For example, mineral oil as a test material would be mostly unabsorbed in the intestine where it would solubilize fat-soluble vitamins, leading to deficiencies of these nutrients. This effect may be eliminated by appropriate fortification of the diet with vitamins A, D, E, and K. Quantities of nutrients to be used for fortifying the diet should be determined experimentally, in relation to the amount of mineral oil (test substance) used. Under-fortification could fail to protect against nutrient deficiencies and over-fortification could lead to altered toxicological responses to xenobiotics and "background" pathology rates. Sufficiently great over-fortifications could produce hypervitaminosis.

Control and test diets should be of the same caloric density and nutritionally (micronutrients) equal to test diets. Selection of appropriate control diets may present particular problems when testing non-caloric food substitutes or food substitutes that interfere with absorption of nutrients. Due to nutrient variations in chow diets from batch to batch, it is preferable to use a semi-purified diet base in these studies.

Additional information can be found in **Chapter IV B 5**, Diets for Toxicity Studies and in **Chapter IV B 1**, General Guidelines for Toxicity Studies.

2. Absorption, Metabolism, Distribution, and Elimination Studies

Studies designed to follow the metabolic path and fate of macro-additives take on particular importance in providing assurance of safety if the conventionally calculated safety factor cannot be used. Greater understanding of the disposition and pharmacokinetics of the additive should help to diminish uncertainties regarding safety. Questions of the following types should be answered through appropriate studies:

- Does the product or its metabolites alter or interfere with absorption, metabolism, or excretion of normal nutrients or metabolic intermediates?

- Does the product or its metabolites alter the action of commonly used drugs?

- Is the product absorbed, metabolized, distributed, stored or excreted differently in man than in test animals?

- Does the product or its metabolites accumulate in tissues, and what are the toxicological consequences if there is accumulation?

- If the product is poorly absorbed, does the high concentration in the gut affect gut morphology, physiology, or biochemistry? Are any changes in the gut morphology or biochemistry associated with the development of neoplasms of the gut?

- Does the product alter the composition or nature of the gut flora? If it does, what are the toxicological consequences of the changes?

3. Impurities and By-products

Because of the anticipated high human consumption levels of macro-additives, there is a concomitant high potential intake of impurities and by-products. Therefore, every effort should be made to identify and quantify the chemical constituents of the product. If any of these raise particular concerns, toxicity testing of the impurity or by-product itself may be recommended. Limits for impurities such as heavy metals, natural toxins, and anti-nutrition factors may need to be specified for the marketed product.

4. Clinical Studies

When animals studies have been completed or when there is reasonable assurance of safety of the macro-additive from animal studies, clinical studies with human subjects may be useful for increasing confidence in the safety of the product for human consumption. For example, humans may suffer subtle adverse effects not detected in animal studies due to differences in physiology or metabolism between animals and humans; human subpopulations (the old, young, and chronically ill) may each react differently to the food substitute. In addition, human studies may help compensate for the fact that conventional methods of calculating the Acceptable Daily Intake (ADI) may not be applicable to the results of standard toxicity studies on macro-additives.

VII D. Enzymes

Commercial enzyme products may be obtained from edible plants and animals and from non-toxigenic, non-pathogenic microorganisms. Questions about the microbial source of the enzyme (see **Chapter VII E**) and the nature and level of enzyme preparation in the food are of concern in evaluating the safety of commercial enzyme products because they influence the type and level of contaminating impurities in the food.

In general, enzyme preparations from organisms with a history of safe use do not require the same level of toxicological testing as enzymes from sources without a history of safe use in food. The safety of commercial enzyme products from sources without a history of safe use in food usually is evaluated on a case-by-case basis, but some generalizations about toxicology tests for these food additives can be made.

Because of the protein nature of enzymes and their susceptibility to digestion when consumed, residues of pure enzymes in processed food would be expected to have only limited toxic potential. If highly purified preparations of microbial enzymes are used in food processing, exposure to the enzymes is usually reduced to the parts-per-billion range. Such a level of exposure would ordinarily be too low to pose a safety concern, and toxicological testing may not be required. An exception to this generalization may occur if review by the Center's chemists results in concern for the presence in the enzyme preparation of a toxic material used in the purification process; however, this is unlikely because of the requirement that food grade chemicals be used in purification.

In most cases, however, commercial enzyme products from microbial sources are only partially purified. A variety of uncharacterized extraneous substances ("impurities") of biological origin may be present in the enzyme preparation at levels comparable to the active ingredient. These substances have no technical effect in food processing, but are allowed to remain in the enzyme products because the impurities do not interfere with enzyme function. In addition, the enzyme preparation may contain multiple enzyme activities that serve a variety of useful functions in processing food. When the types and levels of impurities in commercial enzyme products from microbial sources are considered to be significant, the Agency may recommend that safety be established by appropriate toxicity testing. Such a requirement usually can be met by 90-day toxicity studies in the rat and the dog. However, if review of the safety of the enzyme preparation raises questions about chemical contaminants, stability of the microbial strain, production of toxic products, *etc.*, additional studies may be needed.

Enzyme products may be added directly to the food to be processed (*e.g.*, rennet) or they may be immobilized on an insoluble matrix for use in processing liquid foods. Enzymes are immobilized by secure bonding (usually by means of a chemical reaction) to an insoluble matrix. Liquid food products (*e.g.*, corn syrup) may be processed by passage over a column of the immobilized enzyme. Only negligible amounts of the immobilized enzyme are expected to enter the processed food. Depending on the nature of the immobilization matrix, however, some potential exists for contamination of the processed food by chemicals used in the immobilization process. If the Agency decides that information about the nature of the fixing agent and its potential migration to food raise questions of safety for foods processed by passage over an immobilized microbial enzyme, the Agency will recommend that the immobilized enzyme be subjected to 90-day toxicity studies in the rat and the dog or other appropriate study.

As described in the preceding paragraphs, a variety of factors will be taken into account by the Agency in deciding what information is needed to assess the safety of additives that are enzymes. Before conducting toxicity studies to assess the safety of such additives, petitioners should consult with Agency scientists. A comprehensive review of the safety concerns relating to additives that are enzymes will be issued in a separate publication.

VII E. Microbially Derived Food Ingredients

Microbially derived food ingredients may be food additives (including enzymes), color additives used in foods and substitute foods. A unique concern about the safety of microbially derived food ingredients is the microbial source; except for this concern, the safety of these ingredients will be evaluated as for analogues, non-microbially derived ingredients. A variety of factors will be taken into account by the Agency in deciding what information is needed to assess the safety of microbially derived food ingredients. Before conducting toxicity studies to assess the safety of such ingredients, petitioners should consult with Agency scientists. A comprehensive review of the safety concerns relating to microbial sources will be issued in another publication.

VII F. Advances in the Development of Alternatives to Whole Animal (Vertebrate) Testing

Because animal experimentation has become an emotional issue, it is important to recognize the growing impact of *in vitro* toxicology on the practice of toxicology. Although the field is often termed "alternative," experimental models have been applied to the three "R's" of Russel and Burch: [1] to replace animal models, to reduce the number of animals used, or to refine test methods to minimize stress and suffering to animals.

This section is not intended as a guideline but serves to identify a future direction in methodology. In the context of this document, "alternatives to whole animal (vertebrate) experimentation" refers to *in vitro* tests for potential toxicity that substitute for or replace *in vivo* (whole animal) studies. "*In Vitro*" literally means "in glass", and is interpreted to mean "in a test tube" or "outside of the body".[2] Alternative tests include short-term tests using isolated cells, tissues, and organs and studies involving mathematical modeling, epidemiology, or the use of human volunteers; short-term tests for genetic toxicity (see **Chapter IV C 1**) are excluded.

In practice, alternative tests are used to support the planning and interpretation of whole animal toxicity studies and are not yet used as substitutes for toxicity studies using whole animals. For example, an alternative test may be used 1) to determine the relative biological potency of a series of toxicants at the cellular level, 2) to select the animal model in which to conduct an *in vivo* test by comparing the metabolic properties of a toxicant at the cellular level in several species, and 3) to identify mechanism(s) of toxicity by defining the relationship between exposure to a toxicant and development of various toxicological endpoints at the cellular, subcellular and molecular levels of organization.

Recent advances that have been made in *in vitro* studies with isolated cells, tissues, and organs have directed the scientific community toward developing, validating, and evaluating alternative test systems. The predictive value of a standardized test must be assessed by means of a series of validation studies. Validation can demonstrate that the use of an *in vitro* test is equivalent to the use of an established *in vivo* test or that the *in vitro* test accurately predicts human toxicity. Anticipating a continued increase in the development and use of alternative *in vitro* test systems, [3,4] the Agency encourages the development of approaches that can provide information relevant to the assessment of human risks.

1. Reasons for Developing Alternative Tests

Several reasons to encourage the development of alternative *in vitro* tests are listed below:

- Economy and efficiency: Once established, *in vitro* tests may provide toxicity information in a cost-effective and time-saving manner. Information generated from *in vitro* test systems can be used to increase the efficiency of whole-animal studies and decrease the number of animals used in toxicity testing. The relative simplicity and space-saving characteristics of *in vitro* methods also are viewed as advantages.

- Information about human risk: Human cells, ethically obtained and successfully established *in vitro*, may provide information about a toxicant that is relevant to human risk. For example, a toxicant's mechanism of action or metabolism in human cells can provide the basis for selecting a suitable animal model for long-term toxicity studies.

2. Possible Applications of Alternative Tests

- Isolated cells, tissues, and organs can be prepared and maintained in culture by methods that preserve

properties characteristic of the same cells, tissues, and organs *in vivo*. Using such *in vitro* systems will permit data to be generated under controlled experimental conditions and in the absence of many complicating factors characteristic of experiments with whole animals. For example, the use of cell culture systems will enable the metabolism of a toxicant that occurs in one type of cell (*i.e.*, hepatocyte cells) to be studied separately from a toxic endpoint that occurs in a different cell type.

- Several toxic endpoints may lend themselves to quantification in an *in vitro* test system. Relevant endpoints could be identified by comparing the action of a toxicant at cellular, subcellular or molecular sites with the toxic effects observed in the target organ or tissue *in vivo*. Analysis of a broad spectrum of *in vitro* cellular events may provide information about the *in vivo* progression of a toxic response as a function of toxicant concentration and time.

- Because *in vitro* procedures have the potential to yield reproducible measurements, they theoretically lend themselves to standardization. However, interpreting data obtained from a standardized *in vitro* toxicity test with a reasonable degree of confidence can only occur after potential confounding factors, such as interactions between the test agent and non-cellular components of the test system, have been identified or eliminated. [5]

- The process of validation appears to be key to the full acceptance of alternative tests where the reliability and relevance of procedures are established for specific purposes.[6] While there is much discussion about the framework for this process, several components appear essential to the overall coordination of the validation process, including: scientific consensus on the definition of a validated test, reference chemicals with defined toxicity and general availability, a central repository for test performance data and protocols, an established network of laboratories with the capabilities of method validation, and scientific understanding of the mechanistic basis of the toxicological process involved. An impartial and competent group of scientists from regulatory agencies and the research community could facilitate the implementation of the validation process.

3. Limitations of Alternative Tests

Limitations of *in vitro* tests are well known. For example:

- *In Vitro* test systems are not available for all tissues and organs. In addition, normal systemic mechanisms of absorption, penetration, distribution, and excretion are absent from *in vitro* test systems. *In Vitro* systems lack the complex, interactive effects of the immune, blood, endocrine systems, nervous system, and other integrated elements of the whole animal. Thus, *in vitro* tests cannot be used to study the complex nature of systemic toxicity.

- Validation of new methods is time-consuming and expensive; acceptance of *in vitro* tests as alternatives to traditional toxicity testing in whole animals is expected to be slow. [7] While many schemes have been proposed to expedite these processes, no alternative *in vitro* test presently can replace an *in vivo* toxicity study.

4. Current Use of *In Vitro* Tests

Numerous & diverse *in vitro* tests have been developed. Their importance and use have been discussed in any publications. [8-23] Many of these tests will be improved over time by the introduction of new scientific information and technological advances in *in vitro* toxicology and related fields, such as molecular biology and biotechnology. The Agency encourages the development and use of *in vitro* test systems for planning and interpreting the results from whole animal toxicity studies.

Significant advances have been made in the development of *in vitro* alternatives for ocular safety testing.[24-27] Other *in vitro* systems have been proposed which measure a broad range of endpoints and are now in various stages of validation. The Agency is currently part of an interagency regulatory groups evaluating these proposed alternative test methods.

In Vitro approaches to toxicity testing can provide useful data when integrated with other information about the toxicity of food and color additives used in food. Results of *in vitro* tests can be used to optimize the design of conventional toxicity tests for a particular test substance by helping to determine appropriate dose levels and by helping to decide which species is the best model for man. Such improvements in the design of whole animal toxicity tests may reduce the number of test animals required to produce useful information about the safety of proposed food and color additives used in food.

In Vitro tests can help elucidate the nature of the interaction between test substance and organism at the cellular, subcellular, and molecular levels. Thus, once the critical target organ or organ system has been identified in whole animal studies, *in vitro* tests can focus on the mechanism of action of the test substance at the target site. Information from these studies can assist the Agency in making decisions about the safety of proposed food and color additives used in food by comparing responses observed in human and animal cells and by facilitating extrapolation from high-dose to low-dose responses.

At present, in evaluating a petition for the use of a food or color additive, the Agency considers *in vitro* tests to be useful in helping to identify the mechanism(s) of action of the test substance and to provide information about subtle effects observed *in vitro* that may not be observed in *in vivo* studies

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VII G. Heritable and Somatic Genetic Toxicity

This chapter discusses FDA's interest in direct food additives and color additives used in foods that can cause both heritable and somatic genetic toxicity. While the FDA currently neither recommends specific tests to determine somatic and heritable genetic toxicity, nor regulates food and color additives used in food on the basis of such activities, the Agency has an heightened interest in this area.

1. Rationale for Testing for Heritable and Somatic Genetic Toxicity

Heritable genetic toxicity is chemically-induced damage to the DNA of male and female germ-line cells that is not correctly repaired, so that the damaged gene(s) can be inherited. The consequences of this genetic toxicity has been well documented, and a number of different genetic diseases have been characterized. Somatic genetic toxicity is chemically-induced damage to the DNA of dividing and non-dividing somatic cells (*i.e.* non-germ-line cells). The consequence of somatic genetic toxicity is that chemicals may alter gene functions in rapidly dividing somatic cells (*e.g.* intestinal lining and bone marrow) and in quiescent cells which may be forced to replicate in response to a regenerative or mitogenic stimulus (*e.g.* G_oG_1 peripheral lymphocytes). Genetic damage to these cells can lead to cancer and alteration of critical cellular functions (*e.g.* altered hormone and receptor site functions).

2. Rationale for Selecting a Specific Test Battery

Currently the Agency recommends the use of a battery of genetic toxicity tests (see **Chapter IV C 1 c**) for all chemicals that are direct food additives or color additives used in foods, including chemicals with structures assigned to all three structure categories (see **Chapter III B 2**), as well as chemicals associated with Concern Levels I, II, and III (see **Figure 4** in **Chapter III B 1**). These tests are recommended to evaluate the genetic toxicity of chemicals in order to identify those chemicals that may be direct acting carcinogens (see **Chapter IV C 1**).

Short-term tests for genetic toxicity can also be conducted to evaluate the effects of chemicals on the genetic material of both somatic and germ-line cells, and the tests used for these purposes can overlap those used for predicting carcinogenicity. For example, the data obtained from the *Salmonella typhimurium* reverse mutation assay is not only useful in predicting the potential carcinogenicity of test substances,[1,2,3] but it is also an important means of determining whether a chemical has the potential to damage the genetic material in both germ-line and somatic cells. Although FDA considers the information obtained from the test battery recommended in **Chapter IV C 1** to be useful in assessing a chemical's potential to cause heritable and somatic genetic toxicity, the scientific community has not yet reached a consensus that these indicators are reasonably predictive of human responses.

While FDA does not recommend a unique battery of tests for determining heritable and somatic genetic toxicity, the Agency recognizes that certain types of tests may be useful for this purpose.

Historically, gene mutations in germ line cells have been detected using *in vivo* tests such as the sex-linked recessive lethal assay in *Drosophila melanogaster* and rodents. [4,5,6] Unfortunately, the standard classical assay procedures are not completely satisfactory; each of these tests has one or more of the following limitations:

- standard procedures have a very low sensitivity for detecting known mutagenic chemicals, and the assays fail to detect dose-related increases in chemical activities;

- standard protocols have many deficiencies (*e.g.* they frequently lack concurrent positive controls, multiple test chemical doses are rarely used, *etc.*);

- standard protocols for heritable genetic toxicity cannot simultaneously measure somatic cell toxicity in the same animals; and

- standard methods require large numbers of animals and are very time consuming and expensive.

Thus, two groups of tests may provide a sensitive method for detecting heritable and somatic cell genetic toxicity. First, a battery of tests for germ-line and somatic cell genetic toxicity should include the same short-term genetic toxicity tests used to predict potential carcinogenicity {*e.g. Salmonella typhimurium* reverse mutation assay, *in vitro* ML mutation assay and an *in vivo* cytogenetics assay (see **Chapter IV C 1**)}. Second, a battery of tests for germ-line and somatic cell genetic toxicity also should include the use of transgenic mice. The Agency recognizes that current genetic toxicity tests using transgenic animals do not directly demonstrate heritable genetic toxicity effects; however, chemical-induced genetic toxicity to germ cells demonstrates the potential for this to occur. Since research with several different experimental rodent models has been progressing rapidly, and a variety of transgenic rodents are now commercially available, it may be possible in the future to simultaneously assess chemically-induced genetic damage to germ line cells and to a variety of somatic tissues. The transgenic test system should have several advantages over classical tests for heritable genetic toxicity:

- the investigator can easily manipulate the treatment conditions so that tissue-specific toxicological effects can be compared for different assay protocols;

- the test requires relatively few animals (*i.e.* 2 or 3 animals per treatment group); and

- the test is relatively inexpensive and can be performed in a matter of days.

FDA continues to encourage the scientific community to develop sensitive assays for detecting germ-line and somatic cell genetic toxicity.

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