

TOXICOLOGICAL PRINCIPLES

for the Safety Assessment
of
Direct Food Additives
and
Color Additives Used in Food



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PREFACE



The assurance of safety of all food and color additives is the responsibility of the Food and Drug Administration. Over the last two decades it has become increasingly apparent to those in the government, the public, and the private sectors, that development of information for the assurance of safety can and must be acquired as cost-effectively as possible within the constraints of finite resources. Therefore, it is necessary to provide appropriate guidance regarding the criteria used for food and color additive safety evaluation. Information requirements need to be well defined and commensurate with the potential for the additive to cause safety concerns.

The "safety" of food and color additives is defined in sections 70.3 and 170.3 of the Code of Federal Regulations (21 CFR 170.3) as a reasonable certainty that a substance is not harmful under the intended conditions of use. The following document represents the latest effort on the part of FDA to delineate the sensitivity and rigor of toxicological and other information needed to make safety determinations for direct food additives and color additives used in food. With this document the agency is attempting a better definition of the boundaries of the "reasonable certainty" requirement, while at the same time retaining needed flexibility in this rapidly changing scientific area. The agency is hopeful that the document is sufficiently detailed to stimulate comment and scientific discussion on several key issues including the following: the use of exposure information and molecular structure information; the proper role for short-term tests for carcinogenicity potential; construction of a tiered system for information development; use of data from previously performed toxicological studies; guidelines for developing safety information on new additives; and the use of priority-setting in managing risks from all additives.

On the whole, the specific requirements of this document do not differ greatly from what the agency now requires for making a safety determination on direct food additives or color additives used in food. What is new is that the basic scheme of scientific decision making for the development of that safety information is now structured around a more cost-effective and flexible framework. The document also describes the agency's priority system for all direct food additives and color additives used in food that would allow the agency to better direct its resources to those potential issues that are most likely to benefit the public health. FDA believes that under the proposed system, approvals of new additives can be more timely and efficient and the agency can keep abreast of new developments and maintain a better and more comprehensive overview of issues that affect the public health.

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1. Introduction

During the past two decades, significant changes have taken place both with respect to the technology of food production and processing, including the use of additives, and also with respect to the scientific criteria used to establish the safety of additives.

The following document results from work that the agency has recently undertaken to develop scientific criteria for establishing the safety of new additives, and to assure their continued safety in view of possible trends in their use levels and the constantly evolving scientific criteria for safety evaluation.

The system outlined in this document has been designed to apply to direct food additives and to color additives used in food but not to indirect food additives. Throughout the document, the term "additive" is used to denote both direct food additives and color additives used in food.

The safety review of indirect additives often involves different chemical structure classes, and special problems in estimating consumer exposure, including the possibility of migration of miniscule amounts of chemical substances to food that make them of extremely low or no toxicological concern in terms of food safety or for the purposes of applying legal standards. Therefore, FDA intends to publish a separate system of tiered information requirements for indirect additives. Such a system would have many conceptual elements in common with the present system, but specific toxicological information requirements for indirects may differ somewhat in scope and substance from those for direct additives.

A. Background

1. Changes in regulatory toxicology

The approval of any new food or color additive depends upon the outcome of toxicological tests that are performed prior to marketing. The last quarter century has been a period of change and progress in the fields of regulatory toxicology and analytical chemistry. Though many features of today's toxicological test regimens were also present in 1958, the field has nevertheless advanced in many new areas. (Refs. 1,2) Not only have test requirements become generally more sophisticated, but scientists understand more fully the public health significance of test results.

a) Changes in test requirements

Today, toxicological criteria and standards are generally more rigorous and test endpoints are more sensitively measured than 25 years ago. (Ref. 2) Early investigators of chronic noncarcinogenic toxicity tended to use 10 or fewer test animals per group. (Ref. 3) When the Food and Color Additive Amendments to the Act were passed, procedures for carcinogenicity testing were relatively unsophisticated. Even in 1970, groups of 20 to 30 rodents per sex per dose level were often considered sufficient. (Ref. 4) Only recently

(1976) has the National Cancer Institute developed minimum testing guidelines for determining chemical carcinogenicity. (Ref. 5) Now scientists accept a properly conducted, long-term chronic toxicity test as the definitive model for estimating the carcinogenic risk of food chemicals for humans. (Refs. 2,6) These and other such tests currently used for purposes of federal regulation often initially employ a minimum of 50 rodents per sex per dose in each of 2 species. (Ref. 7). Certain types of tests may employ fewer rodents per group if the number of dose levels is increased. However, little current literature now exists to support use of groups of fewer than 20 animals in chronic studies. (Ref. 8)

Increases in the numbers of test animals have been accompanied by improvements in animal husbandry, and general laboratory techniques, (Ref. 6) leading to an increase in the level of statistical sensitivity of these tests.

Chronic tests, when performed according to modern protocols allow a more thorough investigation of numerous subtle chronic effects, (Ref. 6) and even well-conducted sub-chronic studies in rodents are now capable of detecting the early signs of most chronic effects except cancer. (Refs. 8, 9).

b) Advances in scientific technique and understanding

It is generally agreed that additional research is needed to define more fully the molecular events that are the basis for adverse toxic responses in biological tissues. Nevertheless, significant progress has been made, even since 1970, in understanding fundamental relationships between exposure to chemicals and possible toxic responses in animals and humans. (Ref. 2)

Today there is increased emphasis on understanding the potential of a compound to cause specific types of toxicity such as reproductive, teratological, behavioral or mutagenic effects. For example, FDA often recommends that petitioners include studies of the toxicological pharmacokinetics (including metabolism) of food chemicals in the safety evaluation of food and color additives. Such studies can help provide data for more reliable extrapolations and prediction of human response to food chemicals. Studies of the genetic toxicity of food chemicals are recent additions to the battery of toxicology tests. These and other short-term tests are important tools for predicting the potential for hazard in man and are often used effectively in conjunction with the chronic 2-year tests. (Ref. 2)

A growing base of experimental data now permits at least tentative predictions of toxic potential of compounds based upon knowledge of their molecular structures. (Refs. 10-14) In similar fashion, improvements in the field of analytical chemistry during the last 25 years have enhanced the ability of chemists to detect the presence of chemical substances in foods and to detect impurities and minor constituents in food additives themselves.

2. Changes in the food and in the use of food and color additives

Many changes in the food supply can be associated with fundamental changes in the American lifestyle. The American diet has changed with increasing consumption of processed food, a trend that is apparently accelerating. If processing is defined as "anything the food industry does to food beyond the simplest preparation for sale," well over 50 percent of the food Americans now eat is processed. (Ref. 15) For example, the National Academy of Sciences (NAS) reported that the use of fresh citrus fruits dropped from 32 to 28 pounds annually per capita between 1960 and 1976, while consumption of processed fruit increased from 50 to 90 pounds per capita. The increase in annual soft drink consumption from 200 eight-ounce bottles per person in 1960 to 450 bottles per person in 1976 is another example of this continuing trend. (Ref. 15)

Changing work and leisure time patterns of Americans continue to reinforce the trend toward more meals being eaten away from home. The NAS reported that "expenditures for food away from home increased from one-quarter of the food budget 20 years ago to more than one-third of an approximately \$200 billion food budget in 1977." (Ref. 15) Many of these prepared meals may require the use of additives that facilitate mass distribution from a central preparation facility through retail outlets and food service institutions. The same lifestyle changes have increased the demands for processed "convenience foods."

Much of the interest in these trends has focused upon additives because they are substances intentionally added to food, whose use may be more readily controlled. (Ref. 16(a) and (b))

The President's Science Advisory Committee (1973), Panel on Chemicals and Health, (Ref. 17) cited a number of trends to which the increased use of additives may be attributed. The panel reported that changing patterns of intake have introduced more snack foods and more ready-to-eat foods, most of which contain a variety of additives. Additionally, the population shift from a rural to an urban base, greater interest in foods of ethnic origin, and consumer demands for a variety of foods without seasonal or geographic limitations, all contribute to new or expanded food processing practices. Moreover, economic realities may provide a continuing impetus toward reducing food processing and distribution costs, thus pointing to a continuing increase in the use of additives. (Ref. 17)

3. Issues raised by these changes

The increasing consumption of additives and changes in their toxicological safety criteria have important consequences for the food industry and the public, and for the agency's ability to assure a continued safe food supply. Many of the present food and color additive regulations were issued in the early 1960's. For example, from 1958 to 1967, the agency issued regulations for 303 direct food additives (not including most synthetic flavors); from 1967 to 1975, it issued regulations for only 67 direct food additives. (Ref. 18) Many presently regulated additives were approved for use in food based on scientific knowledge, standards and patterns of consumption that are now almost 20 years old.

Largely because of the types of changes noted above, safety decisions made at one point in time may become progressively incomplete because of the expanding base of knowledge. New, more discriminating tests performed on additives long after approval may raise concerns among the general public and the food industry.

The industry may suffer potential economic uncertainties and the public may be concerned by a perceived lack of relevance of such tests to their own health and safety. (Refs. 19, 20)

Though such instances have occurred relatively infrequently over the years, petitioners for new additives have often had to deal with uncertainty caused by evolving regulatory requirements and scientific knowledge needed for the approval of new additives. Industry has often cited the lack of explicit test guidelines as a source of uncertainty that inhibits new and important food processing developments. (Ref. 19) Testing requirements for certain types of substances have been cited by some as unnecessarily stringent, and by others as not rigorous enough. Many have cited the need for well articulated criteria for demonstrating the safety of additives. (Ref. 18)

The agency recognizes the significance of these changes. When these criteria change over time, safety evaluations may lack consistency, even when performed within the same relative time span. This fact may add to the difficulty of maintaining a comprehensive overview of all issues involving additives, increasing the probability that the "most recent" problem becomes the "most important" to public health.

B. Need for Criteria

Under the FD and C Act, the "safety" of a food or color additive must be established prior to marketing by an evaluation of probable exposure of consumers to the substance, and by evaluation of appropriate toxicological information. FDA regulations define the statutory terms "safe" and "safety" as a "reasonable certainty...that a substance is not harmful under the intended conditions of use." (Ref. 21(a), (b) and (d))

FDA has consistently taken the position that various scientifically valid types of data may properly support a finding that the proposed use of a food additive will cause "no harm" to consumers. For example, section 170.20 of the Code of Federal Regulations (21 CFR 170.20) which sets forth the general scientific criteria that FDA uses in evaluating a food additive petition, cites the "principles and procedures . . . stated in 'current' publications of the National Academy of Sciences, National Research Council" as a guide that the agency uses in its safety evaluation of food additives. (Ref. 21 (c)) NAS has written testing standards from time to time in separate publications for both public and agency use, but these testing requirements have been stated in relatively general terms. (Refs. 2, 22 (a) and (b), 23) In practice, FDA has applied toxicological criteria and exposure information that were current for the time in assessing the safety of each food additive. (Ref. 23) The agency has continuously adjusted food additive testing requirements as necessary to reflect both the steady progress of science and the most current information about population exposure to additives.

FDA is aware of the possible adverse consequences discussed above, that may result if the agency does not routinely publish modifications of its testing requirements. Therefore, the overall criteria the agency now employs for determining the safety of additives ought to be delineated clearly for all to comment on and use. This, of course, needs to be accomplished without forfeiting the flexibility that is required in this area of scientific decision making. The agency contemplates a scheme in which the criteria comprise a set of requirements that anyone may use.

while this scheme does not preclude a petitioner from demonstrating safety by using other types of data elements, a submission using the agency's scheme should normally provide sufficient scientific data to demonstrate safety. Moreover, using this scheme will enable FDA to focus available resources on solving problems that have the greatest importance to the public health. The data base that has accumulated over the years now indeed permits the agency to use such a "principle of commensurate effort" (Ref. 24) in determining additive testing requirements. For such a process to be effective in promoting and protecting public health, however, all elements of safety evaluation including continuous and comprehensive assessment of priority concerns must be related in a unified approach.

FDA believes that this scheme permits the agency to consider the potential adverse effects of consumer exposure, the quantity and quality of existing data, the cost of acquiring data, and the relative priorities for obtaining data, in a systematic way.

It is with these goals in mind that FDA has developed the following document.

C. Outline of the Document - Principal Elements

The following document has a two-fold purpose: First, to delineate the agency's most up-to-date scientific criteria for establishing the safety of direct food additives and color additives used in food, and second, to establish a process for monitoring that safety. The former answers the need for written criteria to help petitioners and the public to understand the scientific decision process by which the agency assures the safety of new food additives. The latter allows the agency to maintain a comprehensive overview of all approved food additives, in order to better assess the public health consequences that inevitably result from the changes that occur with the passage of time.

1. Safety Criteria*

An overall approach to safety assessment criteria may be structured conveniently under four basic premises, as follows:

The first premise is that, because additives are substances that people ingest, the agency should possess at least some toxicological or other biological safety information for each additive intended for addition to the food supply.

The second premise is that the proper level of toxicological verification of safety is dictated by the objective level of agency concern about potential public health consequences of consumer exposure. The same degree of information in terms of quantity and rigor is not required for all additives.

*Throughout this document, the term "criteria" will refer to the collective set of requirements including toxicological and exposure information against which all data on an additive should be compared in order to justify a finding of safety. Criteria include the list of toxicological tests that ultimately should be performed (including their test design parameters and quality evaluations), and all necessary information about the population exposure, molecular structure, purity and specifications of the additive substance. The term "toxicological criteria" refers to only those criteria that have direct bearing on toxicological feeding studies. The term "standards" refers specifically to that sub-set of authoritative principles that would be used to evaluate the quality and quantity of toxicological test data, including the statistical reliability and content of the data produced in new tests (current standards), as well as data that have been reported in previously performed studies (core standards). "Guidelines" is used throughout to designate the detailed test design parameters that the agency suggests for all toxicological tests that are ordinarily used to demonstrate the safety of additives.

The third premise is that some initial level of concern may be determined for any given additive, (even in the absence of toxicological data), by the potential population exposure and an estimate of toxicity based on the additive's molecular structure (see subsection II.B.2 below). An initial concern level such as this should be associated with a set of necessary tests that are sufficient to permit the agency to make an adequate determination of the safety of an additive. In principle, an additive that has undergone the appropriate tests and has shown no toxic effects at appropriate levels of intake would need no further testing to establish a finding of safety.

The fourth premise is that the initial set of tests could be adjusted if necessary whenever toxicological data of sufficient quality indicate the presence of significant adverse effects. The usefulness and validity of all toxicological data must be determined by the conformance of the tests to current standards.

Within the context of these premises, an overall approach to safety assessment criteria requires the following elements:

1. Information about population exposure to substances, (including information on purity and specifications of additives) and toxicological information about the intrinsic ability of each substance to cause adverse toxic responses (at a given exposure level) in humans
2. Standards to assess the quality and quantity of that information
3. Decision elements, a) for deciding whether any further data on a substance are needed, and for selecting appropriate studies, and b) for ranking the order in which selected tests ought to be conducted
4. Guidelines for performing studies so that the necessary amount of useful information is obtained.

The following document sets forth the toxicological safety evaluation criteria that the agency intends to employ in judging the safety of additives.

Section II below discusses the basic concepts that underlie a tiered system for information development (premise 2) including the "Concept of Concern," and the determination of "Levels of Concern" for additives based upon population exposure and potential toxicity (premise 3). The toxicological tests associated with "Levels of Concern" are also described.

Section III (and Appendix II) describes suggested guidelines for the performance of toxicological tests to assure that results will be of sufficiently reliable quality.

Section IV discusses the standards of toxicological test quality which determine whether the results of new tests as well as previously performed tests can be used reliably for predicting the safety of substances to humans.

Sections V. A and B discuss the so-called Selection Decision Elements, which indicate the need for and selection of further tests based on existing toxicological or other scientific information (Premise 4).

2. Updating of Information

As noted in subsections I.A.1 and I.A.2 above, additives, once approved, will tend to vary in the degree to which their data packages compare with current criteria, either because of changes in their exposure levels, availability of new toxicological information, or actual changes in the safety criteria themselves.

Section V.C describes a priority ranking scheme that will allow the FDA to assess safety information about additives on a continuing basis, so that it may devote available resources to only those additives that are of greatest public health importance.

II. Criteria for Assessment of Safety: the Concept of "Concern"

A. Introduction

The degree of effort expended in reducing uncertainty about the safety of an additive ought to relate in some concrete way to the likelihood that the substance poses a potential for health risk to the public (premises 2 and 3 of Section I). Such a "principle of commensurate effort," (Ref. 24) applied to the safety assessment of additives would help to ensure that all the information needed for making initial safety judgments about them may be gathered simultaneously for any number of additives, even when they may range widely in their potential for health risks to the public. Ideally, the initial development of information needed for the safety assessment process should be cast in a tiered system by which more resources can be concentrated on a smaller number of additives of highest probable risk, and less effort (per additive) can be spread over the generally larger number of additives where use levels and/or potential toxicity is minimal. Such a "balanced" system for development of safety information would tend to be more cost-effective than one in which all additives are made to undergo the same regimen of testing irrespective of any other considerations. (Ref. 25)

This section applies these premises to the safety evaluation of additives by introducing a "Concept of Concern," in which the term "Concern" is the primary parameter for establishing a cost-effective system used in gathering necessary safety information. For this purpose the common word "concern" takes on a more specialized meaning with respect to the variables such as exposure and toxicity per unit dose chosen to be parametrically related to it.* Therefore, for the purposes of the following discussion, this idealized, quantitative "Concern" or "Degree of Concern" related to safety judgments will be capitalized throughout.

*The term denoted here as "Concern" may, under certain assumptions, be conceptually related to the term "utility" introduced by von Neumann and Morgenstern for the purpose of making optimum choices and decisions using "maximum expected utility criteria," and under conditions of incomplete knowledge. (Ref. 26).

B. Concept of Concern

1. General

In the review of toxicological information for safety evaluation, two factors are of primary importance: the extent of human exposure, and the toxicological effects on various biological systems (including the effect of the biological system on the additive). These factors determine the extent of the health concerns for the use of any additive.

For the Concern concept to be useful, it must relate simultaneously to each of these two factors (exposure and toxicity). For this purpose, the Degree of Concern can be thought of as a relative measure of the degree to which the use of an additive may present a potential hazard to the public health. It must therefore simultaneously depend on, 1) the degree to which exposure exceeds the level justified on the basis of toxicological information, and 2) The nature and severity of any adverse toxic effects that are predicted to occur on the basis of the same information.

As noted previously in section I. B, an additive is considered safe if there is a reasonable certainty that no harm will result from its use. The exposure level of an additive for which there is reasonable certainty that no harm will result can be determined by appropriate extrapolations from toxicological testing results. For example, a safety factor* applied to the highest "no-adverse effect" level (HNEL) obtained in a toxicity test or an extrapolation from an "effect" level to some societally determined acceptable level of risk+ can be used to determine the acceptable exposure level for use of an additive.

Therefore, for a single type of toxic effect, the Degree of Concern for the use of a food additive can be defined as the degree to which the actual exposure level exceeds the acceptable exposure determined from toxicological information. For example, an additive with a change in use pattern which results in an increase in exposure beyond an acceptable level would have a high Degree of Concern. An additive with new toxicological information which alters its acceptable exposure level may have a high or low Degree of Concern, depending upon the relationship of the actual exposure to this new acceptable exposure level. Because Concern is also a function of the type of toxic response observed, data that point to a more severe type of toxic response may increase the Degree of Concern for a substance regardless of exposure considerations.

- * a). Section 170.22 of the Code of Federal Regulations (21 CFR 170.22) cites the 100-fold safety factor normally applied by FDA to the HNEL.
- b). 100-Fold Margin of Safety. A.J. Lehman, et. al. Quarterly Bulletin of the Association of Food and Drug Officials, January, 1954.
- + See "Policy for Regulating Carcinogenic Chemicals in Food and Color Additives; Advance Notice of Proposed Rulemaking," Federal Register, 47(64):14464-14470, April 2, 1982.

2. Levels of Concern

While the key variables for determining the Degree of Concern are the extent of human exposure (dose) and the toxicity of the additive (nature of effect, target, and magnitude of response per unit dose), present knowledge does not allow these data to be combined in a manner in which a precise mathematically defined Concern function can be derived. Ideally the relative Degree of Concern associated with each additive would be expressed quantitatively in terms of actual measurable parameters and available data for that additive. Should such a quantitative function be derived, quantitative data on exposure and toxicity would, in principle, give a unique and mathematically valid estimate for an actual Degree of Concern.

Although it is not possible at present to directly calculate values for such an idealized quantity, it is possible under certain simplifying assumptions to create broad "Levels of Concern." Such Levels of Concern can be used in constructing a tiered system for determining initial toxicological information needs commensurate with this Concern.

3. The Determination of the Level of Concern for a Compound

Even though existing information bases on a compound do not yet allow a quantitative determination of the actual relative Degree of Concern for an additive, existing data are sufficiently useful to allow qualitative categorization of additives into broad Levels of Concern from which valid safety judgments can nevertheless be made. Some additives or potential additives may have a great deal of adequate toxicological test data, while others, particularly new chemicals, may have very little or none. For the purpose of determining the extent of toxicity testing that may be necessary to reduce uncertainty about the safety of an additive, it is useful to define "Levels of Concern;" that is, broad bands or qualitatively estimated regions of concern, using data that should always be available. In the absence of toxicological data, a compound may be assigned to a Level of Concern based on an estimate of the population exposure and an initial estimate of toxicity from knowledge of its molecular structure.

Compounds can be classified into groups depending upon their molecular structures, and this information, when combined with exposure information by a simple algorithm, can be used to assign additives to Concern Levels. Throughout this process of Concern Level assignment it should be remembered that the initial estimate of toxicity can be refined later by the toxicological information obtained from testing. This level of knowledge based on testing is related to the rigor and sensitivity of the tests employed. For example, if one initially assigns an additive to a high Level of Concern based on structure-activity analogy, then one could later lower that estimated Degree of Concern by revising the estimate of toxicity with more precise information. If the estimate of the degree of toxicity cannot be reduced by further testing, then the only available means to accomplish a reduction of the Degree of Concern would be to reduce exposure.

Exposure

The human exposure to an additive depends upon the nature of its use. The actual level of exposure can be estimated for previously regulated additives using information from consumers' eating patterns and industry use surveys. For newly petitioned compounds, exposure may be estimated from projections based on anticipated uses.

Although reasonably reliable data exist on the concentration of some additives contained in some of the foods eaten, comprehensive estimates of consumer exposure to additives are difficult to obtain for a variety of reasons including the following: the lack of legal authority for agency inspection of industry records; the large number of additives with potential use in processed foods; the increasing multiplicity of processed foods; uncertainties of losses or changes in additives during processing; the complexities of American dietary patterns, with associated regional and cultural variations; and exposure to additives from multiple, including non-food, sources. Additionally, estimates of the degree to which additives can and do serve as alternatives for other additives are difficult to obtain, but could be of importance in determining consumer exposure to additives already in use. Different patterns of consumption associated with various age groups, "average" or "typical" diets versus intakes of special groups within the population, and "per capita" or "mean" versus "90th or 99th percentile" consumption of particular additives are all important considerations in estimating consumer exposure (Ref. 27)

For a new additive, or for new uses of an additive already in current use in the food supply, the law specifies that the agency's safety determination is to be based upon the "probable consumption" of the additive. (Ref. 28) Over the years FDA has devised methods for development of exposure estimation that have generally served well; (Refs. 29, 30) and will not be discussed in detail in this document.

For approved additives FDA continues to maintain exposure data. Such data on current use levels and exposure patterns of approved substances help the agency weigh the safety considerations associated with approving additional new uses of already approved substances, or with estimating the degree of exposure that is likely to occur from a new additive that is functionally equivalent to one or more approved additives. Also, maintaining exposure estimates of approved additives allows the agency to form a comprehensive overview of any relative concerns that might arise, should there be significant changes in consumption patterns of specific additives.

Initial assignment of an additive to a Level of Concern relates to general considerations of safety for the entire population. For this purpose the agency has chosen to use estimates of per capita exposure based on the total poundage of a substance added annually to the U.S. food supply.

More detailed information about the consumption (in mg/kg b.w./day) of the additive by age group, may be used more effectively in final safety determinations where protection of certain more susceptible subgroups of the population becomes a greater consideration.

Structure

In the absence of direct experimental toxicological data, a qualitative estimate of the inherent biological activity of a compound can be inferred from structural similarities to compounds of known biological activity. (Ref. 10) Some authors have published schemes based on this premise and applied them to safety evaluation of chemicals. (Ref. 11) It is now apparent that molecular structure can be used as an aid in initially determining the presumptive level of concern of additives. However, correlations between biological activity and molecular structure are complex, involving physical and chemical properties as well as metabolic pathways. Furthermore, although it would be desirable to apply such correlations to all compounds and all potential toxic effects, the current state of science coupled with the broad range of compounds characteristic of additives, may restrict the inferential process to single categories of toxic responses, such as carcinogenicity, for example.

Therefore, FDA is proposing a scheme for classification of direct additives into only three broad categories of molecular structure: Category C, for those additives whose toxicological potency is likely to be high, Category A, for those likely to be of low toxic potency, and the remainder, Category B for those likely to be of indeterminate or intermediate toxic potency. (Ref. 12) Under this scheme, such structure category assignments could help determine the Level of Concern, and thus the basic level of testing for additives. The process by which structure information can be integrated with exposure estimates to determine the Levels of Concern and testing is described below.

Concern Level Determination

In the past, FDA has required certain types of additives to be tested with varying degrees of rigor and sensitivity on the basis of exposure. Compounds below 0.05 ppm exposure into food were traditionally required to have acute testing (unless other data suggested the need for more extensive testing); compounds of exposure above 0.05 ppm and below approximately 1.0 ppm required subchronic testing; while compounds contributing more than approximately 1.0 ppm to the total diet generally were required to have chronic testing. The present system uses a combination of the estimated toxicity (molecular structure categories A,B, or C) together with similar exposure considerations to define the Concern Levels for additives.

Figure I illustrates how toxicity estimated from molecular structure category can be combined with the above exposure break-points to create three distinct Levels of Concern. Any additive, even in the absence of toxicological feeding studies, may be easily assigned to one of the three such Concern Levels depending on the combination of exposure and estimated toxicity.

4. Minimum Testing Levels for Additives

The extent and type of basic toxicological testing of an additive ought to depend upon the Concern that derives from the additive's potential adverse effects on human health. As noted above, this Concern is derived from several variables: the additive's extent of exposure; its chemical structure; the absorption, distribution and metabolism of the substance; and the observed biological effects. The effect a biological system has upon the additive can either increase or decrease the health concern for the use of the additive. For example, a non-toxic substance may be transformed by the metabolic activity of an organism into a substance of much greater toxic potential. Alternatively, an organism may distribute or metabolize a potentially toxic substance in a manner that protects the target tissue from the chemical (blood-brain barrier, placental barrier, metabolic deactivation).

This system considers these variables in determining toxicological testing requirements. The system requires the most extensive toxicological testing for additives with large exposure and reactive structures, or additives which induce adverse toxicological effects at low doses or after short durations of exposure. Conversely, compounds with low exposure, and unreactive structures, or which induce few adverse effects only at high doses, initially receive less extensive testing. In this way, the greater the health concern for an additive, the greater will be the sensitivity and extent of testing for assessing its safety. The basic testing requirements will be determined by the combination of exposure and chemical structure as depicted graphically in Figure 1. Whether or not a toxic effect is observed in a test depends upon the selection, sensitivity, and rigor of the toxicological tests performed on the additive. The selection of toxicological tests to be performed on an additive is of paramount importance, in that the test determines the sensitivity and extent of toxicological observation which can be made for the additive.

The final extent of testing will be determined by the effects (dose, onset, duration, type, extent, etc.) observed in the basic set of tests. The methods of test selection under such a scheme are described below.

The relationship between exposure, toxicity and concern can be used to determine the rigor and sensitivity with which an additive should be tested. A high Concern Level (as a result of high exposure or

estimated toxicity) would require that the additive be tested with a high level of rigor and sensitivity. Conversely, a low Concern Level (low exposure or estimated toxicity) would require less sensitivity and rigor, if no further toxicity were observed to alter the original estimates.

The use of three such Levels of Concern is convenient because historically toxicological testing needs have been divided as follows into three broad classes, on the basis of duration of exposure: acute, subchronic, and chronic. As test duration increases, the lowest effect dose and the types of effects observed are usually determined with greater sensitivity.

The extent to which adverse effects can be observed is related to the number of subjects and species studied and the number of parameters monitored. As the science of toxicology has progressed, the number of parameters measured and the number of subjects examined have increased (For example, see Section IV for a comparison of the standards which reveal the difference in rigor).

Finally, as data from these initial or minimum tests are obtained, the results can be used to refine or adjust the type, sensitivity, and rigor of subsequent tests, and therefore the precision of the estimate of toxicity.

4) Tests for Each Level of Concern

a) Concern Level III:

The tests for Concern Level III are the most demanding and provide the greatest sensitivity for determination of adverse biological effects. These tests include: carcinogenicity studies in two rodent species, a chronic toxicity study of at least one year duration in a rodent species, a long-term (at least one year in duration) feeding study in a non-rodent species, and a two-generation reproduction study with teratology phase in a rodent species. Results from the reproduction study may be used to indicate the need for teratological or reproductive testing in more generations, or the need to conduct tests employing in utero exposure. The remainder of the battery of tests is sensitive enough to detect nearly all types of observable toxicity including malignant and benign tumors, pre-neoplastic lesions, and most other chronic toxicity. These tests are able to provide a firm basis for development of a safety profile of an additive. In addition to these tests, the results from a battery of short term tests for carcinogenicity potential may be useful in assisting in safety evaluation.

b) Concern Level II

The tests for Concern Level II are of intermediate sensitivity. These tests are sensitive enough to detect most toxic phenomena other than late-developing histopathological changes. The tests for this level of concern include: subchronic feeding studies (usually 90-days duration) in a rodent and non-rodent species, a two-generation reproduction study with a teratology phase in a rodent species, and, because the majority of the late-developing lesions are related to oncogenicity, a set of short-term tests for carcinogenic potential. The results from short-term tests at this level of concern will identify compounds from this level for which chronic testing is necessary. Results from the reproduction study may be used to indicate the need for teratological or reproductive testing in more generations, or the need to conduct tests employing in utero exposure.

c) Concern Level I

The tests for Concern Level I are the least sensitive. They include: a short-term feeding study (usually of 28-days duration) in a rodent species and a battery of short-term tests for carcinogenic potential. The feeding study is sensitive enough to detect any acute, life-threatening toxicity and provide an indication of target organs and doses for toxicity testing of longer duration. The set of short-term tests will indicate the need for further information from toxicity testing of longer duration.

Although not specifically required for any Concern Level, studies of the absorption, distribution, metabolism and elimination characteristics of a test substance are recommended to be conducted prior to the initiation of toxicity studies of longer than 90-days duration. Disposition studies may also provide assistance in the selection of the appropriate rodent or non-rodent species for required toxicity testing.

The tests for each Level of Concern are summarized below:

Tests for Concern Level III Compounds

- a) Carcinogenicity studies in two rodent species.
- b) A chronic feeding study of at least one year in duration in a rodent species (under most circumstance this study is added to one of the carcinogenicity studies and performed as a combined test).
- c) Long-term (at least one year in duration) feeding study in a non-rodent species.
- d) Multigeneration Reproduction study, (minimum of 2 generations) with a teratology phase in a rodent species.

e) Short-term tests for carcinogenic potential that can be used for determining priority for conduct of lifetime carcinogenicity bioassays, and that may assist in the evaluation of results from such bioassays.

Tests for Concern Level II Compounds

- a) Subchronic feeding study (at least 90 days in duration) in a rodent species.
- b) Subchronic feeding study (at least 90 days in duration) in a non-rodent species.
- c) Multigeneration reproduction study (minimum of 2 generations) with a teratology phase in a rodent species.
- d) Short-term tests for carcinogenic potential.

Tests for Concern Level I Compounds

- a) Short-term feeding study (at least 28 days in duration) in a rodent species.
- b) Short-term tests for carcinogenic potential.

5) Summary of Concern Level Assignment Procedures

Exposure levels and their relationships to each structure category and concern level are stated below and graphically summarized in Appendix IV, Figure 1.

Structure Category A

Concern Level III: 1 ppm in the total diet, or 0.025 mg/kg/day or greater.

Concern Level II: 0.05 ppm in the total diet, or 0.0012 mg/kg/day or greater

Concern Level I: Less than 0.05 ppm in the total diet or less than 0.0012 mg/kg/day.

Structure Category B

Concern Level III: 0.5 ppm in the total diet, or 0.0125 mg/kg/day or greater

Concern Level II: 0.025 ppm in the total diet or 0.00063 mg/kg/day or greater

Concern Level I: Less than 0.025 ppm in the total diet, or less than 0.00063 mg/kg/day.

Structure Category C

Concern Level III: 0.25 ppm in the total diet, or 0.0063 mg/kg/day or greater.

Concern Level II: 0.0125 ppm in the total diet, or 0.00031 mg/kg/day or greater

Concern Level I: Less than 0.0125 ppm in the total diet, or less than 0.00031 mg/kg/day.

C. Summary

To this point, a method for determining an initial Level of Concern for the safe use of an additive has been presented. This method is based upon the use of variables related to the exposure and potential toxicity of the additive. It considers that toxicity data may not be available for many additives, and therefore relies on initial estimates of toxicity based on molecular structure analogy. A relationship between Concern Level and base sets of toxicity tests is also described. The system uses information other than testing results for the determination of tests needed for safety evaluation. In the absence of complete information, the system as described so far, allows determination of some base sets of tests, but it does not allow for adjustment of these tests on the basis of observed data (premise 4, Section I). So that the use of laboratory data in the determination of testing can be incorporated into the proposed system, a set of "decision elements" for determination of further testing or reduction in the base set of tests, based on observed data, is presented in Section V below. Taken together, the methods described in Section II and V provide an efficient means for determining the overall testing necessary to evaluate the safety of an additive.

III. Guidelines for Toxicological Tests

A. Introduction

A major difficulty in the preparation of a safety profile for an additive is a lack of common, consistent, and clearly defined testing guidelines for the design and conduct of toxicological studies. Another difficulty is the lack of orderly recording and reporting of the critical information required for assessment of effects observed in toxicological tests. In order to eliminate these difficulties the agency has determined it should identify and publish guidelines for the design, conduct and reporting of such studies. These guidelines should reflect the most up-to-date scientific knowledge relevant to safety evaluation.

Although many agencies regulate the same chemicals, the toxicity testing guidelines developed separately by various health regulatory agencies are not always uniform. The differences in requirements often result in duplication of effort and inefficient use of already scarce testing resources. The Interagency Regulatory Liaison Group (IRLG) Testing Guidelines and Standards Workgroup was established to develop common, consistent, and compatible testing guidelines, quality assurance procedures and other policies relative to the testing of substances. The FDA was a full participating member of the IRLG. Where possible the guidelines presented in Appendix II are consistent with guidelines of other agencies or organizations; it must be emphasized, however, that food additives can present special needs for testing and the guidelines presented in Appendix II reflect such needs. Any modifications of these guidelines would be required on the basis of data obtained from the minimum battery of tests.

B. Guidelines for Conduct of Studies

The proposed guidelines for each of the toxicological tests normally employed in additive evaluation are presented in Appendix II. These guidelines will be revised and updated as appropriate.

Conduct of all studies should include compliance with the FDA Good Laboratory Practices (GLP) Regulations 21 CFR part 58 (43-FR-59986).

1. Acute Oral Toxicity Study:

Although this test is not required for the safety evaluation of a direct food additive, a guideline for this test is included in Appendix II. This guideline is for use when the acute toxicity of a substance is of concern, or when acute toxicity data are needed for the design of longer duration studies.

Acute toxicity is examined to determine the degree of toxicity of a chemical substance (that is, the relationship between dose and adverse effects), to establish its toxicity relative to other chemical substances whose acute toxicity is known, and to determine specific toxic effects and to provide information on the mode of toxic action. A suitably designed acute toxicity study will also provide information from which a median lethal dose (LD₅₀) can be calculated. By studying the effects following administration by different routes, the relative hazards of different pathways of exposure can be assessed. By using animals of both sexes, sex differences in toxic response can be detected.

Acute toxicity studies will thus identify highly toxic chemicals and provide information on the possible hazards which could occur where humans are exposed. The slope of the dose response curve and the type of toxic response in experimental animals are of use in human health hazard evaluation; exposure to single acutely toxic doses of a chemical represents an abnormal or accidental situation for general human exposure.

The guideline for this study is designed for use in acute ingestion tests using rodents, but is adaptable to other species.

Although several accepted methods for determining the LD₅₀ values have been developed, many important observations of toxicity are not represented either by these values or by slopes of dose-response curves for lethality. These observations are integral to an evaluation of acute toxicity and should be observed during the course of an acute toxicity study.

Morbidity and/or pathogenesis may have more toxicological significance than mortality.

The numerical value of the median lethal dose (LD₅₀) is widely used in toxicity classification systems, but it should not be regarded as an absolute number which identifies the toxicity of a chemical substance. LD₅₀ values for the same chemical may vary from study to study and between species or within a species because acute toxicity is influenced by both internal and external factors.

2. Short-Term Oral Toxicity Study: Range-Finding

In the assessment and evaluation of the toxic characteristics of a chemical, the determination of short-term oral toxicity may be carried out after initial information on toxicity has been obtained by acute testing. The short-term test provides information on possible health hazards likely to arise from repeated exposures over a limited period of time.

Short-term oral exposure studies of one month or less are conducted to determine the adverse effects of substances after repeated dosing. This study also serves as a range-finder of the doses which will not cause lethality after many months or years of administration in subchronic or chronic studies. Use of this information allows future subchronic and chronic studies to be designed with realistic doses and with special emphasis on the target organs.

The testing procedures utilized include the oral administration of the test substance in daily graduated doses to several groups of experimental animals, one dose per group for a period of 28 days. During the period of administration the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied, and at the conclusion of the test surviving animals are sacrificed and necropsied.

3. Subchronic Oral Toxicity Studies:

Subchronic studies are designed to determine adverse effects of substances when given in regularly repeated doses over periods ranging from 90 days to 12 months. The intent is to characterize the toxicity of the substance and to define a level that results in "no observed adverse effects." Such a study generally cannot, however, determine carcinogenic potential. The testing procedures utilize a broad screen of measurements which should detect the most likely forms of toxicity which can occur.

While acute toxicity deals with the adverse effects of single doses, a more common form of human exposure to many chemical substances is in the form of repeated doses which do not produce immediate toxic effects. Delayed effects may occur due to accumulation of the chemical in tissues or from other mechanisms, and it is important to identify any potential for these by subchronic testing. In addition, the subchronic

study will provide more detailed information on toxic effects, target organs, reversibility of effects, and an indication of a "no-effect" level.

4. Chronic Toxicity Studies:

The objective of a chronic toxicity study is to determine the effects of a test substance in a mammalian species following prolonged and repeated exposure. Under the conditions of this test, effects which require a long latent period or are cumulative should become manifest. The application of these guidelines should generate data from which one can identify the majority of chronic effects and determine dose response relationships. Ideally, the design and conduct should allow for the detection of general toxicity including neurological, physiological, biochemical, and exposure-related morphological effects. The guidelines suggest the oral route of administration for consideration in evaluating a test substance. Three test dosage levels plus a control group are recommended, with the highest dose requirement differing from that of the carcinogenicity study because at this level some signs of toxicity should be elicited. In discussing the duration of the chronic studies, arguments were offered that in some cases toxicity and life-shortening effects would be missed if the duration was for 12 months only. To allow latitude for appropriate scientific evaluation, it is recommended that the duration of exposure should be for at least 12 months. Daily observations are recommended to minimize loss due to disease, autolysis and cannibalism and to detect the onset and progression of toxic effects. Additional examinations for clinical signs of toxicity including neurological and ocular effects and for hematological and organ function effects as determined from blood and urine analysis are also suggested.

5. Carcinogenicity Studies:

The objective of a long-term carcinogenicity study is to observe test animals over a major portion of their life span for the development of neoplastic lesions during or after exposure to various doses of a test substance administered by an appropriate route. Such an assay requires careful planning and documentation of the experimental design, a high standard of pathology, and unbiased statistical analysis. As part of the base set of tests for Concern Level III compounds, it is recommended that the study of a test

substance be conducted in two species, and by the oral route of administration. Because of the long latent period required for induction and manifestation of tumors, it was generally agreed that treatment of test animals should be started in young animals and continued for the duration of the experiment. The main form of oral administration is dietary. The choice of other methods of administration depends upon the physical and chemical characteristics of the test substance and the form typifying human exposure. Although experimental exposures do not necessarily have to be by the same route as of human exposure in order to be meaningful, possible physiologic and metabolic differences related to routes of absorption and distribution should be considered in assessing their relevance.

Testing at doses and under experimental conditions that permit maximum expression of carcinogenicity is widely accepted in these bioassays. For risk assessment purposes, at least three dose levels should be used, in addition to the concurrent control group. Each dose group and concurrent control group should contain 50 animals of each sex. The highest dose level should be sufficiently high to elicit signs of minimal toxicity without substantially altering the normal life span due to effects other than tumors. The lowest dose should not interfere with normal growth, development and longevity of the animal; it must not cause any other indications of compound-related toxicity. The intermediate dose should be established approximately mid-way between the high and low doses, depending upon the absorption, distribution, metabolism and elimination of the chemical, if known.

It is necessary that the duration of a carcinogenicity test comprise the majority of the normal life span of the animals to be used. The guidelines recommend the termination of the study at 24 months for rodents, but for certain strains of animals with greater longevity and/or low spontaneous tumor rate, termination can be extended to 30 months for rats. A finite period covering the majority of the expected lifespan of the strains is recommended over exposure for the entire lifetime of all animals since the probability is high that, for the great majority of chemicals, induced tumors will occur within a finite observation period.

The evaluation of carcinogenicity bioassay results rests on the extent and accuracy with which organs and tissues of both treated and control animals are examined for morphological

changes. Although a well conducted pathologic examination cannot rescue a poorly designed or conducted bioassay, inadequate pathologic examination can significantly reduce or eliminate the value of an otherwise well conducted experiment. The strength of evidence provided by a bioassay depends on the number of tissues examined. The absence of a carcinogenic effect in a study cannot be assured unless all organ systems have been examined grossly in all animals, and all grossly visible suspect lesions examined microscopically. An attempt should be made to correlate gross observations with the microscopic findings.

Microscopic examination is as essential as a gross necropsy in the proper conduct of a carcinogenicity study. While an all inclusive examination of all tissues is perhaps theoretically desirable, the resource limitations dictate a more selective approach. As a minimum, the following is recommended for microscopic examinations:

- a. All grossly visible tumors or lesions suspected of being tumors should be examined in all groups.
- b. All preserved organs and tissues of (a) all animals that die or are killed during the study, and (b) animals of the highest dose group and controls. While notation should be made of all histopathologic lesions, those which were hyperplastic, preneoplastic and/or neoplastic should be fully described.
- c. If a significant difference is observed in hyperplastic, preneoplastic or neoplastic lesions between the highest dose and control groups, microscopic examination should be made on the particular organs or tissues of all animals in the study.
- d. In case the results of the experiment give evidence for substantial alteration of the animals' normal longevity or for the induction of effects that might affect a neoplastic response, the next lower dose level should be examined as described above.

6. Combined Chronic Toxicity/Carcinogenicity Studies:

This guideline for an oral chronic toxicity/carcinogenicity study is suggested for use with one species, typically the rat. The objective is to obtain data to determine effects of a test substance which would be provided separately

in a carcinogenicity or a chronic toxicity study. In addition to three dosage levels and a concurrent control group, each of which contain at least 50 animals per sex, this guideline recommends three satellite treatment groups of 10 animals per sex. Whereas the high dose for the carcinogenicity phase should not produce toxicity, the highest dose for the satellite treatment groups should be chosen so as to produce overt toxicity without causing excessive mortality. These satellite groups should be retained in the study for at least 12 months. These animals should be scheduled for sacrifice for determination of test substance-related pathology, uncomplicated by geriatric changes. The other three treated groups and the control group would be handled as in the carcinogenicity guidelines. In these guidelines, recommendations are included for periodic observations of signs, onset, and progression of toxic effects, hematological and organ function tests, and clinical examinations for neurological and ocular changes.

7. Reproduction Studies:

The guideline for reproduction testing is designed to provide general information concerning the effects of a test substance on gonadal function, estrous cycles, mating behavior, conception, parturition, lactation, weaning, and the growth and development of the offspring. The study is not designed to determine specific cause and effect in all cases. The study, however, may also provide information about the effects of the test substance on neonatal morbidity, mortality, and preliminary data on teratogenesis and serve as a guide for subsequent special tests. This guideline is for use with substances given orally to rodents. The guideline recommends that the test substance be administered to parental (P) animals prior to their mating, during the resultant pregnancies, and through the weaning of their F₁ offspring. The substance is then administered to selected F₁ offspring during their growth into adulthood, mating, and production of an F₂ generation, up until the F₂ generation is 21 days old. If there is an indication of effects occurring at lower doses, higher incidences, or greater intensity in the second generation as compared to the first, then the study should include a third generation.

8. Teratogenicity Testing in Rat, Mouse, Hamster, and Rabbit:

The purpose of this test is to yield data to help determine the effects of a test substance administered during in utero

development. Treatment by the oral route of administration must be started early enough and continued long enough to include the period of organogenesis for the particular species used (rat, mouse, hamster, rabbit, etc.)

Such a study may also be performed in conjunction with a multigeneration reproduction study as long as the fetuses are exposed continuously through organogenesis. The guideline recommends that the test substance be administered in graduated doses, for at least that part of the pregnancy covering the period of organogenesis, to several groups of pregnant experimental animals, one dose being used per group. Shortly before the expected date of delivery, the mother is sacrificed, the uterus removed, and the contents examined for embryonic or foetal deaths, and live foetuses.

9. Absorption, Distribution, Metabolism and Elimination ("A,D,M,E") Studies:

Data from "A,D,M,E" studies are desirable to aid in the evaluation of test results from other toxicology studies and in extrapolation of data from animals to man.

"A,D,M,E" studies also provide data useful for selecting appropriate dose levels for use in chronic toxicity and carcinogenicity studies by providing information about dose-dependent kinetics.

The time at which it is best to do a "A,D,M,E" study varies with the need for data to evaluate the safety of the test chemical. In certain cases, the initial experiments for determining absorption, distribution and elimination of the test chemical may be done soon after the acute toxicological studies. Further experiments establishing the metabolic fate of the compound may be needed for chemicals which will likely undergo chronic testing. If the results of toxicological studies indicate that further information on the metabolism of the test chemical is needed, identification and characterization of major metabolites in blood and urine should be undertaken. For some purposes, dose-related "A,D,M,E" studies may be carried out. In pregnant animals, a kinetic analysis makes it possible to assess the amount of placental transfer of the parent compound and its metabolites at critical periods of organogenesis in relation to maternal exposure.

10. Short-term Tests for Carcinogenicity Potential:

In the context of this document short-term tests refer to any of several tests which are useful in estimating the carcinogenic potential of a substance. As the name implies, the time required for completion of such tests ranges from a few days to several weeks; the tests utilize cells or organisms which can be grown rapidly and in large numbers. While many of the tests measure mutagenic changes (such as the loss or gain of an enzyme), several have been developed to reflect other endpoints such as chromosomal deletions or rearrangements, nonspecific DNA damage, and cell transformation.

A highly significant correlation has been observed between the positive results of point mutational and DNA repair tests with in vivo bioassays for carcinogenesis. The primary reason for recommending these tests is this strong empirical correlation between positive results in several of these tests and in vivo carcinogenicity of the test compound.

Therefore, positive data from the less time consuming and less expensive short-term tests are considered useful for determining the judicious use of scarce resources for long-term bioassays for carcinogenicity. The assessment of food additive safety will use short-term test data for this purpose.

The correlation between responses in short-term tests and in vivo carcinogenicity is not perfect for any one test, i. e., false positives and negatives do occur in all test systems. However, many of the individual tests have detection sensitivities which overlap with the other tests. Thus by carefully selecting and combining tests one can construct a battery of tests which can be a highly efficient screen for most if not all classes of chemical carcinogens thereby significantly reducing false negatives without substantially increasing false positives.

The agency has surveyed the already broad and still expanding field of short-term tests and selected a battery of tests which we feel can at this time be used as a reliable predictor of potential carcinogenicity. The criteria which we used to select a test for inclusion in the battery of tests were: (1) that the test show a high degree of sensitivity for detection of known animal carcinogens with an acceptable level of false positives; (2) that the test be readily available and reproducible among laboratories; (3) that the test response can be scored and interpreted in a relatively unambiguous manner; (4) that different endpoints are represented; (5) that, in toto, the tests complement one another so that most classes of known carcinogens are detected.

It should be emphasized that the suggested battery of tests is to be used for the detection of potential carcinogenicity. The agency does not take the position that positive results in short-term tests are incontrovertible evidence of carcinogenicity, since there are a multitude of factors operating in the whole animal which serve to modify the effects seen in the short-term tests.

The following provides guidance for the types of tests and acceptable protocols to be found in the literature as well as considerations to be used by the Agency in evaluating submitted test data. Since the use of mutagenicity tests as predictors for carcinogenicity is still under development, the choice of tests should be flexible, depending on the precision of the end point, the extent of correlation and the ease of performance and evaluation of the assay.

The battery of short-term tests recommended includes: (1) a bacterial mutagenesis test, the Ames test is suggested; (2) a mammalian mutagenesis test, the L5178Y mouse lymphoma test for mutants at the TK locus is suggested; and (3) a generalized test for DNA damage, we suggest the Unscheduled DNA Synthesis test in primary rat hepatocytes developed by G. Williams. (Refs. 31, 32) Two additional tests which are quite useful but which seem to lack sufficient commercial availability to be recommended routinely are the mammalian cell transformation tests and the sex-linked recessive lethal (SLRL) mutation test in *Drosophila*.

The Salmonella/Ames bacterial mutagenicity system is suggested because there currently exists an extensive data base on the correlation between results in this test and carcinogenicity as determined by long-term whole animal studies. (Refs. 33-35) These data indicate that mutagenicity in bacteria is a generally reliable indication that a chemical is likely to be carcinogenic in vivo. It appears, however, that there are chemical classes of carcinogens that fail to be detected as mutagens in bacterial assays. For this reason, point-mutational tests in mammalian cells are also recommended as well as the *Drosophila* SLRL test, DNA repair studies, and mammalian cell transformation tests. Although the published data for these latter tests are not as extensive as those for bacterial mutagenesis tests, current indications are that these tests are useful as screens for carcinogenicity.

Mammalian cell transformation is an end-point theoretically related to carcinogenesis and this test does correlate well with known animal carcinogens. In certain instances, transformation tests complement the other tests by their detection of metal and hormonal carcinogens.

Testing in other non-bacteria test systems such as *Drosophila* may be particularly important for additives which are intended for use as antimicrobial or antifungal agents. The bacterial toxicity may make it impossible for testing at doses sufficiently high to allow consideration of negative findings.

There is evidence that some carcinogens do not yield a positive response in the short-term test procedures. Therefore, when a compound is of a structural class for which there is reason to believe that the short-term tests are inadequate as a screen for carcinogenicity, their use to reduce the concern for toxicity will not be accepted and in vivo carcinogenicity testing may be required to satisfactorily reduce the concern for toxicity. All in vitro short-term tests for carcinogenicity should be performed in the presence and in the absence of a metabolic activation system, which is generally derived from rodent liver (or other relevant tissue). *Drosophila* metabolism has been demonstrated to be similar to rodent metabolism in the activation of carcinogens. (Refs. 36-39) Data indicating that the pattern of metabolites produced in the in vitro activation system during the test is similar to that produced in vivo in the target species is useful in interpreting the applicability and significance of the test results.

Due to the rapid advances being made in the field of short-term tests to assess potential for carcinogenesis, it is difficult to develop precise protocols that would be highly recommended for each general test type. At the present time, however, the most convincing bacterial mutagenicity data available are on the histidine-requiring strains of *Salmonella typhimurium* developed in the laboratory of Dr. Bruce N. Ames. Good results appear to be obtained by the procedure given by Ames, et al., (Ref. 40) or with the "pre-incubation" assays described by Yahagi et al., (Ref. 41) or Prival et al., (Ref. 42).

The standard technique for the X-linked recessive lethal test in *Drosophila* and relevant data on chemicals tested are contained in papers by Abrahamson and Lewis (Ref. 43), Vogel (Ref. 36), and Wurgler et al., (Ref. 38).

The induction of DNA repair synthesis in cultured mammalian cells can be detected either by autoradiography (Ref. 44) or less reliably by liquid scintillation counting of extracted DNA (Ref. 45). The preferred system is that which employs primary rat liver cell cultures, which are themselves capable of activating a variety of pro-carcinogens. (Refs. 31, 32)

The most accepted of the several system developed for the assessment of in vitro cell transformation utilize cell lines from the mouse [C3H/10T1/2, Reznikoff et al., (Ref. 46); BALB 3T3, Kakunaga, (Ref. 47)] or from the hamster [BHK-21, Purchase et al., (Ref. 48)]. Other systems, [Syrian Hamster Emryo (SHE), Pienta et al., (Ref. 49)]; [RLV-infected rat cells, Freeman et al., (Ref. 50); Traul et al., (Ref. 51)], while quite interesting, have more limited impact because only a few labs have successfully implemented these tests. The former systems also require a good deal of technical expertise, but most of the factors important for correctly carrying out the tests have been identified and discussed (report 6 in Montesano et al., (Ref. 52); Hollstein et al., (Ref. 53).

The most widely-used test for mutation in cultured mammalian cells is probably the test for mutations at the thymidine kinase locus in mouse lymphoma cells, as described by Clive and Spector (Ref. 54), Clive et al., (Ref. 55), and Amacher et al., (Refs. 56, 57). It also appears that important information on the correlation with carcinogenicity is becoming available on the test for mutations in the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in Chinese hamster ovary cells, as described by O'Neill, et al., (Ref. 58). There are a number of other cell culture/mutagenicity test systems which offer promise but which have, in general, not been validated with enough compounds to be recommended at this time (see the review by Hollstein et al., (Ref. 53). Most (23) of these short-term tests for determining carcinogenic potential are currently being critically evaluated by scientific panels established and supported by the Gene-Tox program of the Environmental Protection Agency (Ref. 59). As these individual test reviews are finished they will be published in Mutation Research. An overall comparison of the utility of the tests will be synthesized by a separate EPA panel. These results should be most useful in selecting the most appropriate tests and establishing minimum test criteria.

Other batteries of tests, besides the one suggested by the agency may be acceptable as supporting evidence to reduce the toxicity concern factor for an additive.

The substitution of tests similar to those listed certainly possible, but in order to be an acceptable must be validated. That is, sufficient data must be in the literature to show that the test is efficient and capable of detecting known carcinogens. As in all testing, the application of the procedures requires an understanding of the limits of each test as well as an understanding of the structure and metabolism of the compound. It is clear that knowledge of a compound's metabolites would suggest more appropriate short-term tests or test details. The following are general considerations on variation in test procedures covered in future test guideline publications.

The agency anticipates that use of these short-term tests will simplify the approval process and reduce the cost to the sponsoring company. Their use is indicated not only for compounds with little exposure and little structural information but whenever a compound is to be tested. Strong positive responses in several short-term tests augurs for a positive bioassay. Given such a result, a company could well drop further development of a compound unless, of course, the compound were of such potential importance that a bioassay would be justified. The Agency is in the process of developing further documentation regarding the selection of tests, the interpretation and weighting of results, and standards for the acceptability of a negative test. These documents will be available for public comment in the future.

IV. Standards for Assessment of the Adequacy of Toxicological Tests.

The quality standards presented in Appendix III will be used to judge the relative quality of toxicological tests used to develop data for the safety profile of an additive. All toxicological tests should have been performed and those tests should meet the current quality standards described before the food additive can be affirmed as safe.

A. Core Quality Standards for Assessment of the Adequacy of Test Results

In the course of evaluating the safety of a new additive or when a concern arises about a previously approved additive, results of previously performed toxicological studies may provide information relevant to a safety judgment, even when such tests may not meet current standards of test sensitivity or rigor. Such older data may provide safety information that can alert the agency to a toxic hazard associated with an additive. Such data can also provide a basis for requesting information needed for future safety evaluations, and can be helpful in setting priorities for determining relative concerns among approved additives. In order to eliminate those studies which are so inadequate as to preclude the use of their data, a set of "core quality standards" is needed. A "core standard" defines the minimum data required for the acceptance of a study. The "core standards" are contained in Appendix III.

The presence of "positive findings" in tests which may be judged inadequate by the application of "Core Standards" may require further review in order to determine whether these findings might be applied for determining future testing needs or priority.

B. Current Standards for Toxicological Test Results

A "current standard" defines the minimum data necessary for the acceptance of a study to establish that a substance caused "no adverse effects."

The new guidelines which assist the investigator in the design of toxicity tests (Appendix II), suggest many of the current quality standards necessary for assessment of safety. These quality standards include such things as demonstration of absorption, number of animals, number of doses, types of clinical tests used, number of tissues

examined, histopathology, etc. The acceptability of future negative toxicological data for safety evaluation of direct food additives is dependent upon the content of the test data meeting these current standards. These quality standards for conduct of a study are incorporated into the current standards in Appendix III.

Another major quality standard is the FDA Good Laboratory Practices (GLP) Regulations (43 FR 59986).

C. Procedures for Application of Toxicity Study Standards

This system is designed for the classification of studies. At times, reviewers may determine that a deficiency of a particular experimental parameter within a study may not seriously compromise the classification of the study as meeting "Core or Current Standards." In this instance, a rationale for the re-classification of that study should be provided by the reviewer. This rationale will be reviewed by a group of agency scientists in order to insure uniformity in application of the standards.

1. Submissions of New Data

- a) Appropriate "Current Standards" should be applied to data developed subsequent to issuance of the "current standards" in Appendix III.
- b) Compliance with the Current Test Standards and GLP regulations for a study will almost certainly result in acceptance of that study by the agency.
- c) Failure to comply with either GLP regulations or "Current Standards" are grounds for rejection of the study for the purpose of safety affirmation.

2. Data Developed Prior to the Issuance of the Current Test Standard

- a) Data that fulfill the requirements of the "Current Standard" will be accepted for the safety affirmation process.
- b) Data failing to meet the "Current Standards" are subject to comparison with the "Core Standards."
- c) Data meeting "Core Standards" can be used for interim safety determination and priority setting for compounds.

- d) Data that fail to meet "Core Standards" will be reviewed for the presence of adverse effects, but may not be used for interim safety determinations.
- e) If compound-related adverse effects in a study not meeting "Core Standards" are determined to be unrelated to the poor quality of the study, the effects will be used for test selection or priority setting for further testing.

D. Scheme for the Sequential Application of the Test Standards for Chronic, Subchronic and Short-term Tests

1. Chronic Studies: Apply Current and Core Chronic Test Standards. If the data meet either standard, then the data will be used as acceptable "Current" or "Core" chronic data. If the requirements are not met, then apply subchronic and short-term standards sequentially. If the chronic data or data from interim sacrifices fulfill one of these standards, then the data can be used to satisfy either the requirements for subchronic or short-term tests.
2. Subchronic Studies: Apply the Subchronic "Current" and "Core" Test Standard. If the data meet the standard, then the study will be used as acceptable "Current" or "Core" subchronic data. If the standards are not met, then apply the short-term test standard. If the subchronic data fulfill the short-term standard, then this test can be used to satisfy the requirements for a short-term study.
3. If a study does not meet the standard for a short-term study, it will be reviewed for the presence of adverse effects but may not be used to fulfill any testing requirements.

V. Decision Elements - Selection and Priority-Setting for Toxicological Testing

A. Introduction

The purpose of this section is two-fold:

- 1) to provide a framework for deciding what further toxicological safety information may need to be developed for an additive, based on evaluation of the data obtained from studies, either those previously conducted or as part of the base set of tests for new additives described in Section II above; and
- 2) to describe a priority-setting scheme for all approved additives by which test selection and conduct can be carried out simultaneously and in a way that is consistent with public health priorities as well as economic limitations and potential administrative constraints.

Section II above described a scheme by which the basic toxicological information needed for the initial additive safety determination can be derived from simple information that is nearly always available for all compounds. However, even when such toxicity information is available and of acceptable quality, an evaluation of this information may raise significant public health questions that suggest the need to develop additional toxicity data, before the agency can make a final safety judgment about an additive. Determining exactly how much information is sufficient, and what the precise nature and sequence of that information development ought to be, is a problem that has long been recognized both by government and industry. Only recently has any substantial headway been made in solving the problem in a way that strikes a balance between the need for flexibility, and the need to at least outline the conceptual steps common to the the great majority of safety evaluations. Possible solutions to this problem have recently been put forward by the Food Safety Council and others. (Refs. 11, 15, and references cited therein, 60)

The approach taken in this document is centered around a series of Decision Elements. These Decision Elements are of two types; namely Selection Elements, and Ranking Elements. The Selection Elements provide a means of selecting, in a stepwise fashion, the toxicological tests best designed to answer specific concerns that arise because of the appearance of adverse effects seen in available data. Selection Elements would be applied whenever a safety determination for any additive needs to be made. Such determinations are a routine part of the premarket approval process for new food additives. They may also be useful in the case of previously approved substances, where there is marked increase in consumer intake, or when new toxicological information gives rise to concerns about the continued safe use of the substance.

The Ranking Elements determine a sequence for the conduct of selected studies which provide for the development of new information for already approved additives. These Ranking Elements ought to apply to all additives simultaneously, while considering a number of pertinent variables in addition to potential public health concerns, such as economic limitations, or administrative or other constraints. Taken together, these Decision Elements provide the framework for a unified system designed to answer the question: "What specific information ought to be developed for which compounds, and with what degree of urgency?" In providing answers to this question we help to accomplish, in an operational sense, the goal cited in Section II above, of assigning for each additive a quantitative measure of the actual relative Degree of Concern that we ought to have for these substances.

B. Selection Elements

Selection Elements provide a basis for determining the need for developing additional specific toxicological information beyond the base set of tests as determined by Concern Level assignment (Section II). Selection Elements are specific to a given type of toxicological information such as carcinogenicity data or reproductive toxicity information.

The following examples illustrate how the Selection Elements might function: A given additive, because of low exposure and intermediate (B) structure category assignment, has fallen into Concern Level I. According to the testing levels given in section II B 4, the initial toxicological information necessary could be derived from a short term 28-day oral toxicity study and a set of short-term tests for determining carcinogenic potential. If the compound causes an adverse effect at a dose less than 2000 times the human exposure level, the Selection Elements identify this compound as a candidate for a sub-chronic, 90-day feeding study to attempt to resolve the impact this finding may have on the ultimate safety judgment on the compound. Alternatively, had the same compound shown instead a potential for carcinogenic activity, a selection element would identify it as a candidate for a carcinogenicity bioassay. Finally had the compound showed no adverse effects at appropriate high intake levels relative to human exposure in the course of the Concern Level I tests, it would require no further testing.

The Selection Elements ought to provide the food additive safety evaluation scheme with the following capabilities: 1) the capability of rapid identification of those substances presenting potential health risks, 2) identification of testing needs for substances, and 3) continual review that focuses on toxicological effects that may have been observed in previous studies and that suggest the need for further testing.

The Selection Elements for determining what further toxicological information needs to be developed for the safety evaluation of additives are as follows:

1. Selection Elements for Performance of a Rodent Short-Term Feeding Study

- o Concern Level I compounds without short-term multiple dose exposure studies will require this study in a single rodent species, preferably the rat, unless data mitigate the requirement.
- o Compounds without data that allow the selection of dosages for conduct of any subchronic or chronic study are recommended for this study, whenever such longer duration studies are required.

2. Selection Decision Elements for Performance of Short-term Tests for Determining Carcinogenicity Potential

- o Concern Level III compounds without at least a "core standard" carcinogenicity study will require this set of short-term tests for determining carcinogenicity testing priority.
- o Concern Level II and I compounds will require these tests, if there are no "core standard" carcinogenicity data available.

These tests should include:

- i. Gene Mutation with and without metabolic activation in *Salmonella typhimurium* strains TA-1535, TA-1537, TA-1538, TA-100 and TA-98,
- ii. Mammalian Cell Mutagenesis Test with and without metabolic activation,
- iii. Unscheduled DNA synthesis, and
- iv. *Drosophila* Recessive Lethal (optional, unless compound has anti-microbial activity).
- v. Mammalian Cell Transformation Test (optional)

3. Selection Decision Elements for Performance of a Rodent Subchronic (90-day) Study

- o Concern Level II compounds without at least a "core" subchronic study in a rodent species will require this study.

- o Concern Level II compounds with a "core" subchronic rodent study but without a study of this type that meets current standards will require this study unless data mitigate the requirement.
 - o Concern Level I compounds with a lowest "effect" level from a rodent short-term study, which is less than 2000 times the human consumption (mg/kg body weight/day), will require this study in a rodent species.
 - o If priority for a required chronic study is such that the delay in initiation of that study would be longer than 3 years, then Concern Level III compounds with a short-term lowest "effect" level which is less than 1000 times the human consumption will require this test.
 - o Compounds without data that allow the selection of dosages for conduct of any chronic study are recommended for this study, whenever such longer duration studies are required.
4. Selection Decision Elements for Performance of a Subchronic (90-day) Study in a Non-rodent
- o Concern Level II compounds without at least a "core" subchronic study in a non-rodent species will require this study.
 - o Concern Level II compounds with a "core" subchronic non-rodent study but without a study of this type that meets "current" standards will require this study unless data mitigate the requirement.
 - o Compounds with a lowest "effect" level from a short-term non-rodent study which is less than 2000 times the human consumption will require this study, if the non-rodent species tested is the most sensitive to this effect and is appropriate for extrapolation to the human.
 - o Compounds without data that allow the selection of dosages for conduct of any, long-term non-rodent study are recommended for this study, whenever such long duration studies are required.
5. Selection Decision Elements for Performance of a Chronic Study in Rodents
- o Concern Level III compounds without at least a "core" rodent chronic study will require this study in a rodent species.

- o Concern Level III compounds with a "core" rodent chronic study but without a study of this type that meets current standards will require this study unless data mitigate the requirement.
 - o Compounds whose lowest "effect" level from a rodent study is less than 1000 times the human consumption will require this study.
 - o Concern Level II compounds whose toxic profile suggests the probability of late occurring toxicity in rodents, which may not be observed or may be poorly quantified in subchronic tests, will require this study.
6. Selection Decision Elements for Performance of a Long-term (at least 1-year) Toxicity Study in a Non-Rodent
- o Concern Level III compounds without at least a "core" long-term, non-rodent study will require this study.
 - o Concern Level III compounds with a "core" long-term, non-rodent study but without a study of this type that meets current standards will require this study unless data mitigate the requirement.
 - o Compounds with a lowest "effect" level from a non-rodent study is less than 1000 times the human consumption require for this study, if the non-rodent species is the most sensitive to the effect and is appropriate for extrapolation to man.
 - o Concern Level II compounds whose toxic profile suggests the probability of late occurring toxicity which may not be observed or be poorly quantified in subchronic tests will require this test.
7. Selection Decision Elements for Performance of Carcinogenicity Bioassay in Rodent Species
- o Concern Level III compounds without at least a "core" carcinogenicity bioassay require this study in two rodent species.
 - o Concern Level III with a "core" carcinogenicity bioassay but without a study of this type that meets current standards will require this study unless data mitigate the requirement.
 - o Compounds with data which indicate treatment-related, focal hyperplasia, metaplasia, or other proliferative lesions will require this study in two rodent species.

- o Compounds with data that indicate treatment-related necrosis or some other progressive irreversible lesions will require this study in two rodent species.
 - o Concern Level II compounds with a finding that they have significant carcinogenic potential, based upon an evaluation of the results from a battery of appropriate short-term tests for carcinogenicity potential, will require this study in at least one rodent species (two species if human exposure is greater than 0.0125 mg/kg body weight/day).
 - o Concern Level I compounds with a finding that they have significant carcinogenic potential, based upon an evaluation of the results from a battery of appropriate short-term tests for carcinogenicity potential, will require at least one rodent (preferably the rat) carcinogenicity study.
8. Selection Decision Elements for Performance of a Two-Generation Reproduction Study with a Teratology Phase
- o Concern Level I compounds with results indicating reproductive organ toxicity will require this test.
 - o Concern Level II and Concern Level III compounds will require this test.
9. Selection Decision Element for Performance of a Reproduction Study of at Least Three-Generation Duration
- o Compounds with results in a two-generation study which show that the lowest "effect" level is less than 1000 times the human consumption, or that the effects are occurring at lower doses in the second-generation, occurring at significantly higher incidences, or are of greater severity, will require a reproduction study for at least three generations.
10. Selection Decision Elements for Performance of Subchronic and Chronic Testing with In Utero Exposure Phase
- The determination of whether the use of the in utero route of exposure is required for either subchronic or chronic toxicity studies is based upon application of the following selection decision elements.
- o The in utero route will be required for compounds whose lowest "effect" level is less than 200-times the human exposure.
 - o Non-nutritive additives whose exposure exceeds 0.25 mg/kg/day in the diet will require testing by the in utero route.

- o Nutritive additives will be considered for possible in utero testing.
- o Compounds with reproductive toxicity or teratogenic activity will be considered for in utero study.
- o Any compound with data indicating differences in affected target organs in in utero studies vs. non-in utero studies which require further study will be considered for in utero exposure.
- o Compounds with other data indicating a need for in utero exposure.

ii. Selection Decision Elements for Performance of Special Toxicological Tests

- a) Any compound with toxicological effects which suggest the need for special studies should be considered for design of special studies. The type of test required should be based on the effects observed.
- b) Teratology with gavage administration of the test substance will be required for:
 - o Compounds whose exposure exceeds 0.625 mg/kg/day in the diet,
 - o Concern Level III compounds whose use may result in "beverage" exposure during pregnancy, or
 - o Compounds with adverse reproductive effects which suggest possible teratogenicity.
- c) Special Behavioral or Neurotoxicological Studies:
 - o Compounds which induce neurotoxic signs, symptoms, or effects in any of the required toxicological tests may require special testing. The type of test(s) required will depend upon the review of data.

A schematic representation of the Selection Elements for each Concern Level (I, II or III) to which a compound is assigned is presented in Appendix IV, Figures 2-4.

C. Ranking Decision Elements

Additives once approved do not always remain static relative to the exposure and toxicological criteria used originally to evaluate their safety. As noted in Section I, exposure may change over time and scientific criteria advance. In addition, new data may become available. Because of the many factors involved, additives may range widely in the degree to which their available data compare to current criteria.

Clearly, not all substances deserve the same level of agency concern. Therefore, some means are necessary that will allow the FDA to assess relative concerns for additives so that it may devote more of its resources to those additives that are of highest potential public health concern. Moreover, the available resources ought to be spread as efficiently as possible among as many substances as possible, and not just on one additive at a time. Effective protection of public health can be made more cost-effective if at least some information can be developed for a number of substances simultaneously. In this way the scheme can produce some toxicological information on a number of compounds while also identifying the occasional compound of special concern for immediate testing and even possibly regulatory activity, if necessary.

Ordering compounds for future development of toxicity information ought to relate directly to the relative public health concern for those substances. Thus, Degree of Concern as defined earlier (Section II) ought to implicitly form the basis for any ranking algorithm. Secondly, the ranking of substances ought to be flexible enough to allow consideration of economic and other constraints, and to provide for the use of expert judgment.

The process of priority ranking chemicals is not a new one. A number of agencies and organizations have performed priority ranking for similar purposes. (Refs. 11, 13, 22(d) and references cited therein, 61-71) FDA has reviewed some of these ranking systems (Ref. 72). The method developed below makes use of some of the principles invoked by earlier workers and also employs techniques and parameters that help to solve problems unique to food additives.

The basis for FDA's priority ranking is a system of Ranking Elements that relate together the exposure, molecular structure, type and severity of toxicological responses for substances in a way that is a representation of the relative degree of concern the substance creates. Overall, the ordering of additives ought to abide by three general principles, as follows:

- First, to determine the actual priorities of the additives, the following four goals must be met:
 - i) Because the priority should implicitly reflect the degree of public health concern for that additive, the consideration of the Level of Concern of a given additive as defined in section II, would be an important goal in determining priority. Generally, substances assigned a high Level of Concern should rank above those assigned a lower Level of Concern.
 - ii) Priority rank should be influenced by the results of previously performed toxicological tests.
 - iii) Priority should generally be highest for those substances that have demonstrated a toxic potential at levels comparable to those actually present in the food supply.
 - iv) For all other substances, and all else being equal, the ranking should be consistent with the notion that higher testing priority should be assigned to compounds that lack basic toxicological information, and a lower priority to those compounds that have shown no overt toxic effects. This hierarchy would have the benefit of ensuring that agency concern is focused also on those substances that lack adequate data.
- Second, because testing criteria ought to be stated in terms of specific toxicological effects, the priority ranking system should permit attention to be focused with varying degrees of scientific sophistication, on a number of substances simultaneously. This may be accomplished by creating a separate priority list for each major type of toxicological study or test type. Thus, while further chronic (lifetime) animal feeding studies may be of high priority for certain additives to resolve questions of long-term effects, other additives

may have a higher priority for shorter term studies to determine dose ranges for an anticipated chronic test, or to resolve a toxic effect of concern where a chronic test would not be necessary or appropriate. Still other additives may have high priority for in vitro and other short term tests for carcinogenicity potential to fulfill only basic testing needs. In short, the mechanism ought to be designed to reflect current concern about many potential toxic responses, not only cancer. The principle is not only to reduce overall concern for all potential toxic phenomena as efficiently as possible, but also to uncover as many unknown and unanticipated hazards as possible from the ranks of untested or lower priority substances.

- Third, the priority ranking system must be designed to allow for the use of expert judgment and must take into account economic and administrative realities. The availability of testing facilities, the cost of obtaining toxicological information, the potential health consequences of postponing the gathering of information, the continuing need for facilities to test new additives, the priorities of the National Toxicology Program, and limitations on FDA scientific personnel must all be taken into account.

Under such a scheme, any information, even that which is developed out of priority sequence, can have the effect of either raising or lowering the priority position of an additive. All reliable information, whatever the source, should be allowed to periodically update the priority list. In this way the application of Ranking Elements provides a current and comprehensive overview of all food additive safety concerns.

Ranking Elements for additives are listed below. (Note that if data on a compound satisfy more than one ranking element for a given test, then the compound should be ranked under the ranking element which gives the highest priority. Each compound should occupy only one position for each test.)

(The "R" value described in the subsequent sections is defined as the ratio of human consumption in mg/kg/day to the lowest dose producing the appropriate compound-related adverse effects in the longest duration, highest quality study available.)

1. Ranking Decision Elements for Compounds Selected for a Short-term Feeding Study are as follows (in descending order)

- a) Compounds which have been selected for a longer duration feeding study, and which lack sufficient data for selection of doses for that study, will be ranked according to ranking decision elements for the carcinogenicity study (elements 7a-c, below) followed by chronic study (element 5a, below), followed by subchronic study (element 3a, below) followed by carcinogenicity study (elements 7d-e, below) finally by chronic study (elements 5b-d, below).

- o) Concern Level I compounds without at least a "core" short-term feeding study will be ranked by a measure of the "effective exposure" of the compound. The higher the "effective exposure," the higher the priority for testing. "Effective exposure" is a function of the expected human consumption adjusted by the chemical structure assignment (A, B, or C). For example, within a set of compounds with the same exposure, any with structure assignments B or C as compared to A would be given highest priority.
 - c) Concern Level I compounds without a "current standard" short-term feeding study will be ranked by the "R" value from the "core" quality short-term feeding study.
- 2. Ranking Decision Elements for Compounds Selected for Short-term Tests for Determining Carcinogenicity Potential are as follows (in descending order)
 - a) Compounds with unresolved positive indications from one of the short-term tests for potential carcinogenicity (see selection decision element 2) will be ranked by expected human consumption.
 - b) Compounds with suspected carcinogenicity potential from some other short-term test will be ranked by human consumption.
 - c) Other compounds requiring these tests will be ranked by "effective exposure".
- 3. Ranking Decision Elements for Compounds Selected for a Subchronic Rodent Study are as follows (in descending order)
 - a) Compounds ((including Concern Level I compounds) with a lowest "effect" level from a rodent short-term study which is less than 2000 times the human consumption) requiring subchronic rodent study without any subchronic data will be priority ranked according to the "R" value from the longest duration "core" study available.
 - b) Compounds which have been selected for a longer duration feeding study in a rodent species and which need subchronic study in order to select doses for the long duration study will be ranked according to the ranking decision elements for carcinogenicity study (elements 7a-c, below) followed by chronic study (element 5a, below) followed by carcinogenicity (elements 7d-e, below) followed by chronic (elements 5b-d, below).

- c) Compounds selected for a subchronic rodent study without any "core" or "current" standard subchronic study; will be priority ranked for performance of a subchronic study in a rodent species according to the "R" value obtained from the available short-term data from a rodent species.
- d) Compounds selected for a subchronic rodent study with a "core" or "current" standard subchronic or long-term study available in a non-rodent species and no subchronic rodent data, will be priority ranked for performance of a subchronic test in the rodent species according to the "R" value obtained from the available data from the subchronic or chronic non-rodent study.
- e) Compounds selected for a subchronic rodent study with a rodent subchronic study that meets "core" quality standards will be priority ranked according to the "R" value obtained from the "core" study.

4. Ranking Decision Elements for Compounds Selected for a Subchronic Non-rodent Study (in descending order)

- a) Compounds with a lowest "effect" level for a short-term non-rodent study which is less than 2000 times the human consumption and for which the non-rodent species has been determined to be the most sensitive species will be ranked according to the "R" value obtained in the short-term non-rodent study.
- b) Compounds which have been selected for a longer duration feeding study in a non-rodent species and need subchronic study in order to select doses for that longer duration study will be ranked according to the decision elements for non-rodent long-term study (elements 6a-c, below).
- c) Compounds selected for a subchronic non-rodent study with "core" or "current" standard subchronic or chronic study in a rodent species but without any non-rodent data will be priority ranked according to the "R" value obtained from the available rodent data.
- d) Compounds selected for a subchronic non-rodent study with a non-rodent subchronic study which meet "core" quality standards will be priority ranked according to the "R" value obtained from the "core" study.

5. Ranking Decision Elements for Compounds Selected for a Chronic Rodent Study *(in descending order)

- a) Compounds with the lowest "effect" level from a rodent study, which is less than 1000 times the human consumption, will be ranked by the "R" value from that study.
- b) Concern Level III compounds without chronic rodent data or long-term non-rodent data will be ranked by the "R" value from the data available.
- c) Concern Level III compounds with a "core" or "current" standard long-term non-rodent study, but without any chronic rodent data will be ranked by the "R" value from the long-term non-rodent study.
- d) Concern Level II compounds whose toxic profile suggests the probability of late occurring toxicity will be ranked by the "R" value for the effect which is suggested.
- e) Concern Level III compounds with "core standard" chronic rodent study will be ranked by the "R" value from that study.

*This study can be combined with a carcinogenicity study.

6. Ranking Decision Elements for Compounds Selected for a Long-term Non-rodent Study (in descending order)

- a) Compounds with the lowest "effect" level which is less than 1000 times the human consumption from a non-rodent study will be ranked by the "R" value from that study.
- b) Concern Level III compounds with a "core" or "current" standard rodent study, but without any non-rodent data will be ranked by the "R" value from the rodent data.
- c) Concern Level II compounds whose toxic profile suggests the probability of late occurring toxicity will be ranked by the "R" value for the effect which is suggested.
- d) Concern Level III compounds with a "core standard" non-rodent study will be ranked by the "R" value from that data.

7. Ranking Decision Elements for Compounds Selected for a Rodent Carcinogenicity Study (in descending order)

Ranking for this study in the first rodent species (usually rat) will be as follows (in descending order):

- a) Compounds with treatment-related focal hyperplasia, metaplasia, or other proliferative lesions from any study will

be ranked by the "R" value for the observed effect. Where possible, the species selected for this first study should be the same as the species in which the proliferative response was observed.

- b) Compounds with human consumption greater than or equal to 3×10^{-4} mg/kg/day,* and with a finding that it has significant carcinogenic potential based upon an evaluation of the results from a battery of appropriate short-term tests for potential carcinogenicity, will be ranked by human consumption in mg/kg b.w./day.
- c) Compounds with treatment-related necrotic or progressive irreversible lesions from any study will be ranked by the "R" value for the observed effect.
- d) Concern Level III compounds without a "core standard" study will be ranked by the "R" value from short-term or subchronic studies.
- e) Compounds with less than 3×10^{-4} mg/kg/day* consumption and with a finding that it has significant carcinogenic potential based upon an evaluation of the results from a battery of appropriate short-term tests for potential carcinogenicity, will be ranked on the basis of human consumption.
- f) Any compounds selected for this test with a carcinogenicity study that meets the "core" quality standards but does not meet the current toxicology testing standards will be repeated with a priority rank based on "R" from the "core quality standard" carcinogenicity study.

* This is an example of a flexible cutoff value, determined by either estimations of potential risk or exposure break-points for Levels of Concern, which may be used to adjust testing priorities (irrespective of potency considerations) for extremely low exposure additives, where public health concerns would be low.

8. Ranking Decision Elements for Compounds Selected for a Rodent Carcinogenicity Study in a Second Species are as follows (in descending order)

Where possible, the selection of the second species and strain should be based on metabolic or toxicologic consideration. Unless metabolic data are available, the mouse is usually recommended as the second species.

- a) Compounds with treatment-related focal hyperplasia, metaplasia, or other proliferative lesions from any study will be ranked by the "R" value for the observed effect.
- b) Compounds with human consumption greater than or equal to 0.0125 mg/kg/day*, and with a finding that it has significant carcinogenic potential based upon an evaluation of the results from a battery of appropriate short-term tests for potential carcinogenicity, will be ranked by human consumption in mg/kg b.w./day.
- c) Compounds with treatment-related necrotic or progressive irreversible lesions from any study will be ranked by the "R" value for the observed effect.
- d) Concern Level III compounds without a "core standard" study will be ranked by the "R" value from short-term or subchronic studies.
- e) Compounds with less than 0.0125 mg/kg/day* but greater than 3×10^{-4} mg/kg/day* consumption and with a finding that it has significant carcinogenic potential based upon an evaluation of the results from a battery of appropriate short-term tests for potential carcinogenicity will be ranked by human consumption.
- f) Compounds selected for this test with a carcinogenicity study that meet the "core" quality standards but do not meet the current toxicology testing guidelines will be repeated with a priority rank based on "R" from the "core quality standard" carcinogenicity study.

* See previous footnote.

9. Ranking Decision Elements for Compounds Selected for a Two-Generation Reproduction Study with a Teratology Phase are as follows (in descending order)

- a) Compounds with reproductive or reproductive organ toxicity in any study will be priority ranked by the "R" value for that toxicity.
- b) Concern Level II and III compounds will be priority ranked by the "R" value from the longest duration study available.

10. Ranking Decision Elements for Compounds Selected for a Three Generation Reproduction Study are as follows (in descending order)

Compounds selected for reproduction study in at least three generations will be ranked by the "R" value for the reproductive effect which resulted in the selection of the compound for this study.

11. Ranking Decision Elements for Compounds Selected for a Gavage Teratology Study are as follows (in descending order)

- a) Compounds selected for a gavage teratology study on the basis of data which suggest potential compound-related teratogenic effects will be ranked by the "R" value for the observed effects.
- b) Concern Level III compounds selected for gavage teratology study will be ranked by the "R" value for available data (the use of an "R" value from a reproduction study is preferred).

12. Ranking Decision Elements for Compounds Selected for Special Toxicity Studies

Where several compounds are selected for the same special study, the compounds will be ranked by the "R" value for the observed compound-related adverse effect which lead to the compounds selection for special study.

13. Ranking Decision Elements for Compounds Selected for an In Utero Exposure Phase

The rank of these compounds should be that for the tests required; those tests should be conducted with an in utero phase.

14. Ranking Decision Element for Compounds with no Toxicity Data

Although compounds with no toxicity data can be selected for various toxicity studies on the basis of Concern Level assignment, initially these compounds should be ranked only for rodent, short-term feeding studies and for short-term tests for determining carcinogenicity potential using "effective exposure" for ranking. For informational purposes these compounds can be

listed at the bottom of the priority list for other selected tests. As appropriate data become available these compounds can be inserted into the proper position on all lists.

D. Special Decision Elements to Select Compounds for an Immediate Review

A special group of decision elements, which will be used to bring compounds with certain effects or "effect" dose levels to attention for special interim review, are described below.

- o Compounds with an LD₅₀ value that is less than 100 times the expected human consumption in mg/kg b.w./day will be identified for special regulatory attention.
- o Compounds with a lowest "effect" dose from a short-term study that is less than 100 times the maximum human exposure in mg/kg b.w./day will be identified for special regulatory attention.
- o Compounds with a lowest "effect" level (from a subchronic study) that is less than 100 times the expected human consumption in mg/kg b.w./day will be identified for special regulatory attention.
- o Compounds with a highest "no-effect" level which is less than 100 times the expected human consumption in mg/kg b.w./day will be identified for special regulatory attention.
- o Compounds with a confirmed proliferative lesion will be presented before the Bureau of Foods' Cancer Assessment Committee for its evaluation.
- o Compounds whose effects on target organs are to be studied by special or non-routine testing methods will be reviewed in order to design an appropriate study.

E. Summary

The combined application of the Decision Elements (both Selection Elements and Ranking Elements) results in the priority matrix shown in Figure 5 of Appendix IV. In this figure, the vertical axis labeled "Concern" reflects a quantitative evaluation of relative degree of health concerns for all additives. Economic and other considerations can be conveniently factored in at this point. To accomplish this the columns of Figure 5 are free to "slide" vertically with respect to one another to respond to the societal determination that one type of data development may be of greater importance or of greater economic feasibility than another. This relative scaling of columns can be extended to individual boxes

within a column by "detaching" them and displacing them vertically from one another as dictated by societal judgments, and economic considerations or other constraints.

The resulting two-dimensional matrix, shown schematically in Figure 5, is a representation of overall relative priority (based on a more quantitative and broader determination of Degree of Concern) for information development on approved substances.

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Appendix I

Chemical Structure Category System

Chemical Structure Category System

Introduction:

The purpose of grouping food additives into chemical structure classes is to estimate the potential toxicity of the additives on the basis of their chemical structures. The structure classes will subsequently be used for assignment to Levels of Concern. Additives will be assigned to one of three structural classes (A, B, C) based on their structural similarities to known toxicants. This assignment initially involves determining the chemical structures of the additives' functional groups and comparing these structures with substances of known toxicity.

Determination of Additive Structures:

The determination of the chemical structures category of an additive should include, where possible, identification of the chemical structure of the additive and any information about known metabolites; predicted metabolites; components of mixtures, such as, fatty acid mixes, components of plant extracts, etc.; and contaminants. For contaminants or secondary components, the quantity in which they are present or predicted should be indicated. Summaries of this information should contain structures, literature references for known metabolites or contaminants, justifications for prediction of metabolism or contamination, and references for contaminant or secondary component content.

Structure Category Assignment Procedures

The structure category assignments are formulated using a qualitative decision tree. After the functional groups of the additive are identified, the decision tree outlined later in this appendix is used to assign the additive to a structure class. Additives with functional groups of high probable toxicity are assigned to category C. Additives of intermediate or unknown probable toxicity are assigned to category B. Additives of low probable toxicity are assigned to category A. With application of the decision tree below, category assignment will be arrived at in a uniform manner. For example, a simple saturated hydrocarbon alcohol like pentanol would be recorded as A, 2. The table and decision tree will enable most assignments to be made; however, there may be cases where the structure is so complex that the decision tree cannot be used. Under these circumstances, structure category assignment can better be made by a structure verification group which may draw upon the complementary expertise of several individuals. If it is known that the functional group of an additive is more or less toxic than the decision tree suggests, then the compound should be assigned to a different category. If a reassignment is made, the change must be justified with referenced literature support.

Structure Category Assignment Verification:

To insure consistency, all structure category assignments will be reviewed by an internal committee on structure-activity relationships. The committee will review only the Structure Category Summary Sheet; therefore, it is essential that all pertinent information and questions concerning the structure assignment of the additive be included on this sheet. Any alterations in category assignment recommended by the verification committee will be discussed with the toxicologist and CSO originally suggesting the structure category change.

Calculation of Adjusted Poundage:

For the purpose of priority ranking, the actual poundage disappearing into the food supply of a food additive may be normalized in order to make a direct comparison of structure type A, B and C materials. This is accomplished by increasing the poundage of a C class additive by a factor of 2 and decreasing the poundage of an A class additive by a factor of 0.5. For mixtures, the percentage of A, B or C components may be adjusted in a similar manner and then summed to give the total adjusted poundage. This adjusted poundage is only a relative figure and will be used only for priority ranking purposes.

Structure Category Assignment

Decision Tree for Food Additive Structure Category Assignment

Tables A, B, and C follow

1. Are 90% (by weight or volume) of the components identifiable for the additive substance(s)?

If No, then assign additive to Structure Category C.

If Yes, then continue.

2. If quantification of secondary components or contaminants for an additive is not available, are any of these functional groups contained in Table C?

If Yes, then assign additive to Structure Group C and calculate the adjusted poundage on this basis.

3. Does 10% (by weight or volume) or more of the total additive mixture, components, and contaminants contain functional groups listed in Table C? For example, an additive is a mixture of 3 components x, y, & z; 90% is x and it is an A structure, component y is 3% of the total mix and it is a C structure, and z is a C structured contaminant accounting for 7% of the total mix. Therefore 10% of the total mix is C structures and thus the additive is given a C assignment; however, the adjusted poundage should be calculated on the basis of the percentages of C or A material present.

If Yes, then assign additive to Structure Group C.

If No, then continue.

4. Are any functional groups of known or predicted metabolites of the additive contained in Table C?

If Yes, then assign additive to Structure Group C.

If No, then continue.

5. Does 10% or more of the additive mixture (components or contaminants) contain functional groups not listed in Table A?

If Yes, then assign additive to Structure Group B.

If No, then continue.

6. Are any functional groups of known or predicted metabolites of the additive not contained in Table A?

If Yes, then assign additive to Structure Group B.

If No, then assign additive to Structure Group A.

7. Is there any evidence of bioaccumulation?

If Yes, then please describe.

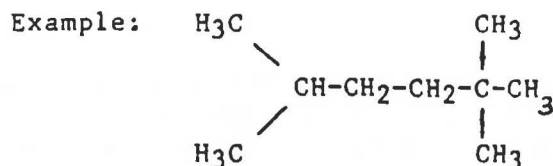
Structure Category Assignment

Sub-structure Tables

TABLE A

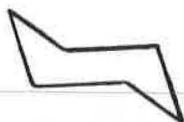
1. Simple aliphatic, non-cyclic hydrocarbons.

These compounds should have NO unsaturation, i.e. no aromaticity, no double or triple bonds.



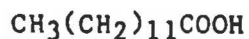
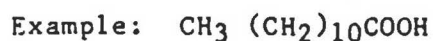
2. Mono-cyclic hydrocarbons (alicyclic) up to a total carbon number of C₂₀. These compounds should have NO unsaturation.

Example:



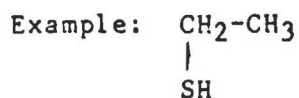
3. Fats, fatty acids or their inorganic salts of alkali metals (Na, K) and alkaline-earth metals (Ca, Mg). Both saturated and unsaturated, non-conjugated compounds.

Carbon length of C₂ to C₃₀.



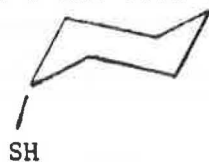
4. Simple aliphatic, non-cyclic (saturated) mono-functional alcohols, ketones, aldehydes, acids, esters, ethers, mercaptans, and disulfides of carbon number greater than or equal to C₂ and less than C₃₀.

These compounds should contain only one functional group and NO unsaturation of the carbon chain.



5. Mono-cyclic hydrocarbons with mono-functional alcohol, ketone, aldehyde, acid, ester, mercaptan, or disulfide substitution or carbon number greater than 6 and less than 20.

Example:



6. Normal human biochemical constituents of carbohydrate and lipid metabolism excluding perhydrophenanthrenes, terpenes, and elecosadienoates (arachidonic acid precursors and metabolites).
7. Endogenous inorganic salts of alkali metals (Na, K) and earth alkaline-metals (Mg,Ca)
8. Conjugation reaction products of Table A substances.
9. Sugars, Polysaccharides, and their metabolites.

Compounds receiving Structure Category A assignments should be metabolized only to compounds also listed on Table A.

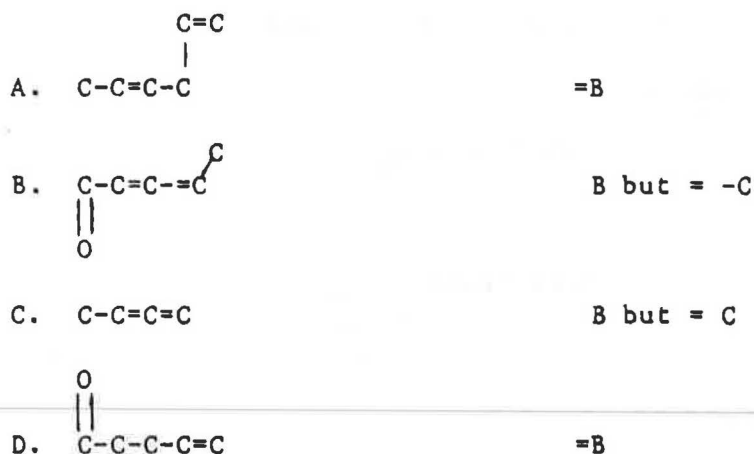
TABLE B

1. Compounds with functional groups not listed in Table A and Table C.

Example: Methanol, Methylesters, Formates, quaternary amines.

2. Non-conjugated olefins, excluding unsaturated fatty acids and fats.

Example:



3. Any multiple functional group containing structure without features listed in Table C.
4. Inorganic salts of Fe, Cu, Mn, Zn, and Sn.
5. Amino acids, unless containing other functional groups listed in Table C.
6. Benzoic Acid and esters, unless substituted with functional groups listed in Table C.
7. Polypeptides and Proteins.
8. Any compound or mixture of undetermined composition, so long as none of the identified portions contain a Table C entry. At least 90% of any mixture (by weight or volume) should be identified, or else a B is assigned.

TABLE C

R = C or H

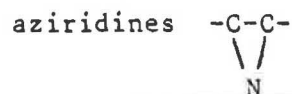
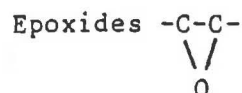
1. Structure not covered by Table C but of high probable toxicity.
2. The structure contains: an organic halogen (C-X), not salts.

X = F, Cl, Br, or I

Example: $\text{CH}_3\text{I} = \text{C}$; CHI salts = C

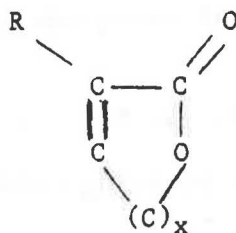
3. Three-membered heterocyclic ring system.

Example:

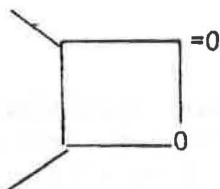


4. α, β -unsaturated lactones

Example:

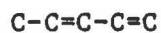


5. 4-membered lactone

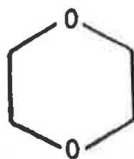


6. (α, β) unsaturated carbonyl function groups (aldehydes, ketones, carboxylic acids, esters), excluding benzoic acid or benzoic ester derivatives.
7. Conjugated alkenes/double bonds and aromatic groups, excluding benzoic acid or benzoic ester derivatives,

Example:



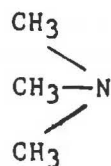
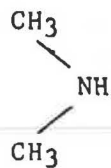
9. 1,4-Dioxane nucleus (six membered cyclic diether)



10. Amides and Imines

11. Amines: including primary, secondary and tertiary amines, aromatic amines and heteroaromatic amines, excluding amino acids.

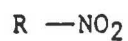
Example: $\text{CH}_3\text{-NH}_2$



but not R_4N^+ Quaternary amines



12. Nitro groups



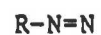
13. N-nitroso groups and C-nitroso groups



14. Nitrilo groups



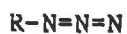
15. Diazo-groups and azo-groups



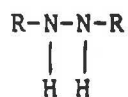
16. Azoxy groups



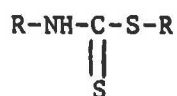
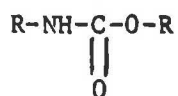
17. Azide groups



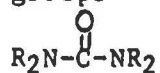
18. Hydrazine groups



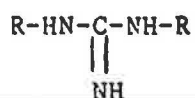
19. Carbamates, thiocarbamides or dithio derivatives



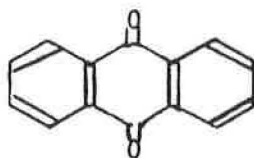
20. Urea groups



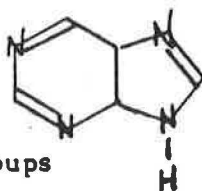
21. Guanidine groups



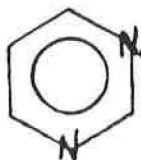
22. Anthraquinone groups



23. Purine groups



24. Pyrimidine groups



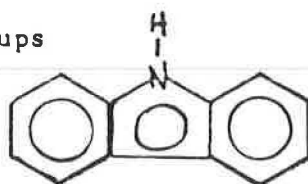
25. Pyrrole groups



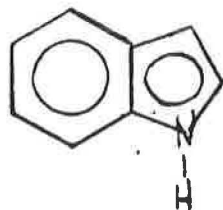
26. Pyrazole groups



27. Carbazole groups



28. Indole groups



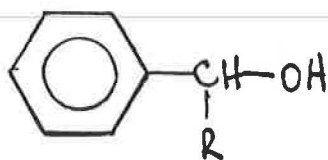
29. Imidazole groups



30. Pyrrolidine groups

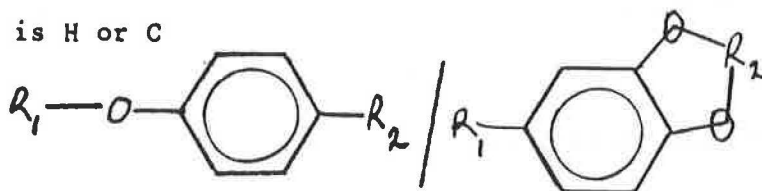


32. Benzylic alcohols, acids, aldehydes and esters



33. "Salfrole-like" structures

R_1 is H or C

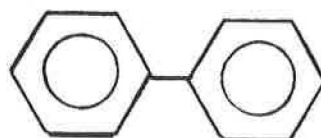
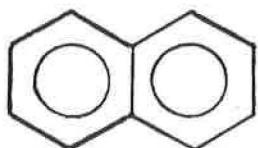


R_2 is C or C=C

34. Polynuclear aromatics (fused)

= Table C-34

≠ Table C-34, but = Table C-7



35. Furan groups



36. Thiazole groups

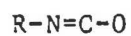


37. Oxazole groups

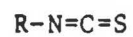


38. Other heterocyclic functional groups.

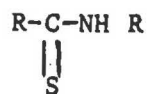
40. Isocyanate groups



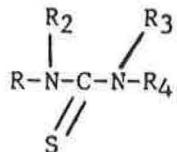
41. Isothiocyanate groups



42. Thioamide groups



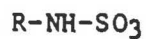
44. Thiourea groups



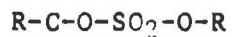
45. Thioether groups



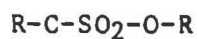
46. Sulfamate groups



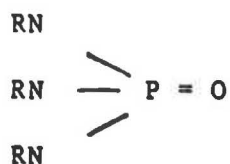
47. Organic Sulfate groups



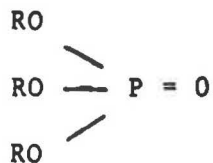
48. Organic sulfonates



49. Phosphoramidate groups



50. Phosphoric ester groups



51. Inorganic Salts not covered by Table A or B

52. Organo-metallics other than those mentioned in Table A or B

Structure Category Summary Sheet

Substance Name: _____
(Main Term)

Additive (or mixture components) Structure(s):

Metabolites:

References:

Contaminants:

References

Example Structure Category Summary Sheet
(page 2)

Substance Name: _____

Quantitative Estimates:

Additive = 100%

Parent Additive Substance

Components

Contaminants

References;

Structure Category Assignment:

Comments:

Primary Reviewer _____ date _____

Secondary Reviewer _____ date _____

Verification _____ date _____

Appendix II

Guidelines For Toxicological Testing

Guideline for Acute Oral LD₅₀ Toxicity Studies

Introduction

This guideline is designed for use in acute ingestion tests using rodents, but is adaptable to other species.

Although several accepted methods for determining LD₅₀ values have been developed, many important determinants of toxicity are not represented either by these values or slopes of dose-response curves for lethality. These determinants are integral to an evaluation of acute toxicity and should be observed during the course of an acute toxicity study. Site and mechanism of action, early or delayed death, and recovery rate may be better indices of toxicity and hazard than LD₅₀ values per se. Morbidity and or pathogenesis may have more toxicological significance than mortality.

The laboratory animals often used for acute toxicity testing are rodents (rat, guinea pig, mouse, gerbil), lagomorphs (rabbit), carnivores (dog, cat), and subhuman primates. Testing may be done in two or more of these species to ascertain qualitative and quantitative differences in response. Similar toxicity in more than one species may increase the predictability of toxicity in man.

For acute oral tests in non-rodents, LD₅₀ values need not be obtained. Evidence of acute toxicity may be developed in range-finding studies using relatively fewer animals than in a typical acute toxicity study using rodent.

This guideline is limited to acute ingestion tests using the rat and the mouse.

I. General Considerations

A. Good Laboratory Practices

Studies should be conducted according to good laboratory practice regulations (e.g., "Nonclinical Laboratory Studies, Good Laboratory Practice Regulations," 43 FR 59986, 22 December 1978).

B. Test Substance

1. The specific substance or mixture of substances to be tested should be determined in consultation with the agency. As far as is practical, composition of the test substance should be known. Information should include the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials to account for the entire test substance.
2. Ideally, the lot of the substance tested should be the same throughout the study. The test sample should be stored under conditions that maintain its stability, strength, quality, and purity from the date of its production until the tests are complete.

C. Animals

1. Recommendations contained in DHEW pub. no. (NIH) 74-23, entitled "Guide for the Care and Use of Laboratory Animals," should be followed for the care, maintenance, and housing of animals.
2. Healthy animals, not subjected to any previous experimental procedures, must be used.
3. The test animal shall be characterized as to species, strain, sex, weight and/or age. Each animal must be assigned an appropriate identification number.
4. Animals may be group-caged for this test unless a specific test guideline or the pharmacological action of the test substance dictates otherwise, but the number of animals per cage should not prevent continued and clear observation of each animal. When signs of morbidity or excitability are observed in group-caged animals during the test, such animals should be moved to separate cages.
5. Animals should be assigned to groups in a random manner so as to minimize bias and to assure comparability of pertinent variables for statistical purposes.

D. Dead Animals, Necropsy, and Histopathology

Animals should be observed as necessary to insure that there is minimal loss due to cannibalism or similar management problems. Where possible, necropsy should be performed soon after an animal is sacrificed or found dead to minimize loss of tissues due to autolysis. When necropsy cannot be performed immediately, the animal must be refrigerated at temperatures low enough to minimize autolysis and not cause freezer burn. If histopathological examination is to be conducted, tissue specimens should be placed in appropriate fixative when they are taken from the animal.

II. Specific Considerations

A. Test Preparation

1. Animals: Laboratory strains of young, adult rats (125-250 g each) and/or mice (20-30 g each) should be used. The weight variation in the animals used in a test should not exceed \pm 20 percent of the new weight. When attempting to estimate hazards to young humans, additional studies designed to consider to developmental stage of the test animal in relation to anticipated human exposure should be performed.
2. Fasting: Prior to administration of the test substance, food should be withheld from rats overnight; for other rodents with higher metabolic rates, a shorter period of fasting is appropriate (for mice 2 to 4 hours)
3. Limit test: A trial test is recommended to establish the need for further testing. If 5 g/kg administered orally to 5 animals of each sex produces no mortality and the expected LD₅₀ is greater than 5 g/kg, no further testing is necessary.
4. Number and sex: At least 10 animals, 5 per sex, should be used at each dose level. Nonpregnant, nulliparous females should be used.
5. Dose levels: At least three and preferably four dose levels should be used to produce toxic effects and mortality rates with a range from 10 to 90% and bracketing the expected LD₅₀. The data should be sufficient to produce a dose response curve and permit an acceptable determination of the LD₅₀.
6. Controls: Controls are generally not required, since dose response during an LD₅₀ may serve as an internal control. If a vehicle or solvent of uncharacterized toxic potential is used, an acute oral toxicity test should be done using the solvent.

B. Test Procedure

1. Route of administration: Ideally, the dose should be administered in a single dose by gavage or capsule. Because of the physical/chemical nature of the test substance, doses may be administered in a suspension or capsules in divided doses over a period of 24 hours.
2. Dosage: The dose is administered via soft rubber or polyethylene catheter or a ball-tip needle. The maximum volume of aqueous solutions that can be given in one dose depends on the rodent's size and should not exceed 2 ml/100g body weight. For non-aqueous liquids and suspensions the volume should not exceed 1 ml/100 gm. When possible, variability in test volume should be minimized, with concentrations being adjusted accordingly.

3. Observation period: The observation period should be at least 14 days. Although a 14-day observation period is sufficient for most compounds, animals demonstrating visible signs of toxicity after 14 days may be held longer.
4. Clinical observations: The animals should be carefully observed frequently during the first day and twice a day thereafter at least 4 hours apart (once each morning and late afternoon). All toxicological and pharmacological signs should be recorded including time of onset, intensity, and duration. The time of death should also be noted. Individual records should be maintained for each animal.
5. Weight change: Animals must be weighed individually on the day the test substance is administered, weekly thereafter, and prior to sacrifice.
6. Necropsy: A complete gross necropsy should be performed on all animals that die during the course of the test. Where significant signs of toxicity are observed consideration should be given to gross necropsy of the animals sacrificed at termination of the test. If the substance will not be subjected to additional acute or multiple dose testing that includes gross necropsy, or if the results of this test are to be used for labeling purposes, complete gross necropsy should be performed on the remaining animals at termination of the test. Microscopic examination of gross lesions should be considered.

III. Data Reporting

A. Identification

Each test report should be signed by the persons responsible for the test and identify:

1. The laboratory where the test was performed by name and address;
2. The inclusive dates of the test; and
3. Each person primarily responsible for separate components of the test including (a) the conduct of the test, (b) pathology, (c) analysis of the data, (d) the writing of the report, and (e) any written or other matter contained in the report.

B. Body of Report

The test report must include all information necessary to provide a complete and accurate description and evaluation of the test procedures and results in the following sections:

1. Summary and conclusions: This section of the test report should contain a brief description of the methods, a summary of the data, an analysis of the data, and a statement of the conclusions drawn. The summary must highlight all positive data or observations and any deviations from control data which may be indicative of toxic effects.
2. Materials: This section of the test report should include, but not be limited to, the following information:
 - (a) Identification of the test substance, so far as practical, including:
 - i. chemical name, molecular structure, and a qualitative and quantitative determination of its chemical composition, including names and quantities of known contaminants and impurities and listing the percentage of unidentifiable materials to account for the entire test sample;
 - ii. manufacturer and lot number of the substance tested, and such information as physical state, pH, stability, and purity; and
 - iii. specific identification of diluents, suspending agents, emulsifiers, or other materials used in administering the test substance.
 - (b) Animal data, including:
 - i. species and strain used and rationale for selection of the strain if other than a common laboratory strain;
 - ii. source of supply of the animals;
 - iii. description of any pre-test conditioning, including quarantine procedures, etc.;
 - iv. description of the method used in randomization of animals to test or control groups; and
 - v. numbers, age and condition of animals of each sex in each test and control group.
 - (c) Data on husbandry should include description of the caging condition including number of animals per cage, diet, bedding material, ambient temperature, humidity, and lighting conditions.

3. Methods

- (a) Deviation from guidelines: This section shall indicate all ways in which the test procedure deviates from these guidelines and shall state the rationale for such deviation.
- (b) Specification of test methods: This section shall include a full description of the experimental design and procedure, the length of the study, and the dates on which the study began and ended.
- (c) Statistical analysis: All statistical methods used should be fully described or identified by reference.
- (d) Data on dosage administration, including:
 - i. all dose levels administered, expressed as mg/kg of body weight;
 - ii. method, frequency, duration, and time of day; and
 - iii. total volume of substance (i.e., test substance plus vehicle) contained in individual dosages.
- (e) Data on observation methods, including:
 - i. duration and
 - ii. method and frequency of observation of the animals.

4. Results

The tabulation of data and individual results must accompany each report in sufficient detail to permit independent evaluation of results.

- (a) Tabulation of the response data (i.e., number of animals dying, number of animals showing signs of toxicity, and number of animals exposed) at each exposure level by sex, and time of death after dosing;
- (b) LD₅₀ values for each test substance calculated at the end of the observation period, with method of calculation specified;
- (c) 95% confidence interval for the LD₅₀ values;
- (d) Slope and significance of the dose-mortality curve for each substance tested; and
- (e) Findings from all clinical observations, necropsy, and histopathological examinations.

5. References

This section of the test report shall include the following information:

(a) Availability of original data, specimens, and samples of the test substance. The location of all original data, specimens, and samples of the test substances which are retained in accordance with the testing requirement.

(b) Literature or references, including, where appropriate, those references for (1) test procedures, (2) statistical and other methods used to analyze the data, (3) compilation and evaluation of results, and (4) the basis upon which conclusions were reached.

IV. Suggested Reading

1. Balazs, T. 1970. Measurement of Acute Toxicity. In: "Methods in Toxicology." G. E. Paget, ed., F. A. Davis Co., Philadelphia, Pa.
2. Hagan, E. C. 1959. Acute Toxicity. In: "Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics." Association of Food and Drug Officials of the United States.
3. Bliss, C. I. 1938. The determination of the dosage mortality curve from small numbers. Quarterly Journal Pharm. Pharmacol. 11:192-216.
4. Litchfield, J. T., Jr. and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Therap. 96:99-115.
5. Thompson, W. R. 1947., Using of moving averages and interpolation to estimate median effective dose. Bacteriological Rev. 11:115-145.

GUIDELINES FOR A SHORT-TERM CONTINUOUS EXPOSURE
ORAL TOXICITY STUDY

Introduction

Short-term, continuous exposure studies of one month or less are conducted to determine the target organs for toxicity after repeated dosing. This study also serves as a range finder to predict the doses which will not cause lethality or undue toxicity after months or years of administration in subchronic studies. Utilization of this information allows future subchronic and chronic studies to be designed with realistic doses and special emphasis on the target organs.

This guideline is for the use primarily for the rat and dog although other species could be used with protocol modifications.

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 - A. Good Laboratory Practices
 - B. Test Substance
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 - E. Diet
 - II. Specific Considerations
 - A. Test Preparation
 - B. Test Procedure
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 - A. Identification
 - B. Body of Report
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 - 4. Results
 - 5. References
 - IV. Suggested Reading
-

I. General Considerations

A. Good Laboratory Practices

Studies should be conducted according to good laboratory practice regulations (e.g., "Nonclinical Laboratory Studies, Good Laboratory Practice Regulations," 43 FR 59986, 22 December 1978).

B. Test Substance

1. The specific substance or mixture of substances to be tested should be determined in consultation with the agency. As far as is practical, composition of the test substance should be known. Information should include the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials to account for the entire test substance.
2. Ideally, the lot of the substance tested should be the same throughout the study. The test sample should be stored under conditions that maintain its stability, strength, quality, and purity from the date of its production until the tests are complete.

C. Animals

1. Recommendations contained in DHEW pub. no. (NIH) 74-23, entitled "Guide for the Care and Use of Laboratory Animals," should be followed for the care, maintenance, and housing of animals.
2. Healthy animals, not subjected to any previous experimental procedures, must be used.
3. The test animal shall be characterized as to species, strain, sex, weight and/or age. Each animal must be assigned an appropriate identification number.
4. Animals may be group-caged for this test unless a specific test guideline or the pharmacological action of the test substance dictates otherwise, but the number of animals per cage should not prevent continued and clear observation of each animal. When signs of morbidity or excitability are observed in group-caged animals during the test, such animals should be moved to separate cages.
5. Animals should be assigned to groups in a random manner so as to minimize bias and to assure comparability of pertinent variables for statistical purposes.

D. Dead Animals, Necropsy, and Histopathology

Animals should be observed as necessary to insure that there is minimal loss due to cannibalism or similar management problems. Where possible, necropsy should be performed soon after an animal is sacrificed or found dead to minimize loss of tissues due to autolysis. When necropsy cannot be performed immediately, the animal must be refrigerated at temperatures low enough to minimize autolysis and not cause freezer burn. If histopathological examination is to be conducted, tissue specimens should be placed in appropriate fixative when they are taken from the animal.

E. Diet

The animal's diet should meet all of the nutritional requirements of the test species. Special attention should be paid to the 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.

II. Specific Considerations

A. Test Preparation

1. Duration of testing: For this study, animals should be exposed to the test substance 7 days per week for 4 consecutive weeks.
2. Species and age: Testing should be routinely performed on young laboratory rats. Dosing of rats shall begin as soon as possible after weaning and acclimation and in any case before the animals are 6 weeks old. If dogs are used, treatment should normally begin at 4 to 6 months of age.
3. Number and sex: Equal numbers of males and females of each species and strain should be used for the test. For studies of up to 30 days in rats, each test group and concurrent control group shall consist of at least 10 animals per sex per group. In dogs, at least 4 per sex per group should be started on test.

4. Number of exposures and concentration level selection

(a) Control group(s): A concurrent control group is required. When a carrier vehicle is used it should be added to the diet at a concentration similar to the maximum given in any dosage group. If there are insufficient data on the toxic properties of the vehicle used in administering the test substance, an additional control group exposed only to the diet should be included. In all other respects, the control group shall be handled and maintained in a manner identical to that used with the test groups. Excessive mortality due to poor management is unacceptable and may be cause to repeat the study. For example, under normal circumstances, mortality in the control group would not be expected to exceed 10%. If more than 5% of the diet is being replaced, a control diet of equivalent nutritional value should be provided.

(b) Dose Group(s): Ideally, four or five but at least three dose levels, in addition to the control(s), should be used. The highest treatment level should result in toxicological changes unless prohibited by exaggerated pharmacological effects or physical-chemical characteristics of the test substance that prevent the use of higher dose levels (i. e., sedation with phenothiazines). The lowest dosage level should be one which does not induce any evidence of toxicity. The middle dose level should be sufficiently high to elicit minimal toxic effects. When possible, dose levels should be expressed in a manner to show relevance to contemplated or anticipated human exposures. Administration of test substance to animals at all dose levels must be done concurrently.

B. Test Procedure

1. Route of administration: The test substance may be administered to the animals in the diet, by stomach tube, in capsules, or in drinking water provided that all animals are treated by the same method. When administered by gavage or in capsules, the doses should be adjusted weekly for changes in body weight. When administered in the diet, the doses may be calculated on the basis of mg of test substance/kg of food. When the test substance is in water, the dose should be calculated as mg/ml.
2. Observations of animals: All toxicological and pharmacological signs shall be recorded daily, including time of onset, intensity, and duration. Estimates shall be made of food consumption (or water consumption when the test substance is administered in the water) every week during the test, and the animals shall be weighed at least weekly. Sufficient surveillance of animals shall be made to insure that not more than 10% of the animals are lost from the test due to cannibalism, misplacement, or similar management problems.

3. Clinical testing: The following determinations should be made at the times indicated below for each type of testing. For rats, these determinations shall be made on at least 5 animals of each sex in each group. For dogs, the measurements should be made on all animals in the study.

(a) Ophthalmological examination: An ophthalmological examination using an ophthalmoscope or equivalent should be made on all-high dose and control animals at least twice during the course of the study. Once prior to administration of the test substance, and at termination of the study. If changes in the eyes are detected, examinations should be conducted on all remaining animals.

(b) Hematology: The following determinations should be made at the end of the testing period: hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte and a measure of clotting potential such as clotting time, prothrombin time, thromboplastin time, or platelet count.

(c) Clinical chemistry: Clinical biochemistry determinations on blood should be carried out at the end of the test period. Test areas which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations are: calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum alanine aminotransferase, serum aspartate aminotransferase, ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumin, creatinine, total bilirubin and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methemoglobin, cholinesterase activity. Additional clinical biochemistry may be employed where necessary to extend the investigation of observed effects.

(d) Urinalyses are considered to be of limited value for most routine short-term toxicological studies.

4. Gross Necropsy

(a) Gross necropsy shall be performed by or under the supervision of a qualified pathologist, preferably the pathologist who performs the histopathological examination.

(b) All test animals must be subjected to a complete gross necropsy.

(c) In addition, organs which should be weighed include the liver, kidneys, testes and adrenals. Prior to being weighed, organs should be carefully dissected and properly trimmed to remove fat and other contiguous tissue in a uniform manner. They should be weighed as soon as possible after dissection to minimize the effects of drying on weight.

(d) Preparation of tissues: Tissues listed below should be fixed in 10% buffered formalin or any other generally recognized fixative, and stained with hematoxylin and eosin, or other appropriate stain for preparation of microscopic slides.

5. Histopathological Examination

For non-Rodents: The following organs and tissues, when present, of all animals should be subjected to microscopic study: All gross lesions, heart, lungs with mainstem bronchi, pancreas, liver, kidneys, testes, ovaries, and spleen.

For Rodents: All gross lesions. In addition, for animals in the control and high-dose level: heart, lung with mainstem bronchi, thyroid and parathyroid, stomach, small and large intestine, uterus, brain, lymph node, adrenals, pancreas, liver, kidneys, testes, ovaries, spleen, and bone marrow. If changes or equivocal results are seen in any of these tissues, then tissues affected should be examined in other dose levels.

III. Data Reporting

A. Identification

Each test report must be signed by the person responsible for the test and identify:

1. The laboratory where the test was performed by name and address;
2. The inclusive dates of the test; and
3. Each person primarily responsible for separate components of the test and the component for which the person is responsible including (a) the conduct of the test, (b) analysis of the data, (c) the writing of the report, and (d) any written or other matter contained in the report.

B. Body of Report

The test report must include all information necessary to provide a complete and accurate description and evaluation of the test procedures and results. Each report must include the following sections:

1. Summary and Conclusions: This section of the test report should contain a tabular summary of the data, an analysis of the data, and a statement of the conclusions drawn from the analysis. The summary must highlight all positive data or observations and any deviations from control data which may be indicative of toxic effects.
2. Materials: This section of the test report shall include, but not be limited to, the following information:
 - (a) Identification of the test substance so far as practical, including:
 - i. chemical name, molecular structure, and a qualitative and quantitative determination of its chemical composition, including names and quantities of known contaminants and impurities, so far as is practical; the determinations shall also include a listing of materials as unknowns, if any, so that the entire test sample is accounted for;
 - ii. manufacturer and lot number of the substance tested, and such information as physical state, pH, stability, and purity; and
 - iii. exact identification of diluents, suspending agents, emulsifiers, or other materials used in administering the test substance.
 - (b) Animal data, including:
 - i. species and strain used and rationale for selection of the strain if other than a common laboratory strain;
 - ii. source of supply of the animals;
 - iii. description of any pre-test conditioning, including quarantine procedures etc.;
 - iv. description of the method used in randomization of animals to test or control groups; and
 - v. numbers, age, and condition of animals of each sex in each test and control group.
 - (c) Data on husbandry should include description of the caging conditions (including number of animals per cage), diet, bedding material, ambient temperature, light cycle and humidity.

3. Methods

- (a) Deviation from guidelines: This section shall indicate all ways in which the test procedure deviates from these guidelines and shall state the rationale for such deviation.
- (b) Specification of test methods: This section shall include a full description of the experimental design and procedure, the length of the study, and the dates on which the study began and ended.
- (c) Statistical analysis: All statistical methods used should be fully described or identified by reference.
- (d) Data on dosage administration, including:
 - i. all dose levels administered, expressed as mg/kg of body weight; when administered in the diet, the ppm of the test substance in the diet should be reported;
 - ii. method and frequency of administration; and
 - iii. total volume of substance (i.e., test substance plus vehicle) contained in individual dosages.
- (e) Data on observation methods, including:
 - i. duration; and
 - ii. method and frequency of observation of the animals.

4. Results

The tabulation of data and individual results must accompany each report in sufficient detail to permit independent evaluation of results, including summaries and tables that show, as appropriate, the relationship of effects to time of dosing, sex, etc.

- (a) Data presented for each animal should include hematology, clinical chemistry, and other tests performed, a description of all toxicological or pharmacological effects and abnormalities, accompanied by the animal's identification number, test group (dose level and sex), and days of study when the signs appeared and disappeared. An attempt should be made to correlate effects observed during the study with post mortem findings. The time of death for animals which die while on test should be reported. When numerical averages are presented, they should be accompanied by an appropriate measure of variability, such as the standard error. All animals placed on study must be accounted for.

(b) Findings from all clinical observations, necropsy, and histopathological examinations.

(c) Evaluation of data: An evaluation of test results, including their statistical analysis, should be made and supplied, based on the clinical findings, the gross necropsy findings, and the histopathological results. This should include an evaluation of the relationship, or lack thereof, between the animal's exposure to the test substance and the incidence and severity of all abnormalities; such abnormalities, include behavioral and clinical abnormalities, tumors and other lesions, organ weight effects, effects on mortality, and any other general or specific toxic effects.

5. References

This section of the test report shall include the following information:

(a) Availability of original data, specimens and samples of the test substance. The location of all original data, specimens, and samples of the test substances which are retained in accordance with the testing requirement.

(b) Literature or references, including, where appropriate, those references for (1) test procedures, (2) statistical and other methods used to analyze the data, (3) compilation and evaluation of results, and (4) the basis upon which conclusions were reached.

IV. Suggested Reading

1. Brodie, B. B. 1964. Of Mice, Microsomes, and Man. The Pharmacologist 6:12-26.
2. Committee for the Revision of NAS publication 1138, Committee on Tox., Nat'l. Res. Counc., Nat'l. Acad. Sci. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. pp. 1-22, 74-85, and 130. Prepared for the Consumer Prod. Safety Comm. Nat'l. Acad. Sci.: Washington, D. C.
3. Cornfield, J. 1954. Measurement and Comparison of Toxicities: The quantal response. In Statistics and Mathematics in Biology pp 327-334. Ed. by O. Kempthorne, T. A. Barieroft, J.W. Gowen, and J.L. Lush. Iowa State College Press: Ames.
4. FAO/WHO Expert Committee on Food Additives. 1958. Procedures for the testing of intentional food additives to establish their safety for use. Wld. Hlth. Org. Tech. Rep. Ser. No. 114, pp. 11-17.

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6. Fitzhugh, O. G. 1949. Procedures for the appraisal of the toxicity of chemicals in foods - subacute and chronic toxicity. Food, Drug, and Cosmetic Law Quarterly, pp. 421-425.
7. Food Protection Committee, National Research Council. 1959. Principles and procedures for evaluating the safety of food additives. NAS-NRC Publ. No. 750, pp. 1-7.
8. Food Protection Committee, National Research Council. 1967. Proceedings of a conference on use of human subjects in safety evaluation of food chemicals - limitations of animal data for predicting safety for man. NAS-NRC Publ. No. 1491, pp. 43-49.
9. Food Protection Committee (Subcommittee on Toxicology), National Research Council. 1970. Evaluating the safety of food chemicals. NAS-NRC Publ., pp. 1-55.
10. Laug, E. P. 1959. Appraisal of the safety of chemicals in food, drugs, and cosmetics - biochemistry. Assoc. of Food and Drug Officials of the U. S., Austin, Texas. pp. 68-74.
11. Smyth, H.F., Jr., C.S. Weil, E.M. Adams & R.L. Hollingsworth. 1952. Efficiency of Criteria of stress in toxicological tests. A.M.A. Arch. Occup. Med. 6:32-36.
12. Weil, C.S. 1962. Applications of methods of statistical analysis to efficient repeated-dose toxicological test. 1. General Considerations and problems involved. Sex differences in rat liver and kidney weights. Toxicol. Appl. Pharmacol 4:561-571.
13. Weil, C.S. 1970. Significance of organ-weight changes in food safety evaluation. In Metabolic Aspects of Food Safety, pp. 419-454. Frances, J.C. Roe, editor. Blackwell Scientific Publications: Oxford and Edinburgh.
14. Weil, C.S. 1973. Experimental design and interpretation of data from prolonged toxicity studies. Pharmacol. and the Future of Man. Proc. 5th Inter. Congr. Pharmacol. 2:2-12.
15. Weil, C.S., and C.P. Carpenter. 1969. Abnormal values in control groups during repeated-dose toxicological studies. Toxicol. Appl. Pharmacol. 14:335-339.
16. Weil, C.S., and D.D. McCollister. 1963. Relationship between short and long-term feeding studies in designing an effective toxicity test. J. Agr. Food Chem. 1:486-491.

GUIDELINE FOR SUBCHRONIC ORAL TOXICITY STUDIES

Introduction

Subchronic studies are designed to determine adverse effects of substances when given in regularly repeated doses over periods ranging from 90 days to 12 months. The intent is to characterize the toxicity of the substance and to define a level that produces "no observed adverse effects". Such a study usually cannot however, determine carcinogenic potential.

The testing procedures recommended in the guideline include a broad screen of measurements which should detect the most likely forms of toxicity which can occur. This guideline is for use with rodents and dogs; if other species are used, some modification of the guideline may be required.

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I. General Considerations

A. Good Laboratory Practices

Studies should be conducted according to good laboratory practice regulations (e.g., "Nonclinical Laboratory Studies, Good Laboratory Practice Regulations," 43 FR 59986, 22 December 1978).

B. Test Substance

1. The specific substance or mixture of substances to be tested should be determined in consultation with the agency. As far as is practical, composition of the test substance should be known. Information should include the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials to account for the entire test substance.
2. Ideally, the lot of the substance tested should be the same throughout the study. The test sample should be stored under conditions that maintain its stability, strength, quality, and purity from the date of its production until the tests are complete.

C. Animals

1. Recommendations contained in DHEW pub. no. (NIH) 74-23, entitled "Guide for the Care and Use of Laboratory Animals," should be followed for the care, maintenance, and housing of animals.
2. Healthy animals, not subjected to any previous experimental procedures, must be used.
3. The test animal shall be characterized as to species, strain, sex, weight and/or age. Each animal must be assigned an appropriate identification number.
4. Animals may be group-caged for this test unless a specific test guideline or the pharmacological action of the test substance dictates otherwise, but the number of animals per cage should not prevent continued and clear observation of each animal. When signs of morbidity or excitability are observed in group-caged animals during the test, such animals should be moved to separate cages.
5. Animals should be assigned to groups in a random manner so as to minimize bias and to assure comparability of pertinent variables for statistical purposes.

D. Dead Animals, Necropsy, and Histopathology

Animals should be observed as necessary to insure that there is minimal loss due to cannibalism or similar management problems. Where possible, necropsy should be performed soon after an animal is sacrificed or found dead to minimize loss of tissues due to autolysis. When necropsy cannot be performed immediately, the animal must be refrigerated at temperatures low enough to minimize autolysis and not cause freezer burn. If histopathological examination is to be conducted, tissue specimens should be placed in appropriate fixative when they are taken from the animal.

E. Diet

The animal's diet should meet all of the nutritional requirements of the test species. Special attention should be paid to the 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 only basal diet may be necessary.

II. Specific Considerations

A. Test Preparation

1. Duration of testing: For this study, animals should be exposed to the test substance 7 days per week for at least 90 consecutive days.
2. Species and age: Testing should be routinely performed on young laboratory rats. Dosing of rats shall begin as soon as possible after weaning and acclimation and in any case before the animals are 6 weeks old. If dogs are used, treatment should normally begin at 4 to 6 months of age.
3. Number and sex: Equal numbers of males and females of each species and strain should be used for the test. At least 20 rats per sex per group and at least 4 dogs per sex per group should be started on test. If interim sacrifices are planned, the number shall be increased by the number scheduled to be sacrificed before completion of the study. The number of animals at the termination of the study must be adequate for a meaningful evaluation of toxicological effects.

4. Number of exposures and concentration level selection

(a) Control group(s): A concurrent control group is required. When a carrier vehicle is used it should be added to the diet at a concentration similar to the maximum given in any dosage group. If there are insufficient data on the toxic properties of the vehicle used in administering the test substance, an additional control group exposed only to the diet should be included. In all other respects, the control group shall be handled and maintained in a manner identical to that used with the test groups. Excessive mortality due to poor management is unacceptable and may be cause to repeat the study. For example, under normal circumstances, mortality in the control group would not be expected to exceed 10%. If more than 5% of the diet is being replaced a control diet of equivalent nutritional value should be provided.

(b) Dose Group(s): At least three dose levels, in addition to the control(s), should be used. The highest treatment level should result in toxicological changes unless prohibited by exaggerated pharmacological effects or physical-chemical characteristics of the test substance that prevent the use of higher dose levels. The lowest dosage level should be one which does not induce any evidence of toxicity. The middle dose level should be sufficiently high to elicit minimal toxic effects. When possible, dose levels should be expressed in a manner to show relevance to contemplated or anticipated human exposures. Administration of test substance to animals at all dose levels must be done concurrently.

B. Test Procedure

1. Route of administration: The test substance may be administered to the animals in the diet, by stomach tube, in capsules, or in drinking water provided that all animals are treated in the same method. When administered by gavage or in capsules, the doses should be adjusted weekly for changes in body weight. When administered in the diet, the doses may be calculated on the basis of mg of test substance/kg of food. When the test substance is in water, the dose should be calculated as mg/ml.
2. Observations of animals: All toxicological and pharmacological signs shall be recorded daily, including time of onset and duration and intensity. Individual records should be maintained for each animal. Estimates should be made of food consumption (or water consumption when the test substance is administered in the water) every week during the test, and the animals shall be weighed at least weekly. Sufficient surveillance of animals shall be made to insure that not more than 10% of the animals are lost from the test due to cannibalism, misplacement, or similar management problems.

3. Clinical testing: The following determinations should be made at the times indicated below for each type of testing. For rats, these determinations shall be made on at least 10 animals of each sex in each group. For dogs, the measurements should be made on all animals in the study.

(a) Ophthalmological examination: An ophthalmological examination using an ophthalmoscope or equivalent should be made on all high-dose and control animals at least twice: once prior to administration of the test substance, and at termination of the study. If changes in the eyes are detected, examinations should be conducted on all remaining animals.

(b) Hematology: The following determinations should be made at the end of the testing period: hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte and a measure of clotting potential such as clotting time, prothrombin time, thromboplastin time, or platelet count.

(c) Clinical chemistry: Blood chemistry measurements should be made at termination of the study. Test areas which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations are: calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum alanine aminotransferase, serum aspartate aminotransferase, ornithine decarboxylase, gamma-glutamyl transpeptidase, urea nitrogen, albumen, blood creatinine, total bilirubin and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methemoglobin, cholinesterase activity. Additional clinical biochemistry may be employed where necessary to extend the investigation for observed effects.

(d) Urinalyses are considered to be of limited value for most routine subchronic toxicological studies, unless specific nephrotoxicity, uric aciduria or oxalo-aciduria is expected.

4. Gross Necropsy

(a) All test animals should be subjected to complete gross necropsy, including examination of the external surfaces, orifices, cranial cavity, carcass, the external and cut surfaces of the brain, spinal cord, and all viscera and glands.

(b) In addition, organs which should be weighed include the liver, kidneys, testes, and adrenal, and thyroid/parathyroid (for the dog). Prior to being weighed, organs should be carefully dissected and properly trimmed to remove fat and other contiguous tissue in a uniform manner. They should be weighed as soon as possible after dissection to minimize the effects of drying on weight.

(c) Preparation of tissues: Tissues listed below (if present) should be fixed in 10% buffered formalin or any other generally recognized fixative, and stained with hematoxylin and eosin, or any other appropriate stain for preparation of microscopic slides.

5. Histopathological Examination

(a) For non-rodents: The following organs and tissues of all animals should be subjected to microscopic study: All gross lesions, brain (at least 3 levels), spinal cord (at least 2 levels), eye, pituitary, salivary gland, heart, thymus, thyroid, parathyroid, lungs, with mainstem bronchi, trachea, esophagus, stomach, small and large intestine, adrenals, pancreas, liver, gall bladder, kidneys, urinary bladder, aorta, gonads, prostate, uterus, spleen, a representative lymph node, bone with marrow, sciatic nerve with skeletal muscle, and mammary gland.

(b) For rodents: All gross lesions should be examined microscopically. In addition, for animals in the control and high dose level and all animal which die during the study: brain (at least 3 levels), spinal cord (at least 2 levels), eye, pituitary, salivary gland, mammary gland esophagus, lungs (with mainstem bronchi), trachea, liver, stomach, small and large intestine, spleen kidneys, thymus, thyroid parathyroid, adrenals, pancreas, urinary bladder, heart, aorta, testes, prostate, ovaries, uterus, a representative lymph node, sternum, and sciatic nerve with skeletal muscle. If changes or equivocal results are seen in any of these tissues, then the same tissue from all of the animals in the other dose groups should be examined.

III. Data Reporting

A. Identification

Each test report should be signed by the persons responsible for the test and identify:

1. The laboratory where the test was performed by name and address;
2. The inclusive dates of the test; and
3. Each person primarily responsible for separate components of the test including (a) the conduct of the test, (b) pathology, (c) analysis of the data, (d) the writing of the report, and (e) any written or other matter contained in the report.

B. Body of Report

The test report must include all information necessary to provide a complete and accurate description and evaluation of the test procedures and results in the following sections:

1. Summary and conclusions: This section of the test report should contain a brief description of the methods, a summary of the data, an analysis of the data, and a statement of the conclusions drawn. The summary must highlight all positive data or observations and any deviations from control data which may be indicative of toxic effects.
2. Materials: This section of the test report should include, but not be limited to, the following information:
 - (a) Identification of the test substance, so far as practical, including:
 - i. chemical name, molecular structure, and a qualitative and quantitative determination of its chemical composition, including names and quantities of known contaminants and impurities and listing the percentage of unidentifiable materials to account for the entire test sample.
 - ii. manufacturer and lot number of the substance tested, and such information as physical state, pH, stability, and purity; and
 - iii. specific identification of diluents, suspending agents, emulsifiers, or other materials used in administering the test substance.
 - (b) Animal data, including:
 - i. species and strain used and rationale for selection of the strain if other than a common laboratory strain;
 - ii. source of supply of the animals;
 - iii. description of any pre-test conditioning; including quarantine procedures, etc.
 - iv. description of the method used in randomization of animals to test or control groups; and
 - v. numbers, age and condition of animals of each sex in each test and control group.
 - (c) Data on husbandry should include description of the caging conditions (including number of animals per cage), diet, bedding material, ambient temperature, humidity, and lighting conditions.

3. Methods

- (a) Deviation from guidelines: This section shall indicate all ways in which the test procedure deviates from these guidelines and shall state the rationale for such deviation.
- (b) Specification of test methods: This section shall include a full description of the experimental design and procedure, the length of the study, and the dates on which the study began and ended.
- (c) Statistical analysis: All statistical methods used should be fully described or identified by reference.
- (d) Data on dosage administration, including:
 - i. all dose levels administered, expressed as mg/kg of body weight;
 - ii. method, frequency, duration, and time of day; and
 - iii. total volume of substance (i.e., test substance plus vehicle) contained in individual dosages.
- (e) Data on observation methods, including:
 - i. duration; and
 - ii. method and frequency of observation of the animals.

4. Results

The tabulation of data and individual results must accompany each report in sufficient detail to permit independent evaluation of results, including summaries and tables that show, as appropriate, the relationship of effects to time of dosing, sex, etc.

- (a) Data presented for each animal should include hematology, clinical chemistry, and other tests performed, a description of all toxicological or pharmacological effects and abnormalities, accompanied by the animal's identification number, test group (dose level and sex), and days of study when the signs appeared and disappeared. An attempt should be made to correlate effects observed during the study with post mortem findings. The time of death for animals which die while on test should be reported. When numerical averages are presented, they should be accompanied by an appropriate measure of variability, such as the standard error. All animals placed on study must be accounted for.

(b) Findings from all clinical observations, necropsy, and histopathological examinations.

(c) Evaluation of data: An evaluation of test results, including their statistical analysis, should be made and supplied, based on the clinical findings, the gross necropsy findings, and the histopathological results. This should include an evaluation of the relationship, or lack thereof, between the animal's exposure to the test substance and the incidence and severity of all abnormalities; such abnormalities include behavioral and clinical abnormalities, tumors and other lesions, organ weight effects, effects on mortality, and any other general or specific toxic effects.

5. References

This section of the test report shall include the following information:

(a) Availability of original data, specimens and samples of the test substance. The location of all original data, specimens, and samples of the test substances which are retained in accordance with the testing requirement.

(b) Literature or references, including, where appropriate, those references for (1) test procedures, (2) statistical and other methods used to analyze the data, (3) compilation and evaluation of results, and (4) the basis upon which conclusions were reached.

IV. Suggested Reading

1. Brodie, B. B. 1964. Of Mice, Microsomes, and Man. The Pharmacologist 6:12-26.
2. Committee for the Revision of NAS publication 1138, Committee on Tox., Nat'l. Res. Council., Nat'l. Acad. Sci. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. pp. 1-22, 74-85, and 130. Prepared for the Consumer Prod. Safety Comm. Nat'l. Acad. Sci.: Washington, D. C.
3. Cornfield, J. 1954. Measurement and Comparison of Toxicities: The quantal response. In Statistics and Mathematics in Biology pp 327-334. Ed. by O. Kempthorne, T. A. Barieroft, J.W. Gowen, and J.L. Lush. Iowa State College Press: Ames.
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9. Food Protection Committee (Subcommittee on Toxicology), National Research Council. 1970. Evaluating the safety of food chemicals. NAS-NRC Publ., pp. 1-55.
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15. Weil, C.S., and C.P. Carpenter. 1969. Abnormal values in control groups during repeated-dose toxicological studies. Toxicol. Appl. Pharmacol. 14:335-339.
16. Weil, C.S., and D.D. McCollister. 1963. Relationship between short and long-term feeding studies in designing an effective toxicity test. J. Agr. Food Chem. 1:486-491.

LONG-TERM TOXICITY IN THE RODENT

PREFACE

This study is designed to determine the adverse effects of substances when given in regularly repeated doses over periods of at least 12 months. The intent is to characterize the toxicity of the substance and to define a dose level that produces no observed adverse effects and higher dose levels that characterize the toxicity of the substance. The testing procedures recommended in the guidelines include a broad screen of measurements which should detect the most likely forms of toxicity which can occur. This guideline is not intended for use as a carcinogenicity guideline, although use of it may reveal data related to carcinogenicity.

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I. General Considerations

A. Good Laboratory Practices

Studies should be conducted according to good laboratory practice regulations (e.g., "Nonclinical Laboratory Studies, Good Laboratory Practice Regulations," 43 FR 59986, 22 December 1978).

B. Test Substance

1. The specific substance or mixture of substances to be tested should be determined in consultation with the agency. As far as is practical, composition of the test substance should be known. Information should include the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials to account for the entire test substance.
2. Ideally, the lot of the substance tested should be the same throughout the study. The test sample should be stored under conditions that maintain its stability, strength, quality, and purity from the date of its production until the tests are complete.

C. Animals

1. Recommendations contained in DHEW pub. no. (NIH) 74-23, entitled "Guide for the Care and Use of Laboratory Animals," should be followed for the care, maintenance, and housing of animals.
2. Healthy animals, not subjected to any previous experimental procedures, must be used.
3. The test animal shall be characterized as to species, strain, sex, weight and/or age. Each animal must be assigned an appropriate identification number.
4. Animals may be group-caged for this test unless a specific test guideline or the pharmacological action of the test substance dictates otherwise, but the number of animals per cage should not prevent continued and clear observation of each animal. When signs of morbidity or excitability are observed in group-caged animals during the test, such animals should be moved to separate cages.
5. Animals should be assigned to groups in a random manner so as to minimize bias and to assure comparability of pertinent variables for statistical purposes.

D. Dead Animals, Necropsy, and Histopathology

Animals should be observed as necessary to insure that there is minimal loss due to cannibalism or similar management problems. Where possible, necropsy should be performed soon after an animal is sacrificed or found dead to minimize loss of tissues due to autolysis. When necropsy cannot be performed immediately, the animal must be refrigerated at temperatures low enough to minimize autolysis and not cause freezer burn. If histopathological examination is to be conducted, tissue specimens should be placed in appropriate fixative when they are taken from the animal.

E. Diet

The animal's diet should meet all of the nutritional requirements of the test species. Special attention should be paid to the 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 only basal diet may be necessary.

II. Specific Considerations

A. Test Preparation

1. Duration of testing: This guideline requires administration of the test substance for at least 12 months. Animals should be exposed to the test substance 7 days per week for 52 weeks.
2. Species and age: Treatment should normally begin during the rapid growth phase as soon as possible after weaning and acclimatization, for rats at about 6 weeks.
3. Number and sex: At least 20 animals/sex/group should be used. If interim sacrifices are planned, then the total number of animals should be increased by the number scheduled to be sacrificed before completion of the study.
4. Number of exposures and dosage level selection
 - (a) Control group(s): A concurrent control group is required. The control group shall be given only the carrier vehicle used in administering the test substance. If there are insufficient data on the toxic properties of the vehicle used in administering the test substance, an additional control group exposed only to the basal diet shall be included.

(b) Dose Group(s): Subchronic range finding studies should be used to insure the proper selection of the dosage regime. (1) When possible, dose levels should be expressed in a manner relevant to contemplated or anticipated human exposures. (2) No dose level should result in an incidence of fatalities which prevents meaningful evaluation of the data. (3) At least three dose levels, in addition to the control(s), should be used. (4) Ideally, the highest treatment level should elicit some signs of toxicity without causing excessive mortality or causing some exaggerated pharmacological effects preventing the use of higher dose levels (i. e., sedation, etc.). (5) The middle dose level should be sufficiently high to elicit minimal toxic effects. (6) The lowest dosage level should not induce any evidence of compound-related toxicity.

B. Test Procedures

1. Route of administration: The test substance may be administered to the animals in the diet, by stomach tube, or in water. If, when the test substance replaces more than 5% of the diet, then a control diet of balanced nutritional value is needed. The same method and route of administration should be used throughout the study. When administered in the diet, the doses may be calculated on the basis of mg of test/kg of food. Doses administered by gavage or in water should be adjusted weekly for changes in body weight. Administration of the test substance to animals at all dose levels and control(s) groups must be done concurrently.
2. Observations of animals: Each animal should be observed at least daily throughout the test period. Body weight and estimated food consumption should be recorded at least once per week for each animal for the first 13 weeks and monthly thereafter. Any behavioral abnormality or any clinical sign of toxicity or pharmacological effects, moribundity, and mortality, should be recorded. Such observations are usually taken at the time of dosing.
3. Ophthalmoscopic examination: An eye examination, using an ophthalmoscope or equivalent, should be on high dose and control animals, performed at the start, every three months thereafter, and at termination of the study. If changes in the eyes are detected, examinations should be conducted on all remaining animals.

4. Clinical testing: If a particular kind of clinical test is required to be repeated during the test period, the test should be performed on the same animal, if possible. The following determinations should be made on at least 10 animals/sex/group in the study at the times indicated:

(a) Hematology: At 3 month intervals and at the end of the testing period the following determinations should be made: hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts, and a measure of clotting potential such as clotting time, prothrombin time.

(b) Clinical chemistry: Blood chemistry measurements should be made at least once before the start of dosing, at 3-month intervals thereafter, and at termination of the study. Test areas which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations are: calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum alanine aminotransferase, serum aspartate aminotransferase, ornithine decarboxylase, gamma-glutamyl transpeptidase, urea nitrogen, albumin, creatinine, total bilirubin and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methemoglobin, cholinesterase activity. Additional clinical biochemistry may be employed where necessary to extend the investigation for observed effects.

(c) Absorption: Evidence of the availability of the test substance to the animals should be provided. If a toxic or exaggerated pharmacological response occurs, absorption can be assumed. If no effects are seen at the highest dose tested, analysis of the concentration blood or serum should be considered.

(d) Urinalyses are considered to be of limited use for long-term toxicity studies in the rodent. If other toxicological data indicate a need for urinalysis, urine samples should be collected prior to the taking of blood samples.

5. Gross Necropsy

(a) All test animals should be subjected to complete gross necropsy, including examination of the external surfaces, orifices, cranial cavity, carcass, the external and cut surfaces of the brain, spinal cord, and all viscera.

(b) The liver, kidneys, testes, and adrenals and thyroid (in non-rodent) should be weighed. Prior to being weighed, organs should be carefully dissected and properly trimmed to remove fat and other contiguous tissue in a uniform manner. They should be weighed as soon as possible after dissection to minimize the effects of drying on weight.

6. Histopathological examination

(a) The following organs and tissues of all animals should be preserved for microscopic study:

Adrenal	Peripheral Nerve
Aorta	Pituitary
Bone	Prostate
Bone Marrow	Rectum
Brain (at least 3 levels)	Representative Lymph Nodes
Caecum	Salivary Glands
Colon	Seminal Vesicle
Corpus and Cervix Uteri	Skeletal Muscle
Duodenum	Smooth Muscle
Esophagus	Spinal Cord (at least 2 levels)
Eyes	Spleen
Gall Bladder (if present)	Sternum
Heart	Stomach
Ileum	Testes
Jejunum	Thymus
Kidneys	Thyroid (Parathyroid)
Liver	Trachea
Lungs	Urinary Bladder
Mammary Glands	All tissues showing abnormality
Ovaries and Fallopian Tube	
Pancreas	

(b) Preparation of tissues: Tissues should be fixed in 10% buffered formalin or any other generally recognized fixative, and stained with hematoxylin and eosin, or other appropriate stain for preparation of microscopic slides.

(c) All gross lesions should be examined microscopically. The liver, lungs, and kidneys of all animals should be examined. The tissues from all animals that died or were killed in extremis during the study and tissues of the highest dose group and controls should be routinely examined. If abnormalities or equivocal results are seen in any of these tissues, then the same tissues from all lower dose groups should be examined. Likewise, if abnormalities are observed in any tissue of an organ system, then the other tissues in that organ system should be microscopically examined in all animals. In the case where results of the experiment give evidence of substantial alteration of the highest dose group animals' survival, then the animals of the next lower dose group should be examined microscopically.

III. Data Reporting

A. Identification

Each test report should be signed by the persons responsible for the test and identify:

1. The laboratory where the test was performed by name and address;
2. The inclusive dates of the test; and
3. Each person primarily responsible for separate components of the test including (a) the conduct of the test, (b) pathology, (c) analysis of the data, (d) the writing of the report, and (e) any written or other matter contained in the report.

B. Body of Report

The test report must include all information necessary to provide a complete and accurate description and evaluation of the test procedures and results in the following sections:

1. Summary and conclusions: This section of the test report should contain a brief description of the methods, a summary of the data, an analysis of the data, and a statement of the conclusions drawn. The summary must highlight all positive data or observations and any deviations from control data which may be indicative of toxic effects.
2. Materials: This section of the test report should include, but not be limited to, the following information:
 - (a) Identification of the test substance, so far as practical, including:
 - i. chemical name, molecular structure, and a qualitative and quantitative determination of its chemical composition, including names and quantities of known contaminants and impurities and listing the percentage of unidentifiable materials to account for the entire test sample;
 - ii. manufacturer and lot number of the substance tested, and such information as physical state, pH, stability, and purity; and
 - iii. specific identification of diluents, suspending agents, emulsifiers, or other materials used in administering the test substance.

- (b) Animal data, including:
 - i. species and strain used and rationale for selection of the strain if other than a common laboratory strain;
 - ii. source of supply of the animals;
 - iii. description of any pre-test conditioning; including quarantine procedures, etc.;
 - iv. description of the method used in randomization of animals to test or control groups; and
 - v. numbers, age and condition of animals of each sex in each test and control group.
- (c) Data on husbandry should include a description of the caging conditions (including number of animals per cage), diet, bedding material, ambient temperature, humidity, and lighting conditions.

3. Methods:

- (a) Deviation from guidelines: This section shall indicate all ways in which the test procedure deviates from these guidelines and shall state the rationale for such deviation.
- (b) Specification of test methods: This section shall include a full description of the experimental design and procedure, the length of the study, and the dates on which the study began and ended.
- (c) Statistical analysis: All statistical methods used should be fully described or identified by reference.
- (d) Data on dosage administration, including:
 - i. all dose levels administered, expressed as mg/kg of body weight;
 - ii. method, frequency, duration, and time of day; and
 - iii. total volume of substance (i.e., test substance plus vehicle) contained in individual dosages.
- (e) Data on observation methods, including:
 - i. duration; and
 - ii. method and frequency of observation of the animals.

4. Results

The tabulation of data and individual results must accompany each report in sufficient detail to permit independent evaluation of results, including summaries and tables that show, as appropriate, the relationship of effects to time of dosing, sex, etc.

(a) Data presented for each animal should include hematology, clinical chemistry, and other tests performed, a description of all toxicological or pharmacological effects and abnormalities, accompanied by the animal's identification number, test group (dose level and sex), and days of study when the signs appeared and disappeared. An attempt should be made to correlate effects observed during the study with post mortem findings. The time of death for animals which die while on test should be reported. When numerical averages are presented, they should be accompanied by an appropriate measure of variability, such as the standard error. All animals placed on study must be accounted for.

(b) Findings from all clinical observations, necropsy, and histopathological examinations.

(c) Evaluation of data: An evaluation of test results, including their statistical analysis, should be made and supplied, based on the clinical findings, the gross necropsy findings, and the histopathological results. This should include an evaluation of the relationship, or lack thereof, between the animal's exposure to the test substance and the incidence and severity of all abnormalities; such abnormalities, include behavioral and clinical abnormalities, tumors and other lesions, organ weight effects, effects on mortality, and any other general or specific toxic effects.

5. References

This section of the test report shall include the following information:

(a) Availability of original data, specimens and samples of the test substance. The location of all original data, specimens, and samples of the test substances which are retained in accordance with the testing requirement.

(b) Literature or references, including, where appropriate, those references for (1) test procedures, (2) statistical and other methods used to analyze the data, (3) compilation and evaluation of results, and (4) the basis upon which conclusions were reached.

IV. Suggested Reading

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2. Committee for the Revision of NAS publication 1138, Committee on Tox., Nat'l. Res. Council, Nat'l. Acad. Sci. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. pp. 1-22, 74-85, and 130. Prepared for the Consumer Prod. Safety Comm. Nat'l. Acad. Sci.: Washington, D. C.
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6. Fitzhugh, O. G. 1949. Procedures for the appraisal of the toxicity of chemicals in foods - subacute and chronic toxicity. Food Drug and Cosmetic Law Quarterly, pp. 421-425.
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15. Weil, C. S., and C.P. Carpenter. 1969. Abnormal values in control groups during repeated-dose toxicological studies. Toxicol. Appl. Pharmacol. 14:335-339.
16. Weil, C. S., and D.D. McCollister. 1963. Relationship between short and long-term feeding studies in designing an effective toxicity test. J. Agr. Food Chem. 1:486-491.

LONG-TERM TOXICITY STUDY IN THE DOG

PREFACE

This study is designed to determine the adverse effects of substances when given in regularly repeated doses over periods of at least 12 months. The intent is to characterize the toxicity of the substance and to define a level that produces "no observed adverse effects". The testing procedures recommended in this guideline include a broad screen of measurements which should detect the most likely forms of toxicity which can occur. This guideline for chronic toxicity studies in dogs is not intended for use as a carcinogenicity guideline, although use of it may produce data related to carcinogenicity.

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I. General Considerations

A. Good Laboratory Practices

Studies should be conducted according to good laboratory practice regulations (e.g., "Nonclinical Laboratory Studies, Good Laboratory Practice Regulations," 43 FR 59986, 22 December 1978).

B. Test Substance

1. The specific substance or mixture of substances to be tested should be determined in consultation with the agency. As far as is practical, composition of the test substance should be known. Information should include the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials to account for the entire test substance.
2. Ideally, the lot of the substance tested should be the same throughout the study. The test sample should be stored under conditions that maintain its stability, strength, quality, and purity from the date of its production until the tests are complete.

C. Animals

1. Recommendations contained in DHEW pub. no. (NIH) 74-23, entitled "Guide for the Care and Use of Laboratory Animals," should be followed for the care, maintenance, and housing of animals.
2. Healthy animals, not subjected to any previous experimental procedures, must be used.
3. The test animal shall be characterized as to species, strain, sex, weight and/or age. Each animal must be assigned an appropriate identification number.
4. Animals may be group-caged for this test unless a specific test guideline or the pharmacological action of the test substance dictates otherwise, but the number of animals per cage should not prevent continued and clear observation of each animal. When signs of morbidity or excitability are observed in group-caged animals during the test, such animals should be moved to separate cages.
5. Animals should be assigned to groups in a random manner so as to minimize bias and to assure comparability of pertinent variables for statistical purposes.

D. Dead Animals, Necropsy, and Histopathology

Animals should be observed as necessary to insure that there is minimal loss due to cannibalism or similar management problems. Where possible, necropsy should be performed soon after an animal is sacrificed or found dead to minimize loss of tissues due to autolysis. When necropsy cannot be performed immediately, the animal must be refrigerated at temperatures low enough to minimize autolysis and not cause freezer burn. If histopathological examination is to be conducted, tissue specimens should be placed in appropriate fixative when they are taken from the animal.

E. Diet

The animal's diet should meet all of the nutritional requirements of the test species. Special attention should be paid to the 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 only basal diet may be necessary.

II. Specific Considerations

A. Test Preparation

1. Duration of testing: Animals should be exposed to the test substance 7 days per week for 52 weeks.
2. Species and age: Treatment of dogs should normally begin at 4 to 6 months of age at which time they should have received appropriate vaccinations.
3. Number and sex: At least 4 male and 4 female dogs per group should be used. If interim sacrifices are planned, the total number of dogs started on study should be increased by the number scheduled to be sacrificed before completion of the study.
4. Number of exposures and selection of dosage levels
 - (a) Control group(s): A concurrent control group is required. The control group shall be given only the carrier vehicle used in administering the test substance. If there are insufficient data on the toxic properties of the vehicle used in administering the test substance, an additional control group exposed only to the basal diet should be included.

(b) Dose Group(s): When possible, dose levels should be expressed in a manner relevant to contemplated or anticipated human exposures. At least three dose levels, in addition to the concurrent control(s), should be used. Ideally, the highest treatment level should elicit some signs of toxicity without causing excessive mortality or causing some exaggerated pharmacological effects preventing the use of higher dose levels (i. e., sedation, etc.). The middle dose level should be sufficiently high to elicit minimal toxic effects. The lowest dosage level should not induce any evidence of compound-related toxicity. No dose level should result in an incidence of fatalities which prevents meaningful evaluation of the data.

B. Test Procedure

1. Route of administration: The test substance may be administered to the animals in the diet, by gavage or in capsules. If the test substance replaces more than 5% of the diet, then a control diet of balanced nutritional value is needed. The same method and route and approximate time of administration should be used throughout the study. When administered in the diet, the dose of the test substance may be calculated on the basis of mg of test substance/kg of food. Doses administered by gavage or in capsules should be adjusted weekly for changes in body weight.
2. Observation of animals: Each dog should be observed throughout the test period, at least daily. Body weight and estimates of food consumption should be recorded for each dog at least once per week for the first 13 weeks and weekly thereafter. All clinical signs of toxicity should be recorded. Such observations are usually taken at the time of dosing.
3. Ophthalmoscopic examination: An ophthalmological examination using an ophthalmoscope or equivalent should be performed at the start, every three months thereafter, and at termination of the study for all animals.
4. Clinical testing: The following determinations should be made on all dogs in the study at the time indicated below. Prior to withdrawal of blood, dogs should be fasted overnight, with samples drawn prior to feeding.

(a) Hematology: The following determinations should be made at least once before start of dosing, at 3-month intervals thereafter, and at the end of the testing period: hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts, platelet counts and a measure of clotting potential such as clotting time or prothrombin time.

(b) Clinical chemistry: Blood chemistry measurements should be made at least once before the start of dosing, at 3-month intervals thereafter, and at termination of the study. Test areas which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations are: calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum alanine aminotransferase, serum aspartate aminotransferase, ornithine decarboxylase, gamma-glutamyl transpeptidase, urea nitrogen, albumin, creatinine, total bilirubin and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methemoglobin, cholinesterase activity. Additional clinical biochemistry may be employed where necessary to extend the investigation for observed effects.

(c) Absorption: Evidence of the availability of the test substance to the animals should be provided. If a toxic or exaggerated pharmacological response occurs, absorption can be assumed. If no effects are seen at the highest dose tested, analysis of the concentration blood or serum should be considered.

(d) Urinalyses are considered to be of limited use for long-term toxicity studies in the dog. If other toxicological data indicate a need for urinalysis, urine samples should be collected prior to the taking of blood samples.

5. Gross Necropsy

(a) All test animals should be subjected to complete gross necropsy, including examination of the external surfaces, orifices, cranial cavity, carcass, the external and cut surfaces of the brain, spinal cord, and all viscera.

(b) Liver, kidneys, testes, thyroid/parathyroid and adrenals should be weighed. Prior to being weighed, organs should be carefully dissected and properly trimmed to remove fat and other contiguous tissue in a uniform manner. They should be weighed as soon as possible after dissection to minimize the effects of drying on weight.

(c) Preparation of tissues: Tissues should be fixed in 10% buffered formalin or any other generally recognized fixative, and stained with hematoxylin and eosin, or other appropriate stain for preparation of microscopic slides.

6. Histopathological examination

The following tissues of all animals should be examined microscopically:

Adrenal	Peripheral Nerve
Aorta	Pituitary
Bone	Prostate
Bone Marrow	Rectum
Brain (at least 3 levels)	Salivary Gland
Caecum	Seminal Vesicle
Colon	Skeletal Muscle
Corpus and Cervix Uteri	Smooth Muscle
Duodenum	Spinal Cord (at least 2 levels)
Esophagus	Spleen
Eyes	Sterum
Gall Bladder	Stomach
Heart	Testes
Ileum	Thymus
Jejunum	Thyroid (Parathyroid)
Kidneys	Trachea
Liver	Urinary Bladder
Lungs	All tissues showing abnormality
Mammary Glands	Representative Lymph Nodes
Ovaries and Fallopian Tube	
Pancreas	

III. Data Reporting

A. Identification

Each test report should be signed by the persons responsible for the test and identify:

1. The laboratory where the test was performed by name and address;
2. The inclusive dates of the test; and
3. Each person primarily responsible for separate components of the test including (a) the conduct of the test, (b) pathology, (c) analysis of the data, (d) the writing of the report, and (e) any written or other matter contained in the report.

B. Body of Report

The test report must include all information necessary to provide a complete and accurate description and evaluation of the test procedures and results in the following sections:

1. Summary and conclusions: This section of the test report should contain a brief description of the methods, a summary of the data, an analysis of the data, and a statement of the conclusions drawn. The summary must highlight all positive data or observations and any deviations from control data which may be indicative of toxic effects.
2. Materials: This section of the test report should include, but not be limited to, the following information:
 - (a) Identification of the test substance, so far as practical, including:
 - i. chemical name, molecular structure, and a qualitative and quantitative determination of its chemical composition, including names and quantities of known contaminants and impurities and listing the percentage of unidentifiable materials to account for the entire test sample;
 - ii. manufacturer and lot number of the substance tested, and such information as physical state, pH, stability, and purity; and
 - iii. specific identification of diluents, suspending agents, emulsifiers, or other materials used in administering the test substance.
 - (b) Animal data, including:
 - i. species and strain used and rationale for selection of the strain if other than a common laboratory strain;
 - ii. source of supply of the animals;
 - iii. description of any pre-test conditioning; including quarantine procedures, etc.;
 - iv. description of the method used in a randomization of animals to test or control groups; and
 - v. numbers, age and condition of animals of each sex in each test and control group.
 - (c) Data on husbandry should include description of the caging conditions (including number of animals per cage), diet, bedding material, ambient temperature, humidity, and lighting conditions.

3. Methods

- (a) Deviation from guidelines: This section shall indicate all ways in which the test procedure deviates from these guidelines and shall state the rationale for such deviation.
- (b) Specification of test methods: This section shall include a full description of the experimental design and procedure, the length of the study, and the dates on which the study began and ended.
- (c) Statistical analysis: All statistical methods used should be fully described or identified by reference.
- (d) Data on dosage administration, including:
 - i. all dose levels administered, expressed as mg/kg of body weight;
 - ii. method, frequency, duration, and time of day; and
 - iii. total volume of substance (i.e., test substance plus vehicle) contained in individual dosages.
- (e) Data on observation methods, including:
 - i. duration; and
 - ii. method and frequency of observation of the animals.

4. Results

The tabulation of data and individual results must accompany each report in sufficient detail to permit independent evaluation of results, including summaries and tables that show, as appropriate, the relationship of effects to time of dosing, sex, etc.

- (a) Data presented for each animal should include hematology, clinical chemistry, and other tests performed, a description of all toxicological or pharmacological effects and abnormalities, accompanied by the animal's identification number, test group (dose level and sex), and days of study when the signs appeared and disappeared. An attempt should be made to correlate effects observed during the study with post mortem findings. The time of death for animals which die while on test should be reported. When numerical averages are presented, they should be accompanied by an appropriate measure of variability, such as the standard error. All animals placed on study must be accounted for.

(b) Findings from all clinical observations, necropsy, and histopathological examinations.

(c) Evaluation of data: An evaluation of test results, including their statistical analysis, should be made and supplied, based on the clinical findings, the gross necropsy findings, and the histopathological results. This should include an evaluation of the relationship, or lack thereof, between the animal's exposure to the test substance and the incidence and severity of all abnormalities; such abnormalities, include behavioral and clinical abnormalities, tumors and other lesions, organ weight effects, effects on mortality, and any other general or specific toxic effects.

5. References

This section of the test report shall include the following information:

(a) Availability of original data, specimens and samples of the test substance. The location of all original data, specimens, and samples of the test substances which are retained in accordance with the testing requirement.

(b) Literature or references, including, where appropriate, those references for (1) test procedures, (2) statistical and other methods used to analyze the data, (3) compilation and evaluation of results, and (4) the basis upon which conclusions were reached.

IV. Suggested Reading

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GUIDELINES FOR ORAL CARCINOGENICITY STUDIES IN RODENTS

Introduction:

This study is designed to determine whether a compound possesses carcinogenic activity when administered to rodents by the oral route.

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1. The specific substance or mixture or substances to be tested should be determined in consultation with the agency. As far as is practical, composition of the test substance should be known. Information should include the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials to account for the entire test substance.
2. Ideally, the lot of the substance tested should be the same throughout the study. The test sample should be stored under conditions that maintain its stability, strength, quality, and purity from the date of its production until the tests are complete.

C. Animals

1. Recommendations contained in DHEW pub. no. (NIH) 74-23, entitled "Guide for the Care and Use of Laboratory Animals," should be followed for the care, maintenance, and housing of animals.
2. Healthy animals, not subjected to any previous experimental procedures, must be used.
3. The test animal shall be characterized as to species, strain, sex, weight and/or age. Each animal must be assigned an appropriate identification number.
4. Animals may be group-caged for this test unless a specific test guideline or the pharmacological action of the test substance dictates otherwise, but the number of animals per cage should not prevent continued and clear observation of each animal. When signs or morbidity or excitability are observed in group-caged animals during the test, such animals should be moved to separate cages.
5. Animals should be assigned to groups in a random manner so as to minimize bias and to assure comparability of pertinent variables for statistical purposes.

D. Dead Animals, Necropsy, and Histopathology

Animals should be observed as necessary to insure that there is minimal loss due to cannibalism or similar management problems. Where possible, necropsy should be performed soon after an animal is sacrificed or found dead to minimize loss of tissues due to autolysis. When necropsy cannot be performed immediately, the animal must be refrigerated at temperatures low enough to minimize autolysis and not cause freezer burn. If histopathological examination is to be conducted, tissue specimens should be placed in appropriate fixative when they are taken from the animal.

E. Diet

The animal's diet should meet all of the nutritional requirements of the test species. Special attention should be paid to the 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.

II. Specific Considerations

A. Test Preparation

1. Duration of Testing: Animals should be exposed to the test substance 7 days per week for at least 104 consecutive weeks. If a strain of animals with increased longevity and low spontaneous tumor incidence is used, then the maximum duration of the study should be extended. Studies of greater than 130 weeks duration are not recommended.
2. Species and Age: in selecting the rodent species and strain it is important to consider particular susceptibilities. There is no scientific rationale to recommend inbred, outbred or hybrid strains over any others. The important consideration is that animals come from well-characterized and healthy colonies. A good knowledge of the tumor profile of the animal strain throughout its life-span is desirable in order to evaluate the results of the experiment. Preference in strain selection should generally be given to strains with a low incidence of spontaneous tumors. Non-inbred strains often have unpredictable background tumor incidence.

Dosing of Animals should begin as soon as possible after weaning and acclimation and in any case before the animals are 6 weeks old.

3. Number and Sex of Test Animals:

- a) Each test group and concurrent control group should consist of at least 50 males and 50 females. Control animals should have been housed, fed, and handled exactly as the test animals and should be caged to preclude airborne or other contamination by the test substance.
- b) If interim sacrifice(s) are included in the study, the initial number of animals per group should be increased by the number of animals scheduled for interim sacrifice(s).
- c) Criteria for Acceptable Negative Lifetime Study:
 - i. 25 rats per sex per dose should survive at least 24 months: 25 mice or hamsters per sex per dose should survive at least 18 months.
 - ii. No more than 10% of any group (animals or tissues) should be lost due to autolysis, cannibalism or management problems.
- d) Criteria for Termination of a Study, Under Special Circumstances:
 - i. For studies with mice or hamsters (criteria c, i above having been met) termination of the study should occur, when, after 18 months, the number of surviving animals in any group reaches 10 per sex. Each sex should be treated independently.

- ii. For studies with rats (criteria c, i above having been met) termination of the study should occur when, after 24 months, the number of surviving animals in any group reaches 10 per sex. Each sex should be treated independently.

4. Number of Exposures and Selection of Dosage Levels

a) Control groups(s): A concurrent control group is required. The control group should be given only the carrier vehicle used in administering the test substance. If there are insufficient data on the toxic (including carcinogenic) properties of the vehicle used in administering the test substance, an additional control group exposed only to the diet should be included. In all other respects, the control group shall be handled and maintained in a manner identical to that used with the test groups.

b) Dose groups(s): Subchronic range-finding studies should be used to insure the proper selection of the dosage regime.

The highest dose level should be sufficiently high to elicit signs of minimal toxicity without substantially altering the normal life-span due to effects other than tumors. Signs of toxicity are those that may be indicated by alterations in serum enzyme levels or slight depression of body weight gain (less than 10 percent).

The lowest dose should not interfere with normal growth, development, and longevity of the animal and it must not otherwise result in any indication of toxicity. In general, this should not be lower than 10% of the high dose.

The intermediate dose should be established approximately mid-way between the high and low doses, depending upon the pharmacokinetic properties of the chemical, if known.

Exception to the section of the high dose:

- (1) No dose level of the test substance should exceed 5% of the total diet for non-nutritive additives.
- (2) Nutritive additives may be fed up to a dose which does not cause significant nutritional deficit.
- (3) If significant differences in the pharmacokinetic or metabolic profile of test substance are demonstrated between the high dose and lower doses, then an optional dose may be included in the study. This optional dose should approximate the maximum dose which yields pharmacokinetics similar to the lower doses. The number of animals in the special dose group should be increased to provide approximately the same sensitivity as the high-dose group.

B. Test Procedures

1. Route of Administration

The test substance should be administered to the animals in their diet, dissolved in their drinking water or by stomach tube. The same method of oral administration should be used for all animals throughout the study. When administered in the diet, the dose of the test substance may be calculated on the basis of mg of test substance/kg of food. Doses administered by gavage or in water should be adjusted weekly for changes in body weight up to about 13 weeks and monthly thereafter.

2. Observations

Careful observations should be performed to detect onset and progression of all toxic effects as well as to minimize loss of tissue due to diseases, autolysis, or cannibalism. Careful daily examination is essential with, at a minimum, observation in the morning and afternoon (with intervals of at least six hours).

Clinical signs and mortality should be recorded for all animals. Special attention must be paid to tumor development; the time of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumor should be recorded.

During the course of the study, clinical signs may suggest the need for other clinical determinations (e.g. urinalysis) or post-mortem examinations.

Body weights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every 4 weeks thereafter.

Food intake should be determined weekly during the first 13 weeks of the study and then at approximately three-month intervals unless health status of body weight changes dictate otherwise.

3. Hematology

Erythrocyte counts and total differential leukocyte counts should be made at 6, 12 and 18 months and prior to terminal sacrifice for all animals.

4. Gross Necropsy

All test animals should be subjected to a complete gross necropsy, including examination of external surfaces, orifices, cranial and oral cavities and the organs contained therein, carcass, and all viscera.

5. Histopathological examination

(a) The following organs and tissues of all animals should be preserved for microscopic study:

Adrenal	Ovaries and Fallopian Tube
Aorta	Pancreas
Bone Marrow	Peripheral Nerve
Brain (at least 3 levels)	Pituitary
Caecum	Prostate
Colon	Rectum
Corpus and Cervix Uteri	Representative Lymph Nodes
Duodenum	Salivary Glands
Esophagus	Seminal Vesicle
Eyes	Skeletal Muscle
Eyes & Contiguous Harderian Gland	Smooth Muscle
Exorbital Lacrimal Gland	Spinal Cord (at least 2 levels)
Gall Bladder (if present)	Spleen
Heart	Sternum
Ileum	Stomach
Jejunum	Testes
Kidneys	Thymus
Liver	Thyroid (Parathyroid)
Lungs	Trachea
Mammary Glands	Urinary Bladder
Nasal Turbinates	Vagina
	Zymbals Gland
	All tissues showing abnormality

(b) Preparation of tissues: Tissues should be fixed in 10% buffered formalin or any other generally recognized fixative, and stained with hematoxylin and eosin, or other appropriate stain for preparation of microscopic slides.

(c) All gross lesions should be examined microscopically. The liver, lungs, and kidneys of all animals should be examined. The tissues from all animals that died or were killed in extremis during the study and tissues of the highest dose group and controls should be routinely examined. Likewise, if abnormalities or equivocal results are observed in any tissues, then the same tissues from all lower dose groups should be examined. Likewise, if abnormalities are observed in any tissue of an organ system, then the other tissues in that organ system should be microscopically examined in all animals. In the case where results of the experiment give evidence of substantial alteration of the highest dose group animals' survival, then the animals of the next lower dose group should be examined microscopically.

III. Data Reporting

A. Identification

Each test report should be signed by the persons responsible for the test and identify:

1. The laboratory where the test was performed by name and address;
2. The inclusive dates of the test; and
3. Each person primarily responsible for separate components of the test including (a) the conduct of the test, (b) pathology, (c) analysis of the data, (d) the writing of the report, and (e) any written or other matter contained in the report.

B. Body of Report

The test report must include all information necessary to provide a complete and accurate description and evaluation of the test procedures and results in the following sections:

1. Summary and conclusions: This section of the test report should contain a brief description of the methods, a summary of the data, an analysis of the data, and a statement of the conclusions drawn. The summary must highlight all positive data or observations and any deviations from control data which may be indicative of toxic effects.
2. Materials: This section of the test report should include, but not be limited to, the following information:
 - (a) Identification of the test substance, so far as practical, including:
 - i. chemical name, molecular structure, and a qualitative and quantitative determination of its chemical composition, including names and quantities of known contaminants and impurities and listing the percentage of unidentifiable materials to account for the entire test sample.
 - ii. manufacturer and lot number of the substance tested, and such information as physical state, pH, stability, and purity; and
 - iii. specific identification of diluents, suspending agents, emulsifiers, or other materials used in administering the test substance.

(b) Animal data, including:

- i. species and strain used and rationale for selection of the strain if other than a common laboratory strain;
 - ii. source of supply of the animals;
 - iii. description of any pre-test conditioning; including quarantine procedures, etc.
 - iv. description of the method used in randomization of animals to test or control groups; and
 - v. numbers, age and condition of animals of each sex in each test and control group.
- (c) Data on husbandry should include a description of the caging conditions (including number of animals per cage), diet, bedding material, ambient temperature, humidity, and lighting conditions.

3. Methods

- (a) Deviation from guidelines: This section shall indicate all ways in which the test procedure deviates from these guidelines and shall state the rationale for such deviation.
- (b) Specification of test methods: This section shall include a full description of the experimental design and procedure, the length of the study, and the dates on which the study began and ended.
- (c) Statistical analysis: All statistical methods used should be fully described or identified by reference.
- (d) Data on dosage administration, including:
 - i. all dose levels administered, expressed as mg/kg of body weight;
 - ii. method, frequency, duration, and time of day;
 - iii. total volume of substance (i.e., test substance plus vehicle) contained in individual dosages.
- (e) Data on observation methods, including:
 - i. duration; and
 - ii. method and frequency of observation of the animals.

4. Results

The tabulation of data and individual results must accompany each report in sufficient detail to permit independent evaluation of results, including summaries and tables that show, as appropriate, the relationship of effects to time of dosing, sex, etc.

(a) Data presented for each animal should include hematology, clinical chemistry, and other tests performed, a description of all toxicological or pharmacological effects and abnormalities, accompanied by the animal's identification number, test group (dose level and sex), and days of study when the signs appeared and disappeared. An attempt should be made to correlate effects observed during the study with post mortem findings. The time of death for animals which die while on test should be reported. When numerical averages are presented, they should be accompanied by an appropriate measure of variability, such as the standard error. All animals placed on study must be accounted for.

(b) Findings from all clinical observations, necropsy, and histopathological examinations.

(c) Evaluation of data: An evaluation of test results, including their statistical analysis, should be made and supplied, based on the clinical findings, the gross necropsy findings, and the histopathological results. This should include an evaluation of the relationship, or lack thereof, between the animal's exposure to the test substance and the incidence and severity of all abnormalities; such abnormalities, include behavioral and clinical abnormalities, tumors and other lesions, organ weight effects, effects on mortality, and any other general or specific toxic effects.

5. References

This section of the test report shall include the following information:

(a) Availability of original data, specimens and samples of the test substance. The location of all original data, specimens, and samples of the test substances which are retained in accordance with the testing requirement.

(b) Literature or references, including, where appropriate, those references for (1) test procedures, (2) statistical and other methods used to analyze the data, (3) compilation and evaluation of results, and (4) the basis upon which conclusions were reached.

IV. Suggested Reading

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13. Weil, C.S. 1970. Significance of organ-weight changes in food safety evaluation. In Metabolic Aspects of Food Safety, pp. 419-454. Frances, J.C. Roe, editor. Blackwell Scientific Publications: Oxford and Edinburgh.
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GUIDELINE FOR COMBINATION OF CHRONIC ORAL TOXICITY
AND CARCINOGENICITY STUDIES IN THE RODENT

Introduction

The objective of a chronic toxicity study is to determine the adverse effects of a substance following prolonged, repeated exposure. Ideally, the design and conduct of this study should allow for the detection of neoplastic effects and carcinogenic potential of the test substance as well as its general toxicity. The intent of this guideline is to allow for combination of chronic toxicity study and the carcinogenicity study into a single procedure.

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I. General Considerations

A. Good Laboratory Practices

Studies should be conducted according to good laboratory practice regulations (e.g., "Nonclinical Laboratory Studies, Good Laboratory Practice Regulations," 43 FR 59986, 22 December 1978).

B. Test Substance

1. The specific substance or mixture of substances to be tested should be determined in consultation with the agency. As far as is practical, composition of the test substance should be known. Information should include the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials to account for the entire test substance.
2. Ideally, the lot of the substance tested should be the same throughout the study. The test sample should be stored under conditions that maintain its stability, strength, quality, and purity from the date of its production until the tests are complete.

C. Animals

1. Recommendations contained in DHEW pub. no. (NIH) 74-23, entitled "Guide for the Care and Use of Laboratory Animals," should be followed for the care, maintenance, and housing of animals.
2. Healthy animals, not subjected to any previous experimental procedures, must be used.
3. The test animal shall be characterized as to species, strain, sex, weight and/or age. Each animal must be assigned an appropriate identification number.
4. Animals may be group-caged for this test unless a specific test guideline or the pharmacological action of the test substance dictates otherwise, but the number of animals per cage should not prevent continued and clear observation of each animal. When signs of morbidity or excitability are observed in group-caged animals during the test, such animals should be moved to separate cages.
5. Animals should be assigned to groups in a random manner as to minimize bias and to assure comparability of pertinent variable for statistical purposes.

D. Dead Animals, Necropsy, and Histopathology

Animals should be observed as necessary to insure that there is minimal loss due to cannibalism or similar management problems. Where possible, necropsy should be performed soon after an animal is sacrificed or found dead to minimize loss of tissues due to autolysis. When necropsy cannot be performed immediately, the animal must be refrigerated at temperatures low enough to minimize autolysis and not cause freezer burn. If histopathological examination is to be conducted, tissue specimens should be placed in appropriate fixative when they are taken from the animal.

E. Diet

The animal's diet should meet all of the nutritional requirements of the test species. Special attention should be paid to the 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.

II. Specific Considerations

A. Test Preparation

1. Duration of Testing: Animals should be exposed to the test substance 7 days per week for at least 104 consecutive weeks. If a strain of animals with increased longevity and low spontaneous tumor incidences is used then the maximum duration of the study should be extended but it is not recommended to exceed 130 weeks. For assessment of chronic toxicity additional treated and concurrent control satellite groups are included in the study. The satellite groups of dosed animals and concurrent control animals should be retained in the study for at least 12 months.
2. Species Selection: In selecting the rodent species and strain, it is important to consider particular susceptibilities. There is no scientific rationale to recommend inbred, outbred or hybrid strains over any others. The important consideration is that animals come from well-characterized and healthy colonies. A good knowledge of the tumor profile of the animal strain throughout its life span is desirable in order to evaluate the results of the experiment. Preference in strain selection should generally be given to strains with a low incidence of spontaneous tumors. Non-inbred strains often have unpredictable background tumor incidence.

Typically the rat has been used for a combined chronic toxicity carcinogenicity assessment. However, other species may be used. Where available, the strain selected should be susceptible to the carcinogenic or toxic effects of the class of substances being tested provided it does not have a background too high for meaningful assessment.

3. Number and sex of test animals

(a) Each test group and concurrent control group should consist of at least 50 males and 50 females. Satellite treatment groups for evaluation of toxicity should contain at least 10 animals of each sex; the satellite control group should also contain 10 animals of each sex. If other interim sacrifice(s) are included in the study, the initial number of animals per group should be increased by the number of animals scheduled for interim sacrifice(s).

(b) Dosing of animals should begin as soon as possible after weaning and acclimation and in any case before the animals are 6 weeks old.

(c) Criteria for Acceptable Negative Lifetime Study

- i. Twenty-five rats per sex per dose should survive at least 24 months. Twenty-five mice or hamsters per sex per dose should survive at least 18 months.
- ii. No more than 10% of any group (animals or tissues) should be lost due to autolysis, cannibalism or management problems.

(d) Criteria for Termination of a Study, Under Special Circumstances:

- i. For studies with mice or hamsters (criteria c, i above having been met) termination of the study should occur, when, after 18 months, the number of surviving animals in any group reaches 10 per sex. Each sex should be treated independently.
- ii. For studies with rats (criteria c, i above having been met) termination of the study should occur when, after 24 months, the number of surviving animals in any group reaches 10 per sex. Each sex should be treated independently.

4. Number of exposures and selection of dosage levels

(a) Control group(s): A concurrent control group is required. The control group should be given only the carrier vehicle used in administering the test substance. If there are insufficient data on the toxic, including carcinogenic, properties of the vehicle used in administering the test substance, an additional control group exposed only to the diet should be included. The animal's diet should meet all of the nutritional requirements of the test species.

Special attention should be paid to the 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 only basal diet may be necessary.

(b) Dose group(s): Subchronic range-finding studies should be used to insure the proper selection of the dosage regime.

The high-dose level of the carcinogenicity assessment phase should be sufficiently high to elicit signs of minimal toxicity without substantially altering the normal life-span of the animals due to effects other than tumors. Signs of toxicity are those that may be indicated by alterations in serum enzyme levels or slight depression of body weight gain (less than 10 percent).

The lowest dose should not interfere with normal growth, development, and longevity of the animal, and it must not otherwise cause any indication of toxicity.

The intermediate dose should be established approximately mid-way between the high and low doses. Exceptions may depend upon the pharmacokinetic properties of the chemical.

For chronic toxicologic assessment, additional treated and concurrent control satellite groups are included in the study. The highest dose for satellite animals should be chosen so as to produce toxicity in order to elucidate a toxicological profile of the test substance. The lowest dose for satellite animals should not cause any indication of toxicity.

5. Exception to the selection of the high dose

(1) In general, no dose level of the test substance should exceed 5% of the total diet for non-nutritive additives. However, nutritive additives may be fed at higher doses as long as range-finding studies show that the high dose does not cause significant nutritional imbalance in the test animal.

(2) If significant differences in the pharmacokinetic or metabolic profile of the test substance are demonstrated between the high dose and lower doses, then an optional dose may be included in the study. This optional dose should approximate the maximum dose which yields similar pharmacokinetics. The number of animals in the special dose group should be increased to provide approximately the same sensitivity as the high-dose group.

b. Test Procedures

1. Route of Administration

The test substance should be administered to the animals in their diet, dissolved in their drinking water or by gavage. The same method of oral administration should be used for all animals throughout the study. When administered in the diet, the dose of the test substance may be calculated on the basis of mg of test substance/kg of food. Doses administered by gavage or in water should be adjusted weekly for changes in body weight up to about 13 weeks and monthly thereafter.

2. Observations

Observations should be made at a minimum, in the morning and afternoon (with intervals of at least six hours).

Clinical signs should be recorded for all animals. Special attention must be paid to tumor development: the time of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumor should be recorded.

Body weights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every 4 weeks thereafter.

Food intake should be determined weekly during the first 13 weeks of the study and then at approximately three month intervals, unless health status or body weight changes dictate otherwise.

3. Ophthalmoscopic examination

An eye examination, using an ophthalmoscope or equivalent, should be on high dose and control animals, performed at the start, every three months thereafter, and at termination of the study. If changes in the eyes are detected, examinations should be conducted on all remaining animals.

4. Clinical testing

If a particular kind of clinical test is required to be repeated during the test period, the test should be performed on the same animal, if possible. The following determinations should be made on at least 10 animals/sex/group in the study at the times indicated:

(a) Hematology: At 3-month intervals and at the end of the testing period the following determinations should be made: hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts, and a measure of clotting potential such as clotting time, prothrombin time.

(b) Clinical chemistry: Blood chemistry measurements should be made at least once before the start of dosing, at 3-month intervals thereafter, and at termination of the study. Test areas which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations are: calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum alanine aminotransferase, serum aspartate aminotransferase, ornithine decarboxylase, gamma-glutamyl transpeptidase, urea nitrogen, albumin, creatinine, total bilirubin and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methemoglobin, cholinesterase activity. Additional clinical biochemistry may be employed where necessary to extend the investigation for observed effects.

(c) Absorption: Evidence of the availability of the test substance to the animals should be provided. If a toxic or exaggerated pharmacological response occurs, absorption can be assumed. If no effects are seen at the highest dose tested, analysis of the concentration blood or serum should be considered.

(d) Urinalyses are considered to be of limited use for long-term toxicity studies in the rodent. If other toxicological data indicate a need for urinalysis, urine samples should be collected prior to the taking of blood samples.

5. Gross Necropsy

(a) All test animals should be subjected to complete gross necropsy, including examination of the external surfaces, orifices, cranial cavity, carcass, the external and cut surfaces of the brain, spinal cord, and all viscera.

(b) The liver, kidneys, testes, and adrenals should be weighed. Prior to being weighed, organs should be carefully dissected and properly trimmed to remove fat and other contiguous tissue in a uniform manner. They should be weighed as soon as possible after dissection to minimize the effects of drying on weight.

6. Histopathological examination:

(a) The following organs and tissues of all animals should be preserved for microscopic study:

Adrenal	Ovaries and Fallopian Tube
Aorta	Pancreas
Bone Marrow	Peripheral Nerve
Brain (at least 3 levels)	Pituitary
Caecum	Prostate
Colon	Rectum
Corpus and Cervix Uteri	Representative Lymph Nodes
Duodenum	Salivary Glands
Esophagus	Seminal Vesicle
Eyes	Skeletal Muscle
Eyes & Contiguous Harderian Gland	Smooth Muscle
Exorbital Lacrimal Gland	Spinal Cord (at least 2 levels)
Gall Bladder (if present)	Spleen
Heart	Sternum
Ileum	Stomach
Jejunum	Testes
Kidneys	Thymus
Liver	Thyroid (Parathyroid)
Lungs	Trachea
Mammary Glands	Urinary Bladder
Nasal Turbinates	Vagina
	Zymbals Gland
	All tissues showing abnormality

(b) Preparation of tissues: Tissues should be fixed in 10% buffered formalin or any other generally recognized fixative, and stained with hematoxylin and eosin, or other appropriate stain for preparation of microscopic slides.

(c) All gross lesions should be examined microscopically. The liver, lungs, and kidneys of all animals should be examined. The tissues from all animals that died or were killed in extremis during the study and tissues of the highest dose group and controls should be routinely examined. Likewise, if abnormalities or equivocal results are observed in any tissues, then the same tissues from all lower dose groups should be examined. Likewise, if abnormalities are observed in any tissue of an organ system, then the other tissues in that organ system should be microscopically examined in all animals. In the case where results of the experiment give evidence of substantial alteration of the highest dose group animals' survival, then the animals of the next lower dose group should be examined microscopically.

III. Data Reporting

A. Identification

Each test report should be signed by the persons responsible for the test and identify:

1. The laboratory where the test was performed by name and address;
2. The inclusive dates of the test; and
3. Each person primarily responsible for separate components of the test including (a) the conduct of the test, (b) pathology, (c) analysis of the data, (d) the writing of the report, and (e) any written or other matter contained in the report.

B. Body of Report

The test report must include all information necessary to provide a complete and accurate description and evaluation of the test procedures and results in the following sections:

1. Summary and conclusions: This section of the test report should contain a brief description of the methods, a summary of the data, an analysis of the data, and a statement of the conclusions drawn. The summary must highlight all positive data or observations and any deviations from control data which may be indicative of toxic effects.
2. Materials: This section of the test report should include, but not be limited to, the following information:
 - (a) Identification of the test substance, so far as practical, including:
 - i. chemical name, molecular structure, and a qualitative and quantitative determination of its chemical composition, including names and quantities of known contaminants and impurities and listing the percentage of unidentifiable materials to account for the entire test sample.
 - ii. manufacturer and lot number of the substance tested, and such information as physical state, pH, stability, and purity; and
 - iii. specific identification of diluents, suspending agents, emulsifiers, or other materials used in administering the test substance.

(b) Animal data, including:

- i. species and strain used and rationale for selection of the strain if other than a common laboratory strain;
 - ii. source of supply of the animals;
 - iii. description of any pre-test conditioning; including quarantine procedures, etc.
 - iv. description of the method used in randomization of animals to test or control groups; and
 - v. numbers, age and condition of animals of each sex in each test and control group.
- (c) Data on husbandry should include a description of the caging conditions (including number of animals per cage), diet, bedding material, ambient temperature, humidity, and lighting conditions.

3. Methods

- (a) Deviation from guidelines: This section shall indicate all ways in which the test procedure deviates from these guidelines and shall state the rationale for such deviation.
- (b) Specification of test methods: This section shall include a full description of the experimental design and procedure, the length of the study, and the dates on which the study began and ended.
- (c) Statistical analysis: All statistical methods used should be fully described or identified by reference.
- (d) Data on dosage administration, including:
 - i. all dose levels administered, expressed as mg/kg of body weight;
 - ii. method, frequency, duration, and time of day;
 - iii. total volume of substance (i.e., test substance plus vehicle) contained in individual dosages.
- (e) Data on observation methods, including:
 - i. duration; and
 - ii. method and frequency of observation of the animals.

4. Results

The tabulation of data and individual results must accompany each report in sufficient detail to permit independent evaluation of results, including summaries and tables that show, as appropriate, the relationship of effects to time of dosing, sex, etc.

(a) Data presented for each animal should include hematology, clinical chemistry, and other tests performed, a description of all toxicological or pharmacological effects and abnormalities, accompanied by the animal's identification number, test group (dose level and sex), and days of study when the signs appeared and disappeared. An attempt should be made to correlate effects observed during the study with post mortem findings. The time of death for animals which die while on test should be reported. When numerical averages are presented, they should be accompanied by an appropriate measure of variability, such as the standard error. All animals placed on study must be accounted for.

(b) Findings from all clinical observations, necropsy, and histopathological examinations.

(c) Evaluation of data: An evaluation of test results, including their statistical analysis, should be made and supplied, based on the clinical findings; the gross necropsy findings, and the histopathological results. This should include an evaluation of the relationship, or lack thereof, between the animal's exposure to the test substance and the incidence and severity of all abnormalities; such abnormalities, include behavioral and clinical abnormalities, tumors and other lesions, organ weight effects, effects on mortality, and any other general or specific toxic effects.

5. References

This section of the test report shall include the following information:

(a) Availability of original data, specimens and samples of the test substance. The location of all original data, specimens, and samples of the test substances which are retained in accordance with the testing requirement.

(b) Literature or references, including, where appropriate, those references for (1) test procedures, (2) statistical and other methods used to analyze the data, (3) compilation and evaluation of results, and (4) the basis upon which conclusions were reached.

IV. Suggested Reading

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13. Weil, C.S. 1970. Significance of organ-weight changes in food safety evaluation. In Metabolic Aspects of Food Safety, pp. 419-454. Frances, J.C. Roe, editor. Blackwell Scientific Publications: Oxford and Edinburgh.
14. Weil, C. S. 1973. Experimental design and interpretation of data from prolonged toxicity studies. Pharmacol. and the Future of Man. Proc. 5th Inter. Congr. Pharmacol. 2:2-12.
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GUIDELINES FOR REPRODUCTION TESTING WITH A TERATOLOGY PHASE

INTRODUCTION

This guideline for reproduction testing is designed to provide general information concerning the effects of a test substance on gonadal function, estrous cycles, mating behavior, conception, parturition, lactation, and weaning. It is not designed to determine specific cause and effect in all cases. The study may also provide preliminary information about the effects of the test substance on neonatal morbidity, mortality, and teratogenesis and serve as a guide for subsequent tests. This guideline is for use with substances given orally to rats and mice. It contains optional procedures for the inclusion of more than one litter per generation and extension of the study to a third generation.

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I. General Considerations

A. Good Laboratory Practices

Studies should be conducted according to good laboratory practice regulations (e.g., "Nonclinical Laboratory Studies, Good Laboratory Practice Regulations," 43 FR 59986, 22 December 1978).

B. Test Substance

1. The specific substance or mixture of substances to be tested should be determined in consultation with the agency. As far as is practical, composition of the test substance should be known. Information should include the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials to account for the entire test substance.
2. Ideally, the lot of the substance tested should be the same throughout the study. The test sample should be stored under conditions that maintain its stability, strength, quality, and purity from the date of its production until the tests are complete.

C. Animals

1. Recommendations contained in DHEW pub. no. (NIH) 74-23, entitled "Guide for the Care and Use of Laboratory Animals," should be followed for the care, maintenance, and housing of animals.
2. Healthy animals, not subjected to any previous experimental procedures, must be used.
3. The test animal shall be characterized as to species, strain, sex, weight and/or age. Each animal must be assigned an appropriate identification number.
4. Animals may be group-caged for this test unless a specific test guideline or the pharmacological action of the test substance dictates otherwise, but the number of animals per cage should not prevent continued and clear observation of each animal. When signs of morbidity or excitability are observed in group-caged animals during the test, such animals should be moved to separate cages.
5. Animals should be assigned to groups in a random manner so as to minimize bias and to assure comparability of pertinent variables for statistical purposes.

D. Dead Animals, Necropsy, and Histopathology

Animals should be observed as necessary to insure that there is minimal loss due to cannibalism or similar management problems. Where possible, necropsy should be performed soon after an animal is sacrificed or found dead to minimize loss of tissues due to autolysis. When necropsy cannot be performed immediately, the animal must be refrigerated at temperatures low enough to minimize autolysis and not cause freezer burn. If histopathological examination is to be conducted, tissue specimens should be placed in appropriate fixative when they are taken from the animal.

E. Diet

The animal's diet should meet all of the nutritional requirements of the test species. Special attention should be paid to the 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.

II. Specific Considerations

A. Test Preparation

1. Animals: This guideline is for use with the rat or mouse. If other species are used, appropriate modifications of this guideline will be necessary. Strains with low fecundity should not be used.
2. Sex and age: For an adequate assessment of fertility, both male and females must be studied. All test and control animals must be weaned and acclimated before treatment begins.
3. Number of animals: Each test and control group should contain at least 20 males and at least 20 pregnant females at or near term. In order to achieve this it may be necessary to start with 30 animals per sex per group in the first parental groups (P) and 25/sex/group in the parents (F_1) of the F_2 generation.
4. Controls: Appropriate controls are required. The control group should be treated in all respects as the treated groups, except for exposure to the test substance. For dietary studies, the control group would normally be fed only the basal diet. However, if a vehicle must be used in administering the test substance, then a vehicle control is

necessary. A single volume of vehicle should be used in all treatment groups, and this volume should also be given to the control group. If there are insufficient data on the toxic properties of the vehicle used in administering the test substance or the vehicle has not been characterized in terms of its influence on either the test substance toxicity or bioavailability, then at least a vehicle and basal diet control is necessary. The use of uncharacterized vehicles should be avoided.

B. Test Procedure

1. Duration of dosing: The first parental animals, $P_1(F_0)$ will be exposed to the test substance up to the weaning of their F_1 offspring. The F_1 animals should be dosed up to birth of the F_2 offspring. Consideration should be given to the production of two litters per generation. If there are indications of poor reproductive performance in the controls, then a second litter per generation is essential for the proper conduct of this study. Table I presents the dosing and breeding schedule for rats, which follows:

(a) Dosing of males

- i. Daily dosing of the P_1 males should begin soon after they are weaned and acclimated. Males should be dosed during growth and for at least one complete spermatogenic cycle in order to elicit any adverse effects on spermatogenesis by the test substance. P_1 males should continue receiving daily doses of the test substance during the mating period of about 3 weeks.
- ii. F_1 males should receive daily doses of the test substance from weaning through the mating period.
- iii. If a two litter per generation design is to be used, then the P_1 males need not be dosed for a full spermatogenic cycle (10 weeks) prior to the mating to produce the F_{1a} litter. Alternatively, they may be dosed for a period of two weeks (similar to that of P_1 females) prior to their first mating.

TABLE I
APPROXIMATE DOSING AND BREEDING SCHEDULE

Approx. Time

<u>(Weeks)</u>	<u>P₁ (F₀)</u>	<u>F₁</u>
0	P ₁ birth	
6	P ₁ weaned; P ₁ males dosing begins;	
14	P ₁ females dosing begins;	
16-19	P ₁ mating;	
19-22		F ₁ born; P ₁ males dosing ends at week 19*
22-25		F ₁ weaned; P ₁ females dosing ends at weaning of the F ₁ *; F ₁ male and female dosing begins
31-34		F ₁ mating; F ₁ male dosing ends at Week 34 *
34-37		F ₂ born, F ₁ female dosing ends at Week 37**

* If a second litter per generation is produced then at least 10 days should separate weaning of the first litter and the mating period for production of the second.

** For a teratology assessment phase, a second mating of the F₁ animals should be conducted. Dosing of F₁ males and females should continue, and at least 10 days, following the weaning of the F_{2a} litter, should elapse prior to mating for the F_{2b}. All of the F₁ dams should be sacrificed about 1 day prior to term and the fetuses studied as described in the teratology guideline.

(b) Dosing of females

- i. Daily dosing of the P₁ females should begin after they are mature and about 2 weeks prior to mating. Females should be dosed for at least two complete estrous cycles in order to elicit any adverse effects on oogenesis or estrus by the test substance. After mating, each P₁ female should continue receiving daily doses of the test substance throughout pregnancy and until its offspring (including second litter, if necessary) are weaned (3 weeks post-partum).
- ii. The F₁ females should receive daily doses of the test substances until they are sacrificed (see Section E).

(c) Mating: For each mating, one female should be placed with a male from the same dose group until pregnancy occurs or 3 weeks have elapsed. Each morning the females must be examined for presence of sperm. Day 0 of pregnancy is defined as the day a vaginal plug and/or sperm are found. For mating the F₁ offspring, 2 males and 2 females are selected by randomized stratification from each litter from cross-mating with the other F₁ offspring of the same dose group to produce the F₁ generation. Sibling matings must be avoided. F₁ males and females not selected for mating are sacrificed upon weaning as described below.

(d) Standardization of number of pups per litter: All litters of more than 10 pups should be culled to 10 in a random manner.

(e) Times of sacrifice - males

- i. All P₁ males should be sacrificed at the end of the 3-week mating period (normally Week 19, if a second litter is necessary Week 26).
- ii. F₁ males selected for mating should be sacrificed at the end of the 3-week mating period of the F₁ generation. F₁ males not selected for mating can be sacrificed when weaned.
- iii. F₂ males naturally delivered should be retained to determine 24-hour post-partum survival rate and then sacrificed unless production of a third generation is necessary.

(f) Times of sacrifice - females

- i. The P_1 females should be sacrificed upon weaning of their F_1 offspring. The duration of gestation should be calculated from day 0 of pregnancy. 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 preserved and studied for possible defects, evidence of live birth and cause of death. Live pups should be weighed and counted at birth and days 1, 4 and 21 after birth. Physical behavioral abnormalities observed in the dams or offspring must be recorded.
 - ii. The F_{1a} females (dams of the F_{1a} litter) in test and control groups should be continued on the test substance and be allowed to litter normally. Signs of difficult, delayed, or prolonged labor should be reported. The duration of gestation should be calculated from day 0 of pregnancy. 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 preserved and studied for possible defects, evidence of live birth and cause of death. Live pups should be weighed and counted at birth and days 1 and 24 hours after birth. Physical behavioral abnormalities observed in the dams or offspring must be recorded.
 - iii. The F_{1b} females (dams of the F_{1b} litter) from each test and control group should be weighed, killed and examined late in pregnancy (around one day prior to term). These F_1 females should be examined for number and distribution of embryos in each uterine horn, embryos undergoing resorption, malformed fetuses, and any other abnormal condition (according to teratology guideline).
 - iv. Naturally delivered F_2 females should be retained to determine post-partum-survival rate and then sacrificed unless production of a third generation is necessary.
2. Dosage: At least three dosage levels should be tested in addition to the control. Unless limited by the physical/chemical nature of biological effects of the test substance, the highest dose level should ideally induce toxicity but not mortality in the P_1 animals. In actual practice, mortality may be encountered making attainment of this ideal difficult. The low dose should not induce any observable adverse effects. For P_1 males, the dosage administered to each animals may be based on the individual

animals's weekly body weight. For females, the dosage administered to each animals may be based on the individual animal's weekly body weight or during pregnancy it may be based on the body weight at day 6 of pregnancy. Dosage may be also determined on the basis of a percentage of the test substance in the diet.

3. Route of administration: The test substance should be administered in the diet, or drinking water, unless the chemical or physical characteristics or use pattern of the test substance suggest a more appropriate method of administration such as by stomach tube. When administered in the diet, the dose of the test substance may be calculated on the basis of mg of test substance/kg of food. Doses administered by gavage or in water should be calculated and adjusted weekly on the basis of body weight. The test substance should be administered at approximately the same time each day.
4. Animal care: Food and water should be provided ad libitum. Near parturition, pregnant females must be caged separately in delivery or maternity cages and may be provided with nesting materials.
5. Observation: Throughout the test period, each animal must be observed at least once daily. Pertinent behavioral changes, food consumption, and all signs of toxicity, including mortality, must be recorded. These observations should be reported individually for each animal.
6. Weight changes:
 - (a) P₁ males and females selected should be weighed on the first day of dosing and weekly thereafter.
 - (b) F₁ males and females should be weighed at birth and on days 4, and 21 and weekly thereafter.
 - (c) Naturally delivered F₂ males and females should be weighed at birth and at 24 hours after birth.
7. Gross Necrospy
When sacrificed, each animal should be subjected to complete gross necropsy with special attention paid to the organs of the reproductive system. Dead or moribund pups should be examined for defects.
8. Histopathology

The vagina, uterus, ovaries, testes, epididymides, seminal vesicles, prostate, and target organs of all P and F₁ animals selected for mating should be preserved for microscopic examination. In the rare event that these organs have not been examined in other multiple dose studies, they should be

microscopically examined in all high-dose and control animals. Organs showing abnormalities or equivocal results should be examined in all other P and F₁ animals selected for mating. In these instances microscopic examination should be made of all tissues showing gross pathological changes.

III. Data Reporting

A. Identification

Each test report should be signed by the persons responsible for the test and identify:

1. The laboratory where the test was performed by name and address;
2. The inclusive dates of the test; and
3. Each person primarily responsible for separate components of the test including (a) the conduct of the test, (b) pathology, (c) analysis of the data, (d) the writing of the report, and (e) any written or other matter contained in the report.

B. Body of Report

The test report must include all information necessary to provide a complete and accurate description and evaluation of the test procedures and results in the following sections:

1. Summary and conclusions: This section of the test report should contain a brief description of the methods, a summary of the data, an analysis of the data, and a statement of the conclusions drawn. The summary must highlight all positive data or observations and any deviations from control data which may be indicative of toxic effects.
2. Materials: This section of the test report should include, but not be limited to, the following information:
 - (a) Identification of the test substance, so far as practical, including:
 - i. chemical name, molecular structure, and a qualitative and quantitative determination of its chemical composition, including names and quantities of known contaminants and impurities and listing the percentage of unidentifiable materials to account for the entire test sample.
 - ii. manufacturer and lot number of the substance tested, and such information as physical state, pH, stability, and purity; and

- iii. specific identification of diluents, suspending agents, emulsifiers, or other materials used in administering the test substance.
- (b) Animal data, including:
 - i. species and strain used and rationale for selection of the strain if other than a common laboratory strain;
 - ii. source of supply of the animals;
 - iii. description of any pre-test conditioning;
 - iv. description of the method used in randomization of animals to test or control groups; and
 - v. numbers, age and condition of animals of each sex in each test and control group.
- (c) Data on husbandry should include description of the caging condition including number of animals per cage, diet, bedding material, ambient temperature, humidity, and lighting conditions.

3. Methods

- (a) Deviation from guidelines: This section shall indicate all ways in which the test procedure deviates from these guidelines and shall state the rationale for such deviation.
- (b) Specification of test methods: This section shall include a full description of the experimental design and procedure, the length of the study, and the dates on which the study began and ended.
- (c) Statistical analysis: All statistical methods used should be fully described or identified by reference.
- (d) Data on dosage administration, including:
 - i. all dose levels administered, expressed as mg/kg of body weight;
 - ii. method, frequency, duration, and time of day; and
 - iii. total volume of substance (i.e., test substance plus vehicle) contained in individual dosages.
- (e) Data on observation methods, including:

- i. duration; and
- ii. method and frequency of observation of the animals.

4. Results:

The tabulation of data and individual results must accompany each report in sufficient detail to permit independent evaluation of results.

(a) The data presented should include the following information:

- species/breed used;
- toxic response data by sex, dose and litter fertility indices, length of gestation, etc.;
- time of death during the study or whether animals survived to termination;
- toxic or other effects on reproduction, offspring, postnatal growth, etc.;
- the time of observation of each abnormal sign and its subsequent course;
- body weight data for P_1 , F_1 , and F_2 animals;
- necropsy findings;
- a detailed description of microscopic findings; and
- statistical treatment of results where appropriate.

(b) Data may be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals pregnant, the types of change and the percentage of animals displaying each type of change.

All numerical results should be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used.

- (c) An evaluation of test results, including their statistical analysis, should be made and supplied, based on the clinical findings, the gross necropsy findings, and the microscopic results. This should include an evaluation of the relationship, or lack thereof, between the animal's exposure to the test substance and the incidence and severity of all abnormalities, tumors and other lesions, organ weight effects, effects of mortality, and any other general or specific toxic effects.

In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered.

5. References

This section of the test report shall include the following information:

- (a) Availability of original data, specimens and samples of the test substance. The location of all original data, specimens, and samples of the test substances which are retained in accordance with the testing requirement.
- (b) Literature or references, including, where appropriate, those references for (1) test procedures, (2) statistical and other methods used to analyze the data, (3) compilation and evaluation of results, and (4) the basis upon which conclusions were reached.

IV. Suggested Reading

1. Goldenthal, E.I., Guidelines for Reproduction Studies for Safety Evaluation of Drugs for Human Use. Drug Review Branch, Division of Toxicological Evaluation, Bureau of Science, FDA, 1966.
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GUIDELINES FOR A THREE GENERATION REPRODUCTION
TOXICITY STUDY WITH OPTIONAL TERATOLOGY PHASE

I. Introduction

This guideline for reproduction testing is designed for substances with data indicating the need for reproductive toxicity evaluation in at least three generations. It should provide further information concerning the effects of a test substance on gonadal function, estrous cycles, mating behavior, conception, parturition, lactation, and weaning and is not designed to determine specific cause and effect in all cases. The study will also provide information about the effects of the test substance on neonatal morbidity, mortality, and teratology. This guideline is for use with substances given orally to rodents.

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I. General Considerations

A. Good Laboratory Practices

Studies should be conducted according to good laboratory practice regulations (e.g., "Nonclinical Laboratory Studies, Good Laboratory Practice Regulations," 43 FR 59986, 22 December 1978).

B. Test Substance

1. The specific substance or mixture of substances to be tested should be determined in consultation with the agency. As far as is practical, composition of the test substance should be known. Information should include the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials to account for the entire test substance.
2. Ideally, the lot of the substance tested should be the same throughout the study. The test sample should be stored under conditions that maintain its stability, strength, quality, and purity from the date of its production until the tests are complete.

C. Animals

1. Recommendations contained in DHEW pub. no. (NIH) 74-23, entitled "Guide for the Care and Use of Laboratory Animals," should be followed for the care, maintenance, and housing of animals.
2. Healthy animals, not subjected to any previous experimental procedures, must be used.
3. The test animal shall be characterized as to species, strain, sex, weight and/or age. Each animal must be assigned an appropriate identification number.
4. Animals may be group-caged for this test unless a specific test guideline or the pharmacological action of the test substance dictates otherwise, but the number of animals per cage should not prevent continued and clear observation of each animal. When signs of morbidity or excitability are observed in group-caged animals during the test, such animals should be moved to separate cages.
5. Animals should be assigned to groups in a random manner so as to minimize bias and to assure comparability of pertinent variables for statistical purposes.

D. Dead Animals, Necropsy, and Histopathology

Animals should be observed as necessary to insure that there is minimal loss due to cannibalism or similar management problems. Where possible, necropsy should be performed soon after an animal is sacrificed or found dead to minimize loss of tissues due to autolysis. When necropsy cannot be performed immediately, the animal must be refrigerated at temperatures low enough to minimize autolysis and not cause freezer burn. If histopathological examination is to be conducted, tissue specimens should be placed in appropriate fixative when they are taken from the animal.

E. Diet

The animal's diet should meet all of the nutritional requirements of the test species. Special attention should be paid to the 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.

III. Specific Considerations:

A. Test Preparation

1. Animals: This guideline is for use with the rat or mouse. If other species are used, appropriate modifications of this guideline will be necessary. Strains with low fecundity should not be used. Animals used for testing should not have been subjected to previous compound administration in other experiments.
2. Sex and age: For an adequate assessment of fertility, both males and females must be studied. All test and control animals should be weaned and acclimated before dosing begins. Animals should be sufficiently mature at the start of dosing.
3. Number of animals: Each test and control group should contain at least 20 males and at least 20 pregnant females at or near term. The objective is to produce enough pregnancies and offspring to assure a meaningful evaluation of the potential of the substance to affect mating, pregnancy, lactation, growth and development of the offspring from conception to maturity. In order to achieve this objective it may be necessary to start with 30 animals/sex/group in the P generation and 25/sex/group in the F₁ generation.
4. Test groups: At least three treatment groups and one vehicle control group should be used., If there are insufficient data on the toxic properties of the vehicle used in administering the test substance, a negative control group exposed only to the diet must also be included. In all other respects the control groups must be handled and maintained in a manner identical to that used with the groups given the test substance.

B. Test Procedure

1. Duration of dosing: A total of six litters are examined; two litters in the first, second, and third generations, with an optional additional litter in the F₂ or F₃ litters, for teratology. The first parental animals will be exposed to the substance continuously through the weaning of their F_{1A} offspring. After the F_{1A} pups are weaned, the parental animals are allowed to rest for at least 10 days and then remated to produce F_{1B}. Randomly selected weanling pups are continued on the control or test compound and mated to produce the following generation. Selection of animals to produce the next generation should be done with the aid of a table of random numbers and should not be based on weight or fitness. F_{2A} and F_{2B} are produced from mating of F_{1B} animals and are treated in the same manner as F_{1A} and F_{1B}. Randomly selected animals from F_{2B} are mated to produce the third generation. F_{3A} animals are weaned and either necropsied or used for a longer term toxicity study. F_{3B} animals are produced and treated in the same manner as F_{1A}.

(See Figure I)

2. Dosage: At least three dosage levels must be tested in addition to the controls. Ideally, unless limited by the physical/chemical nature or biological effects of the test substance, the highest dose level should induce toxicity but not mortality in P animals. The low dose should not induce any observable adverse effects. For P males, the dosage administered to each animal may be based on the individual animal's body weight adjusted weekly for changes in body weight. For females, the dosage administered to each animal maybe based on the individual animal's body weight adjusted weekly for changes in body weight, except during pregnancy when it should be based on the body weight at Day 6 of pregnancy. Dosage levels for both males and females may also be based upon a percentage of the test compound in the diet.

(a) Dosing of males

- i. Daily dosing of the P males should begin soon after they are weaned and acclimated. Males should be dosed during growth and for at least one complete spermatogenic cycle (approximately 56 days in the mouse and 70 days in the rat) in order to elicit any adverse effects on spermatogenesis by the test substance. P males should continue receiving daily doses of the test substance during the remaining periods required to obtain the desired matings.
- ii. F₁, F₂, and F₃ males will be exposed to the test substance in utero during the nursing period and throughout weaning and maturation.

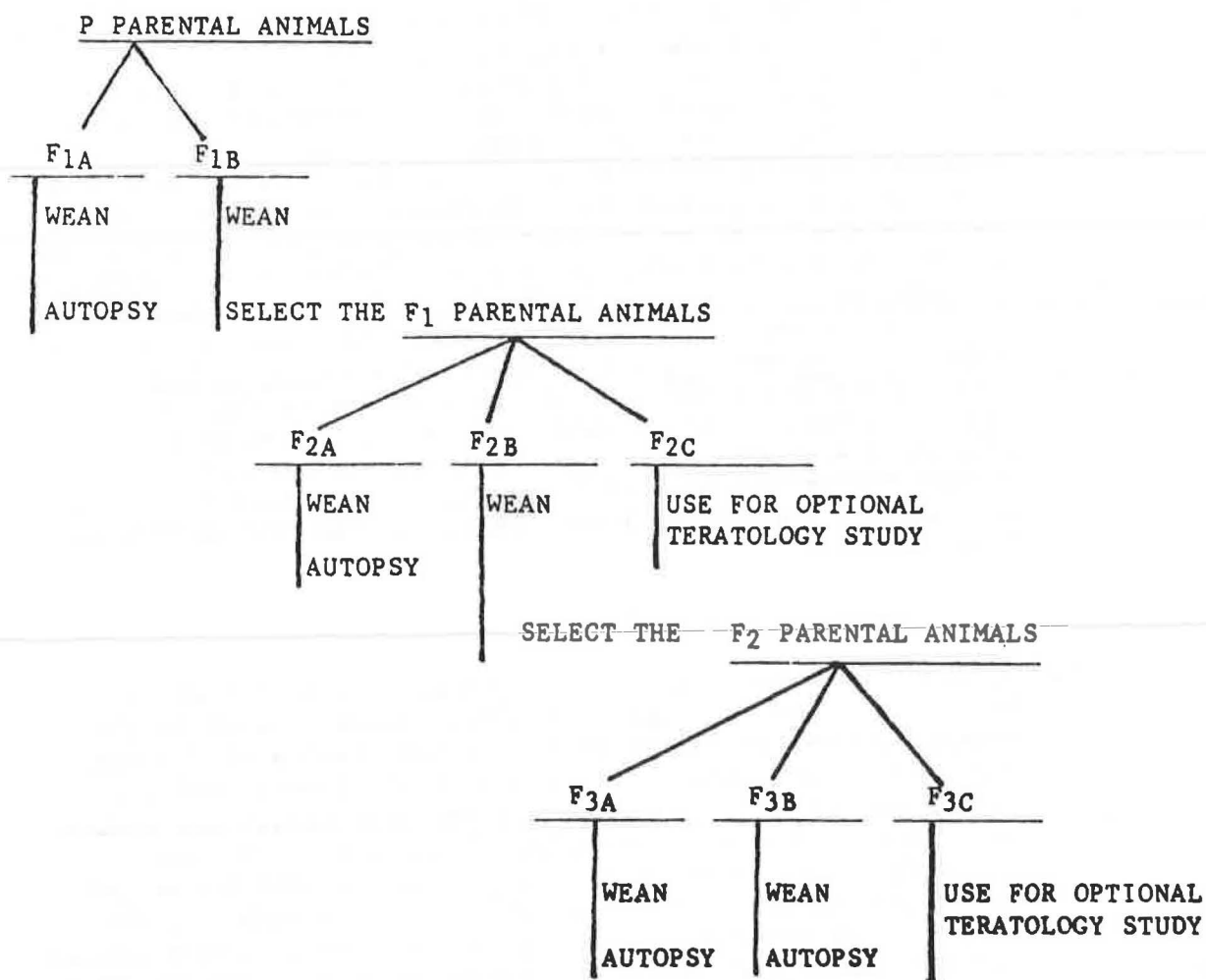


Figure 1. Protocol for 3-generation reproduction and teratology study

(b) Dosing of females

- i. Daily dosing of the P females should begin after they are mature and about 2 weeks prior to mating. Females should be dosed for at least two complete estrous cycles in order to elicit any adverse effects on oogenesis or estrus by the test substance. After mating, each P female should continue receiving daily doses of the test substance throughout pregnancy, until its offspring are weaned (3 weeks post partum), and continuously throughout all subsequent matings.
- ii. F₁, F₂, and F₃ females will be exposed to the test substance in utero, through nursing, and throughout weaning and maturation.

(c) Mating:

For each mating, one female should be placed with a male from the same dose group. For mating the F₁ and F₂ offspring, 2 males and 2 females are selected by randomized stratification from each litter for cross-mating with the other F₁ or F₂ offspring of the same dose group to produce the F₂ or F₃ generation. Sibling matings must be avoided. F₁ males and females not selected for mating are sacrificed upon weaning as described below. Day 0 of pregnancy is defined as the day a vaginal plug and/or sperm are found. On day 4, all litters of more than 10 pups should be culled to 10 in a random manner.

(d) Times of sacrifice - males

- i. All parental P₁ (F₀) males should be sacrificed at the end of the mating period for their last litter.
- ii. F₁ and F₂ males selected for mating should be sacrificed at the end of the period for mating of the following generation. F₁, F₂, and F₃ males not selected for mating should be sacrificed at weaning.

(e) Times of sacrifice - females

- i. Each P₀ (F₀) female should be sacrificed when its last F₁ offspring is weaned.
- ii. F₁ and F₂ females selected for mating should follow the same schedule for sacrifice as the P₁ females.
- iii. F₁, F₂, and F₃ females not selected for mating should be sacrificed at weaning.

3. Teratology phase: Either the F_{2C} or F_{3C} litter can be used to determine fetotoxic effects. If a teratology study is to be done, pregnancy is timed by the vaginal smear method described. Approximately 24 hours prior to delivery, the dams are killed, and caesarean sections are performed. The uterus is opened and examined for the presence of early and late deaths and corpora lutea are counted. The live fetuses are removed, weighed, sexed, and examined for gross malformations. To discover visceral abnormalities, one-half the fetuses can be dissected. The remaining half of the fetuses can be cleared and stained for the detection of skeletal anomalies.
4. Route of administration: The test substance should be administered by diet or drinking water or unless the chemical or physical characteristics or use pattern of the test substance suggest a more appropriate method of administration such as by stomach tube. The method of administration must be the same for controls and test groups. When administered in the diet, the dose of the test substance may be calculated on the basis of mg of test substance/kg of food. Doses administered by gavage or in water should be calculated and adjusted weekly on the basis of body weight. For females during pregnancy of dose may be based on the body weight at day 6 of pregnancy. The test substance should be administered at approximately the same time each day.
5. Animal care: Food and water should be provided ad libitum. Near parturition, pregnant females must be caged separately in delivery or maternity cages and may be provided with nesting materials.
6. Observation: Throughout the test period, each animal must be observed at least once daily by an appropriately trained observer. Pertinent behavioral changes, food consumption, and all signs of toxicity, including mortality, must be recorded. These observations should be reported individually for each animal. Pregnant females in test and control groups should be continued on the test substances and be allowed to litter normally. Signs of difficult, delayed, or prolonged labor should be recorded. The duration of gestation should be calculated from Day 0 of pregnancy. 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 preserved and studied for possible defects and cause of death. Live pups should be weighed and counted at birth and days 4, and 21 after birth. Physical or behavioral abnormalities observed in the dams or offspring must be recorded.

7. Weight changes:

- (a) P males and females selected should be weighed on the first day of dosing and weekly thereafter.
- (b) F₁, F₂, and F₃ males and females used for mating should be weighed at birth and on days 1, 4, and 21 and weekly thereafter.
- (c) F₁, F₂, and F₃ males and females not used for mating should be weighed at birth and on days 1, 4, and 21 after birth.

8. Gross Necropsy

When sacrificed, each animal should be subjected to complete gross necropsy with special attention paid to the organs of the reproductive system. Dead or moribund pups should be examined for defects.

9. Histopathology

The vagina, uterus, ovaries, testes, epididymus, seminal vesicles, prostate, and target organs of all P and F₁ animals should be preserved for microscopic examination. In the rare event that these organs have not been examined in other multiple dose studies, they should be microscopically examined in all high-dose and control animals. Organs showing abnormalities or equivocal results should then be examined in all other P and F₁ animals. In these instances microscopic examination should be made of all tissues showing gross pathological changes.

IV. Data Reporting

A. Identification

Each test report should be signed by the persons responsible for the test and identify:

- 1. The laboratory where the test was performed by name and address;
- 2. The inclusive dates of the test; and
- 3. Each person primarily responsible for separate components of the test, including (a) the conduct of the test, (b) pathology, (c) analysis of the data, (d) the writing of the report, and (e) any written or other matter contained in the report.

B. Body of Report

The test report must include all information necessary to provide a complete and accurate description and evaluation of the test procedures and results in the following sections:

1. Summary and conclusions: This section of the test report should contain a brief description of the methods, a summary of the data, an analysis of the data, and a statement of the conclusions drawn. The summary must highlight all positive data or observations and any deviations from control data which may be indicative of toxic effects.
2. Materials: This section of the test report should include, but not be limited to, the following information:
 - (a) Identification of the test substance, so far as practical, including:
 - i. chemical name, molecular structure, and a qualitative and quantitative determination of its chemical composition, including names and quantities of known contaminants and impurities and listing the percentage of unidentifiable materials to account for the entire test sample.
 - ii. manufacturer and lot number of the substance tested, and such information as physical state, pH, stability, and purity; and
 - iii. specific identification of diluents, suspending agents, emulsifiers, or other materials used in administering the test substance.
 - (b) Animal data, including:
 - i. species and strain used and rationale for selection of the strain if other than a common laboratory strain;
 - ii. source of supply of the animals;
 - iii. description of any pre-test conditioning;
 - iv. description of the method used in randomization of animals to test or control groups; and
 - v. numbers, age and condition of animals of each sex in each test and control group.

- (c) Data on husbandry should include description of the caging condition including number of animals per cage, diet, bedding material, ambient temperature, humidity, and lighting conditions.

3. Methods

- (a) Deviation from guidelines: This section shall indicate all ways in which the test procedure deviates from these guidelines and shall state the rationale for such deviation.
- (b) Specification of test methods: This section shall include a full description of the experimental design and procedure, the length of the study, and the dates on which the study began and ended.
- (c) Statistical analysis: All statistical methods used should be fully described or identified by reference.
- (d) Data on dosage administration, including:
 - i. all dose levels administered, expressed as mg/kg of body weight;
 - ii. method, frequency, duration, and time of day; and
 - iii. total volume of substance (i.e., test substance plus vehicle) contained in individual dosages.
- (e) Data on observation methods, including:
 - i. duration; and
 - ii. method and frequency of observation of the animals.

4. Results

The tabulation of data and individual results must accompany each report in sufficient detail to permit independent evaluation of results.

- (a) The data presented should include the following information:
 - species/breed used;
 - toxic response data by sex, dose and litter fertility indices, length of gestation, etc.;
 - time of death during the study or whether animals survived to termination;

- toxic or other effects on reproduction, offspring, postnatal growth, etc.;
 - the time of observation of each abnormal signs and its subsequent course;
 - body weight data for P₁, F₁, and F₂ animals;
 - necropsy findings;
 - a detailed description of microscopic findings;
 - statistical treatment of results where appropriate.
- (b) Data may be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals pregnant, the types of change and the percentage of animals displaying each type of change.

All numerical results should be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used.

- (c) An evaluation of test results, including their statistical analysis, should be made and supplied, based on the clinical findings, the gross necropsy findings, and the microscopic results. This should include an evaluation of the relationship, or lack thereof, between the animal's exposure to the test substance and the incidence and severity of all abnormalities, tumors and other lesions, organ weight effects, effects of mortality, and any other general or specific toxic effects..

In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered.

5. References

This section of the test report shall include the following information:

- (a) Availability of original data, specimens and samples of the test substance. The location of all original data, specimens, and samples of the test substances which are retained in accordance with the testing requirement.
- (b) Literature or references, including, where appropriate, those references for (1) test procedures, (2) statistical and other methods used to analyze the data, (3) compilation and evaluation of results, and (4) the basis upon which conclusions were reached.

IV. Suggested Reading

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Guideline for Teratogenicity Testing
in Rat, Hamster, Mouse, and Rabbit

INTRODUCTION

The purpose of this test is to yield data to help determine whether a test substance is potentially embryotoxic and/or teratogenic. Treatment must be started early enough and continued long enough to include the period of major organogenesis for the particular species used.

This guideline is for use with substances given orally to the rat, hamster, mouse, or rabbit.

Such a study can also be combined with a multigeneration reproduction study as long as the fetus is exposed during organogenesis.

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I. General Considerations

A. Good Laboratory Practices

Studies should be conducted according to good laboratory practice regulations (e.g., "Nonclinical Laboratory Studies, Good Laboratory Practice Regulations," 43 FR 59986, 22 December 1978).

B. Test Substance

1. The specific substance or mixture of substances to be tested should be determined in consultation with the agency. As far as is practical, composition of the test substance should be known. Information should include the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials to account for the entire test substance.
2. Ideally, the lot of the substance tested should be the same throughout the study. The test sample should be stored under conditions that maintain its stability, strength, quality, and purity from the date of its production until the tests are complete.

C. Animals

1. Recommendations contained in DHEW pub. no. (NIH) 74-23, entitled "Guide for the Care and Use of Laboratory Animals," should be followed for the care, maintenance, and housing of animals.
2. Healthy animals, not subjected to any previous experimental procedures, must be used.
3. The test animal shall be characterized as to species, strain, sex, weight and/or age. Each animal must be assigned an appropriate identification number.
4. Animals may be group-caged for this test unless a specific test guideline or the pharmacological action of the test substance dictates otherwise, but the number of animals per cage should not prevent continued and clear observation of each animal. When signs of morbidity or excitability are observed in group-caged animals during the test, such animals should be moved to separate cages.
5. Animals should be assigned to groups in a random manner so as to minimize bias and to assure comparability of pertinent variables for statistical purposes.

D. Dead Animals, Necropsy, and Histopathology

Animals should be observed as necessary to insure that there is minimal loss due to cannibalism or similar management problems. Where possible, necropsy should be performed soon after an animal is sacrificed or found dead to minimize loss of tissues due to autolysis. When necropsy cannot be performed immediately, the animal must be refrigerated at temperatures low enough to minimize autolysis and not cause freezer burn. If histopathological examination is to be conducted, tissue specimens should be placed in appropriate fixative when they are taken from the animal.

E. Diet

The animal's diet should meet all of the nutritional requirements of the test species. Special attention should be paid to the 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.

II. Specific Considerations

A. Test Preparation

1. Animals: Species commonly used are the rat, mouse, hamster and rabbit. The preferred species are the rat and the rabbit. Commonly used laboratory strains should be employed. The strain should not have low fecundity and should preferably be characterized for its response to teratogens. All test and control animals should be young, mature, pregnant females of uniform age, size, and parity.
2. Test groups: At least three test groups and one vehicle control group should be used. When the test substance is administered in a vehicle, the vehicle only should be administered to the controls. If no vehicle is used, then the controls should be sham treated. 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. In all other respects, the control must be handled and maintained in a manner identical to that used with the groups given the test substance.
3. Number of animals: Sufficient numbers of animals should be bred to assure that each test group and the vehicle control group will consist of at least 20 pregnant rats or mice, or at least 12 pregnant rabbits. These are the minimum numbers of pregnant animals at or near term. The objective is to assure that sufficient pups are produced to permit evaluation of the teratogenic potential of the substance.

B. Test Procedure

1. Duration of test: Day 0 is defined as the day a vaginal plug and/or sperm are found or insemination is performed. The test substance should be administered daily beginning soon after implantation and continuing well into the period of fetal development. * Traditionally the period of dosing for rats and mice has been from day 6 through 15, for the hamster from day 4 through 14, and for the rabbit from day 7 through 18. These periods of dosing are acceptable. An alternative method, however, is to extend the period of dosing in these species to about 1 day before the expected delivery date.

For substances that cause enzyme induction, or are highly toxic, shorter dosage periods may be appropriate.

Fetuses shall be delivered by hysterotomy about one day prior to term.

Care should be taken to insure that all animals are delivered at about the same stage of fetal development.

2. Dose levels: At least three dosage groups and a control group should be used. To select the appropriate dose levels, a pilot or trial study is advisable. It is not always necessary to carry out a trial study in pregnant animals; comparison of the results from a trial study in non-pregnant, and a main study in pregnant animals will establish if the test substance is more toxic in pregnant animals. If a trial study is carried out in pregnant animals, the dose producing embryonic or fetal lethalties should be determined. Unless limited by the physical/chemical nature or biological properties of the substance, the highest dosage level should ideally induce some overt maternal toxicity such as slight weight loss, but not a significant reduction in average litter size as compared to untreated controls or more than 10 per cent maternal deaths. The low dose level should not induce observable effects attributable to the test substance. The intermediate dose(s) should be located geometrically between high and low dose levels. The dosage administered may be based on the individual animal's body weight on the first day of substance administration or the animals may be weighed daily and the dosage adjusted accordingly.

3. Route of Administration: The test substance or vehicle should be administered by oral intubation at approximately the same time each day, unless the chemical or physical characteristics, or pattern of human exposure to the test substance suggest a more appropriate route of administration.
4. Animal care: Food and water should be provided ad libitum.
5. Observation: Throughout the test period, each animal should be observed at least once daily. Pertinent behavioral changes, and all signs of toxicity, including mortality, should be recorded. Any dam showing signs of imminent, abortion or premature delivery may be sacrificed on the date such signs are observed. The observations should be reported individually. Dams should be weighed at the start of substance administration (day 6 or 7), at the time of sacrifice, and at least weekly between these times. Weekly measurements of food consumption should be made.
6. Necropsy: At the time of sacrifice or death during the study, the dam should be examined macroscopically for any structural abnormalities or pathological changes which may have influenced the pregnancy. Immediately after sacrifice or death, the uterus should be removed and the contents examined for embryonic or fetal deaths and the number of live fetuses. It is usually possible to estimate the time of death in utero where this has occurred. In rats and rabbits the number of corpora lutea may be determined. The sex of the fetuses should be determined and they should be weighed individually, the weights recorded, and the mean fetal weight derived. Following removal, each fetus should be examined externally. For rats, mice and hamsters, one-third to one-half of each litter should be prepared and examined for skeletal anomalies, and the remaining part of each litter should be prepared and examined for soft tissue anomalies using appropriate methods. For rabbits, each fetus should be examined by careful dissection for visceral anomalies and then examined for skeletal anomalies.
7. Statistical Analysis: Values from the control and test group should be compared statistically. The following are suggested but others may be substituted. Anomalies may be compared by chi-square methods or the binomial expansion method. Maternal body weight gains and weight of fetuses may be compared to those of controls by F-test and Student's t-test. Fetal survival and incidence of abnormalities per litter may be compared by non-parametric, rank-order methods. Other statistical methods may be substituted.

III. Data Reporting

A. Identification

Each test report should be signed by the persons responsible for the test and identify:

1. The laboratory where the test was performed by name and address;
2. The inclusive dates of the test; and
3. Each person primarily responsible for separate components of the test including (a) the conduct of the test, (b) pathology, (c) analysis of the data, (d) the writing of the report, and (e) any written or other matter contained in the report.

B. Body of Report

The test report must include all information necessary to provide a complete and accurate description and evaluation of the test procedures and results. Each report must include the following sections:

1. Summary and conclusions: This section of the test report should contain a brief description of the methods, a summary of the data, an analysis of the data, and a statement of the conclusions drawn. The summary must highlight all positive data or observations and any deviations from control data which may be indicative of toxic effects.
2. Materials: This section of the test report should include, but not be limited to, the following information:
 - (a) Identification of the test substance, so far as practical, including:
 - i. chemical name, molecular structure, and a qualitative and quantitative determination of its chemical composition, including names and quantities of known contaminants and impurities and listing the percentage of unidentifiable materials to account for the entire test sample.
 - ii. manufacturer and lot number of the substance tested, and such information as physical state, pH, stability, and purity; and

iii. specific identification of diluents, suspending agents, emulsifiers, or other materials used in administering the test substance.

(b) Animal data, including:

- i. species and strain used and rationale for selection of the strain if other than a common laboratory strain;
- ii. source of supply of the animals;
- iii. description of any pre-test conditioning (such as quarantine procedures);
- iv. description of the method used in randomization of animals to test or control groups; and
- v. numbers, age and condition of dams in each test and control group.

(c) Data on husbandry should include description of the caging condition including number of animals per cage, diet, bedding material, ambient temperature, humidity, and lighting conditions.

3. Methods

(a) Deviation from guidelines: This section shall indicate all ways in which the test procedure deviates from these guidelines and shall state the rationale for such deviation.

(b) Specification of test methods: This section shall include a full description of the experimental design and procedure, the length of the study, and the dates on which the study began and ended.

(c) Statistical analysis: All statistical methods used should be fully described or identified by reference.

(d) Data on dosage administration, including:

- i. all dose levels administered, expressed as mg/kg of body weight;
- ii. method, frequency, duration, and time of day; and
- iii. total volume of substance (i.e., test substance plus vehicle) contained in individual dosages.

(e) Data on observation methods, including:

- i. duration; and
- ii. method and frequency of observation of the animals.

4. Results:

The tabulation of data and individual results must accompany each report in sufficient detail to permit independent evaluation of results.

(a) The following information should be included:

- Time of observation of each abnormal sign and its subsequent course.
- Age (or weight) at the start of the test.
- Body weights and body weight changes based on the carcass weight.
- Signs of resorptions.
- Toxic response data.
- Time of death.
- Pregnancy and litter data.
- Fetal data (Litter identification, live/dead, soft tissue and skeletal defects).

(b) Evaluation of the results should include:

- an evaluation of the relationship, if any, between exposure to the test substance and the anomalies, and
- an indication of the dosage level at which no toxic effects attributable to the test substance appeared.

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Guideline for the Design of an In Utero Exposure Phase
for Addition to Subchronic, Chronic or Carcinogenicity Guidelines

I. Introduction:

Under certain circumstances (see section V) an in utero exposure phase may be required for subchronic, chronic or carcinogenicity studies. This guideline suggests the design of such an in utero exposure phase.

II. General Considerations:

A. Good Laboratory Practices

Studies should be conducted according to good laboratory practice regulations (e.g., "Nonclinical Laboratory Studies, Good Laboratory Practice Regulations," 43 FR 59986, 22 December 1978).

B. Test Substance

1. The specific substance or mixture of substances to be tested should be determined in consultation with the agency. As far as is practical, composition of the test substance should be known. Information should include the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials to account for the entire test substance.
2. Ideally, the lot of the substance tested should be the same throughout the study. The test sample should be stored under conditions that maintain its stability, strength, quality, and purity from the date of its production until the tests are complete.

C. Animals

1. Recommendations contained in DHEW pub. no. (NIH) 74-23, entitled "Guide for the Care and Use of Laboratory Animals," should be followed for the care, maintenance, and housing of animals.
2. Healthy animals, not subjected to any previous experimental procedures, must be used.

3. The test animal shall be characterized as to species, strain, sex, weight and/or age. Each animal must be assigned an appropriate identification number.
4. Animals may be group-caged for this test unless a specific test guideline or the pharmacological action of the test substance dictates otherwise, but the number of animals per cage should not prevent continued and clear observation of each animal. When signs of morbidity or excitability are observed in group-caged animals during the test, such animals should be moved to separate cages.
5. Animals should be assigned to groups in a random manner so as to minimize bias and to assure comparability of pertinent variables for statistical purposes.

D. Dead Animals, Necropsy, and Histopathology

Animals should be observed as necessary to insure that there is minimal loss due to cannibalism or similar management problems. Where possible, necropsy should be performed soon after an animal is sacrificed or found dead to minimize loss of tissues due to autolysis. When necropsy cannot be performed immediately, the animal must be refrigerated at temperatures low enough to minimize autolysis and not cause freezer burn. If histopathological examination is to be conducted, tissue specimens should be placed in appropriate fixative when they are taken from the animal.

E. Diet

The animal's diet should meet all of the nutritional requirements of the test species. Special attention should be paid to the 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.

III. Specific Considerations:

A. Preparations

1. Animals: This guideline is for use with the rat or mouse. If other species are used, modifications of this guideline will be necessary. Strains with low fecundity should not be used.
2. Age: All test and control parental animals should be weaned and acclimated before treatment begins.

3. Number: The number of animals/sex suggested in the guideline to which the in utero phase is to be added should serve as a guide for determining the number of animals/group for mating. No more than one animal/sex/litter should be included in any group. For example, the subchronic oral toxicity guideline suggests that each group contain 20 animals/sex. Therefore, at least 20 litters/group are necessary in the in utero phase. Thus one may begin dosing of 25 animals/sex/group in order to obtain sufficient litters for the 90-day phase of the subchronic study.
4. Treatment Duration: The P (parental) animals should receive the test substance for a minimum of 4 weeks prior to mating. Exposure should be continuous throughout pre-mating, mating, gestation and lactation until weaning of the F₁ animals.
5. Dose Level Selection: In general, the dose selection criteria should be the same as the guideline to which the in utero phase is to be added. However, as a result of maternal or fetal toxicity it is often necessary to use lower doses during the in utero phase of the study in order to produce sufficient offspring for the post-weaning phase. It is strongly recommended that the selections of doses be based on the results of pilot studies. Results from absorption, distribution, metabolism, and elimination studies also should provide guidance in selection of a proper dosage regimen.
6. Standardization of number of pup per litter: the litter size should be reduced by random methods, stratified by sex to 8 animals/litter (4/sex if possible).
7. Selection of F₁ Animals: One animal per sex per litter should be randomly selected. Each F₁ animal should be individually identified and its parent's identity recorded.
8. Observations - Parental Animals: The animals should be examined daily. Any pharmacological and toxicological effects should be recorded. Body weight and food consumption should be recorded weekly for the females during gestation and lactation.
9. F₁ Animals: Viability checks and observations of general appearance should be made daily, and the presence of dead pups recorded. The pups should be counted on days 0, 4, 14, 21 of lactation. The pups should be weighed as a litter on days 0, 4 (before and after culling), and 14, but should be weighed individually on day 21. Number of pups per sex should be recorded on days 4 (before and after culling), and 14, and the sex of the individual pups should be recorded on day 21.

10. Termination of P animals and F₁ animals not selected for the post-weaning phase: These animals may be sacrificed after weaning of the F₁. If toxic signs or reproductive toxicity are observed, consideration should be given to gross necropsy of these animals.
11. Data Reporting: Litter mates should be identified. Other data should be reported as described in the test guideline used for the post-weaning phase.

**Guideline for Determination of the Absorption,
Distribution, Metabolism and Elimination
Characteristics of Food Additives.**

Glossary of Terms

Throughout the body of this document, numerous terms are used to describe the various aspects of the study of the action of food additives that have been introduced into living systems. A glossary of terms is included to aid in avoiding misinterpretation of the intent of various sections. Most of the following definitions have been adapted from the following references:

Journal of Pharmacokinetics and Biopharmaceutics 1:3-4, 1973.

Federal Register, 42 #5, Part III, January 7, 1977.

Absorption	-	The process or processes by which an administered compound enters the systemic circulation of the body.
Bioavailability	-	The rate and extent to which the administered compound is absorbed from a formulation and becomes accessible to the site of action and/or reaches the general circulation.
Biopharmaceutics	-	The study of the biological factors influencing bioavailability in animals and man.
Biotransformation	-	The process or processes by which the administered compound is structurally changed in the body by either enzymatic or non-enzymatic reactions; i.e., the resultant product of the reaction is of a different composition of matter or is a different configuration than the administered compound.
Distribution	-	The process or processes by which the absorbed compound and/or its metabolite or metabolites circulate and partition with various tissues in the body.
Dose Proportionality	-	Relationship between increasing doses of compound and measured parameters.
Disposition	-	The study of the absorption, distribution, biotransformation and excretion of compounds.

Excretion	-	The process or processes by which the administered compound and/or its biotransformation product or products are eliminated from the body.
Kinetics	-	The rate of any and all processes.
Metabolite Characterization	-	The determination of some of the physical- chemical parameters of the biotransformation product or products.
Metabolite Identification	-	The unequivocal identification of the biotransformation product. Usually, but not always, this will include a comparison with a synthetic reference.
Metabolite Profile	-	Chromatographic pattern and/or aqueous/non-aqueous partitioning (single or multiple) of the biotransformation products of the administered compound.

1. Introduction

Data from studies of the absorption, distribution, excretion and metabolism characteristics, referred to as disposition studies, of a test chemical are desirable to aid in the evaluation of test results from other toxicology studies and in the extrapolation of those results from animals to man. Flexibility is needed in the conduct of disposition studies, and therefore, the design of such studies will depend on the characteristics of the test chemical being investigated. The main purpose of disposition studies is to produce data which aid in the design and interpretation of other toxicological studies.

By providing information about dose-dependent kinetics, disposition studies should provide data useful for selecting appropriate dose levels for use in carcinogenicity, chronic, subchronic or reproduction toxicity studies.

Biochemical measurements related to metabolism may be included in a disposition study.

2. Purpose of study

In addition to the general purposes stated above, a disposition study may be performed for the following purposes: 1) to determine the amount and rate of absorption of the test chemical at different dose levels; 2) to determine the pattern of distribution of the test chemical among tissues, organs and fluid compartments at different dose levels, after single and repeated dosages, and the reversible binding of the test chemical to tissue sites and plasma proteins; 3) to determine the pattern and the rates of metabolism at different dose levels; 4) to determine the rates of excretion at different dose levels, after single and repeated dosages; 5) to determine covalent binding of the chemical with tissue at different dose levels; and 6) to determine induction of metabolizing enzymes and depletion of glutathione at different dose levels.

3. Conduct of study:

The following guideline elements are intended to provide assistance in the design of disposition studies and are not intended to specify requirements. Ideally such studies should be designed with the specific purpose of the study in mind.

(a) Animals

Disposition studies should be carried out using the same animal species and strain as those being used for most other toxicological studies on the same chemical.

Since certain biotransformation pathways are known to differ substantially among species. Toxic responses that differ as a result of these differing pathways may invalidate a given species as a proper toxicologic model for a specific compound. Preliminary studies may be performed in several species to develop information on comparative metabolism; this information may help in the selection of species for subsequent toxicity tests.

Furthermore, in toxicity studies, the test animal is exposed to the parent compound as well as to its metabolites. Thus, these studies constitute a safety assessment of the metabolites as well as the parent compound for the species involved which may make testing the metabolite separately unnecessary. An exception is the situation in which the major metabolite(s) observed in humans is not found in significant amounts in the a species used for toxicity evaluations. In such a case, toxicity testing of the metabolite(s) may be called for.

(b) Test groups

The number of animals used should be sufficient to indicate the variability in disposition parameters to be expected within a given species. For qualitative answers to specific questions, fewer animals can be used; e.g., in studies of biliary secretion.

Usually 4 young adult animals of each sex in each test group should be used. Alternatively, disposition studies may be done using one sex first and later in the second sex to verify result. For specific purposes, a comparative study using very young animals may provide information about the effects of age on the toxicokinetics. If disposition studies during pregnancy are needed, animals with defined or timed pregnancies should be used.

(c) Dosage

Several dose levels should be used to determine the relationship of dose level to toxicity. Ideally, there should be a low dose that corresponds to a no-effect level, an intermediate dose, and an upper dose at which there may be changes in the metabolic pattern, or at which toxic effects occurred in repeated dosage studies.

Absorption, tissue distribution and elimination should be determined after single administration of a range of doses. Ideally, the metabolite pattern and the potential for induction of metabolizing enzymes should be determined after repeated dosages.

Changes in Disposition Related to Dose Level - Initially a wide range of doses is given to the test species to establish the limits of tolerated doses. Disposition studies may detect changes in pharmacokinetic parameters across a dose range which may be reflected in disproportionate changes in toxicologic response.

When doses increased to the limit of practicality do not produce overt toxicity and the drug has been demonstrated to show dose- dependent kinetics, dose selection may be done on the basis of disposition studies.

Multiple Dose: Accumulation and Induction - It is common to observe changes in toxic response as multiple-dose toxicity studies proceed. Similarly, blood/plasma concentration following a single dose may not correspond to or predict steady state drug concentrations observed under multiple-dose regimens. Disposition studies conducted under multiple-dose conditions can indicate whether factors such as accumulation or induction are involved. The observed changes in response under multiple-dose conditions may be related to these factors.

(d) Route of administration

The disposition study should be done using the same route as that being considered for use in the longer-term toxicity studies. For determining the amount of absorption and the pattern of distribution and elimination soon after the administration of a substance, the intravenous administration of the test chemical for comparison purposes is useful. In some circumstances attention should be given to differences in the dispositions between the administration of the test chemical in the feed and by gavage.

(e) Housing conditions

The temperature and the relative humidity of the experimental animal rooms should be controlled and stable for the duration of the experiment. Where lighting is artificial, a constant light cycle should be used. Animals should be acclimatized to their environment prior to the experiment.

(f) Test chemical

Although studies may be done using mass balance measurements with "unlabelled" or "labelled" forms of the test chemical, use of radiochemically pure chemicals facilitates disposition studies. The use of radiochemicals allows easy measurement of the percentage of parent compound recovered and the recovery of its metabolites in the tissues, body fluids and excreta. However, measurement of radioactivity confirms only the presence of the radioisotope, not the chemical itself, or its metabolites. The conclusive identification of a chemical, and its metabolites, requires the use of analytical methods such as gas chromatography, mass spectrometry, nuclear magnetic resonance spectroscopy and liquid chromatography. The radiolabel (when appropriate, preferably ^{14}C) should be positioned in the chemical to provide the most information about the fate of the compound. When vehicles are used, attention must be given to the possibility that they may interfere with the kinetics of the test chemical.

4. Observations

(a) Absorption

The rate of absorption may be best estimated by determining the concentration of the chemical in the blood, plasma or serum at different times after exposure. In determining enteronepatic circulation of the test chemical, studies employing biliary cannulation may be necessary.

(b) Distribution

Concentrations of test chemical in the tissues and organs should be measured at the time of sacrifice. Samples of tissues (or organs) should be subjected to solvent extraction at different pHs using non-polar and polar solvents. These extracts are then assayed for the test compound and possible metabolites. In addition, useful information may be developed using radioautographic studies, and studies of reversible binding of the test chemical to plasma proteins.

(c) Metabolism

For determining the extent of biotransformation, urine samples and fecal extracts should be analyzed by suitable chromatographic techniques. Major metabolites of the chemical should be identified by appropriate methods. It is of importance to determine the metabolite pattern of the test chemical also after repeated dosages (i.e., at "steady state"). It can be advantageous to perform preliminary studies in-vitro to obtain information about the pathways of metabolism.

Incorporation of radioactivity into non-extractable tissue residues may occur via normal intermediary metabolism. Measurements of such incorporation can also indicate the formation of reactive intermediates (covalently, macromolecular-bound adducts).

Induction of metabolizing enzymes may alter the dispositions of the test chemical and thereby affect its toxicity. Thus, it may be helpful to establish the "enzyme-inducing" potential of the test chemical on various enzyme systems such as the cytochrome P-450 oxidation system.

Depletion of endogenous sulphydryl substances in organs such as liver and kidney provide an indication of conjugation of the test chemical with glutathione and related compounds. Knowledge of this depletion may be useful in evaluating toxic effects related to the formation of reactive intermediates.

(d) Excretion

When determining excretion of the test chemical by laboratory animals, the use of individual metabolism cages is recommended for collection of urine and fecal samples. The concentration of test chemical and major metabolites in urine, feces and in expired air should be measured several times after exposure until about 95% of the administered dose has been excreted, or until a constant rate of excretion has been reached.

5. Timing of study

The time at which it is best to do a disposition study varies with the need for data to evaluate the safety of the test chemical. In certain cases, the initial experiments for determining absorption, distribution and excretion of the test chemical may be done soon after the acute toxicological studies. Further experiments establishing the metabolic fate of the compound may be needed for chemicals which will likely undergo chronic testing. If the results of toxicological studies indicate that further information on the metabolism of the test chemical is needed, identification and characterization of major metabolites in blood and urine should be undertaken. For some purposes, dose-related disposition studies may be carried out using pregnant or nursing animals. A kinetic analysis makes it possible to assess the amount of placental transfer of the parent compound and its metabolites at critical periods of organogenesis in relation to maternal exposure.

The purpose of conducting the drug disposition studies in relation to subchronic toxicity studies is to develop information to aid in interpretation of the subchronic studies and to help design the chronic toxicity studies. For this reason, the disposition studies outlined in the above guidelines should be accomplished prior to the initiation of the long-term chronic toxicity studies.

Appendix III

Standards for Performance of Toxicity Studies

Appendix III
CORE STANDARDS

Core Standard for Acute Oral Toxicity Studies

1. Acute oral toxicity studies shall have been conducted in a mammalian species.

Core Standard for Short-Term Exposure Studies

1. Test Duration: Animals shall have been exposed for at least fourteen days with multiple exposures. Animals receiving the test substance by the oral route of exposure shall have been dosed at least 5 consecutive days per week for 2 consecutive weeks.
2. Animal Species: There are no restrictions as long as the test animals are healthy and from an identified mammalian species.
3. Age: Young adult animals should have been used.
4. Number of Animals: Each treatment group shall contain at least 4 rodents or 2 non-rodents of the same sex surviving at termination of the study.
5. Controls: A concurrent control group is not normally required. In the absence of a concurrent control information, attribution of effects to intercurrent disease is not acceptable.
6. Dose Group(s): At least two levels of the test substance shall have been used. A dose level should produce some toxicological effect, unless limited by the physical or chemical characteristics of the test substance. Only the test substance or vehicle shall have been administered to the animals.
7. Route of Administration: The test substance shall have been administered by the oral route. The oral route includes administration in the diet, drinking water, by capsules, or by stomach tube. All animals shall have been treated by the same method.
8. Data Requirements
 - a. Initial and final body weights shall have been reported.
 - b. Mortality shall have been reported.
 - c. Where possible, all animals shall have been subjected to gross necropsy.

Core Standard for Subchronic Oral Toxicity Studies

1. Test Duration: Period of treatment (exposure to the compound) shall have been at least 90 days. Animals receiving the test substance by oral route exposure shall have been dosed at least 5 consecutive days per week for approximately 13 consecutive weeks.
2. Animal Species: Healthy animals from an identified mammalian species shall have been used.
3. Age: Young adult animals should have been used.
4. Number of Animals: Each group shall have consisted of at least 5 animals per sex for rodents or 2 animals per sex for non-rodents at termination of the study.
5. Control Group(s): A concurrent control group is required.
6. Dose Group(s): At least two dose levels should have been used. A dose level should have produced some toxicological effect unless limited by the physical or chemical properties of the test substance. Only the test substance or vehicle should have been administered to the animals.
7. Route of Administration: The test substance shall have been administered by the oral route. The oral route includes administration in the diet, drinking water, capsules or by stomach tube. All animals shall have been treated by the same method.
8. Data Requirements
 - a. Initial and final body weights shall have been reported.
 - b. Mortality shall have been reported.
 - c. Erythrocyte and leukocyte counts shall have been performed.
 - d. Where possible all animals shall have been subjected to gross necropsy.
 - e. The liver, kidneys, and where present testes shall have been weighed for at least 5 animals per sex in the high dose and controls for rodents; for non-rodents, 2 animals per sex.
 - f. At least 5 animals/sex for rodents (for non-rodents, all animals) in the high dose and control shall have had the following tissues microscopically examined: liver, gonads, kidneys, spleen, stomach or intestine, and heart.

Core Standard for Chronic Oral Toxicity Studies in Rodents or Non-rodent Mammals

1. Test Duration: The period of treatment (exposure to the compound) should be at least 18 months for rodent oncogenicity studies and 6 months for rodent or dog toxicity studies. Animals receiving the test substance by oral exposure methods shall be dosed at least 5 days per week.
2. Age and Condition: Animals shall be healthy and the species shall be identified.
3. Number of Animals
 - a) Rodents: At least 10 rodents/sex/group shall have survived at least 18 months for oncogenicity studies and 6 months for toxicity studies and data from those animals shall be available for evaluation, except in the high dose group where compound related mortality may have occurred. If compound related toxicity occurred the high dose level need not have 10 animals/sex survive 18 months (6 months for toxicity).
 - b) Non-Rodents: At least 3 non-rodent mammals/sex group shall have survived at least 6 months for toxicity studies and data from those animals shall be available for evaluation, except in the high dose where compound related mortality may have occurred.
4. Control Group(s): A concurrent control group is required.
5. Dose Group(s): At least two dose levels shall be used. Where possible some dose levels shall have produced toxicological effects, unless limited by the physical or chemical characteristics of the test substance. Only the test substance or vehicle shall be administered to the animals.
6. Route of Administration: The test substance shall be administered by the oral route, which includes administration in the diet, in drinking water capsules, or by stomach tube. All animals shall be subjected to the same mode of administration.
7. Data Requirements
 - a. Initial, mid-study, and final body weights shall be reported.
 - b. Mortality shall be reported.

- c. At least 5 rodents/sex and 3 dogs/sex in the high dose and control groups shall have had the following determinations made after at least 6 months of dosing (18 months for carcinogenicity): erythrocyte and leukocyte counts, for both oncogenicity and toxicity studies, and hemoglobin levels for toxicity studies.
- d. Where possible, all animals shall be subjected to gross necropsy.
- e. The following organs, where present, shall be weighed for at least 5 animals/sex in the high-dose and control groups: liver, testes and kidneys.
- f. All tissues showing gross changes shall be examined microscopically.
- g. At least 10 rodent/sex (3 dogs/sex) in the high dose (if unforeseen mortality occurs the next dose level shall also be examined) and control shall have had the following tissues microscopically examined: liver, uterus, gonads, lungs, kidneys, spleen, stomach, intestine, adrenals, heart, pancreas, and thyroid. Tissues from other dose groups shall be examined if changes were observed in tissues from the high-dose group.

CURRENT STANDARDS

For the standards a nutrition deficit is defined as: A greater than 10 percent weight loss in the adjusted dietary controls vs basal dietary controls.

Current Standard for Acute Oral Toxicity Studies

1. Species: Young adult rats or mice of both sex are required.
2. Data Requirements: The test shall provide data that is sufficient to determine the slope of the mortality dose response curve and the LD₅₀ value with 95% confidence limits for both sexes.
3. Observation period: Animals shall be observed for a period of at least 14 days post dosing.

Current Standard for Short-Term Oral Exposure Studies

1. Test Duration: Animals shall be exposed to the test substance 7 days per week for 4 consecutive weeks.
2. Species: The rat, the mouse, or the dog shall be the species tested. Species other than the three mentioned above may be used, if adequate justification is available to demonstrate the appropriateness of that species. The strain of test animals used shall be identified.
3. Age: Dosing of rodents shall begin as soon as possible after weaning and acclimation and in any case before the animals are 6 weeks old. Dogs shall not be less than 3 months and not more than 6 months of age at initiation of dosing.
4. Number of Animals: For an acceptable study, data from at least 10 animals/sex/ group for rodents and 4 animals/sex/group for non-rodents shall be available for evaluation at termination of the study.
5. Control Group(s): A concurrent control group is required. When a carrier vehicle is used, it shall be added to the diet at a concentration similar to the maximum given in any dosage group. If there are insufficient data on the toxic properties of the vehicle used in administering the test substance, additional toxicity studies on the vehicle shall be conducted. In all other respects, the control group shall be handled and maintained in a manner identical to that used with the test groups. If more than 5% of the diet is being replaced, a control diet of equivalent nutritional value shall be provided.

6. Dose Group(s): Administration of test substance to animals at all dose levels shall be done concurrently. At least three dose levels shall be used. Where possible the highest treatment level (not to exceed 5% of the diet for non-nutritive additives) shall result in toxicological changes, unless prohibited by the physical/chemical or biological properties of the test substance. The lowest dosage level shall be one in which there is no observed toxicity.
7. Diet: A diet known to provide adequate nutrition for the species tested shall be used.
8. Route of Administration: The test substance shall be administered by the oral route, which includes administration in the diet, in drinking water, by stomach tube or in capsules, provided that all animals are treated by the same method. The doses shall be calculated on the basis of mg of test substance per kg body weight and adjusted weekly.
9. Observations of Animals: Toxicological and pharmacological signs shall be recorded daily. Where possible these shall include time of onset, intensity, and duration. Estimates shall be made of food consumption (or water consumption when the test substance is administered in the water) every week during the test, and the animals shall be weighed once a week.
10. Clinical Testing: The following determinations shall be made at the times indicated below for each type of testing. For rodents, these determinations shall be made on at least 5 animals of each sex in each group. For dogs, the measurements shall be made on all animals in the study.
 - a. Ophthalmological Examination: If changes in the eyes are detected, detailed examinations shall be conducted on all animals. In dogs, pretest examinations shall be performed.
 - b. Hematology: The following determinations shall be made at the end of the testing period: hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts. Measurement of intrinsic and extrinsic clotting potential shall be performed.
 - c. Clinical Chemistry: Blood chemistry determinations shall be made at the end of the testing period. Appropriate tests that assess electrolyte balance, carbohydrate metabolism, liver function, and kidney function shall be performed.

11. Gross Necropsy:

- a. All test animals shall be subjected to a complete gross necropsy.
- b. All tissues listed under Histopathological Examination shall be saved from all animals in the study.
- c. No more than 10% of a tissue of any group shall be lost to autolysis, cannibalism or management problems.

12. Organ Weights: Organs that shall be weighed include: liver, kidneys, adrenals and testes.

13. Histopathological Examination

- a. All gross lesions
- b. For rodents, tissues from all animals in the control and high-dose group shall be examined. If changes are seen in any of the examined tissues, then the same tissues from all animals in the lower dose groups shall be examined. The following tissues are required and shall be examined: liver, kidneys, spleen, heart, adrenals, ovaries, uterus, thyroid/parathyroid, bone marrow, testes, lungs (with mainstem bronchi), stomach, small and large intestine, pancreas, lymph node, and brain.
- c. For non-rodents, the above tissues from all animals shall be examined.

Current Standard for Subchronic Oral Exposure Studies

1. Test Duration: Animals shall be exposed to the test substance for at least 90 consecutive days.
2. Species: The rat, the mouse or the dog shall be the species tested. The rat and the dog are the species of choice. Species other than the three mentioned above may be used, if adequate justification is available to demonstrate the appropriateness of that species. The strain of test animals used shall be identified.
3. Age: Dosing of rodents shall begin as soon as possible after weaning and acclimation and in any case before the animals are 6 weeks old. Dogs shall not be less than 3 months and not more than 6 months of age at the initiation of dosing.
4. Number of Animals: For an acceptable study, data from at least 20 animals/sex/group for rodents and 4 animals/sex/group for non-rodents shall be available for evaluation.
5. Control Group(s): A concurrent control group is required. When a carrier is used, it shall be added to the diet at a concentration similar to the maximum given in any dosage group. If there are insufficient data on the toxic properties of the vehicle used in administering the test substance, an additional toxicity study on the vehicle shall be conducted. In all other respects, the control group shall be handled and maintained in a manner identical to that used with the test groups. If more than 5% of the diet is being replaced, then a control diet of equivalent nutritional value is needed.
6. Dose Group(s): Administration of test substance to animals at all dose levels shall be done concurrently. At least three dose levels shall be used. Where possible the highest treatment level (not to exceed 5% of the diet for non-nutritive additives) shall result in toxicological changes unless prohibited by the physical/chemical characteristics of the test substance. The lowest dosage level shall be one in which there is no observed toxicity.
7. Diet: A diet known to provide adequate nutrition for the species tested shall be used.
8. Route of Administration: The test substance shall be administered by the oral route. The test substance shall be administered to the animals in the diet, in drinking water, by gavage, or in capsules, provided that all animals are treated by the same method. The doses shall be calculated on the basis of mg of test substance per kg of body weight.

9. Observation of Animals: Toxicological and pharmacological signs shall be recorded daily. These shall include time of onset, intensity, and duration. Estimates shall be made of food consumption (or water consumption when the test substance is administered in the water) every week during the test, and the animals shall be weighed weekly.
10. Clinical Testing: The following determinations shall be made at the times indicated below for each type of testing. For rodents, these determinations shall be made on at least 10 animals of each sex in each group. For dogs, the measurements shall be made on all animals in the study.
 - a. Ophthalmological Examination: If changes in the eyes are detected, detailed examinations shall be conducted on all animals. In dogs, pretest examinations shall be performed.
 - b. Hematology: The following determinations shall be made at the end of the testing period: Hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts. Measurement of clotting potential such as clotting time, prothrombin time, thromboplastin time, or platelet count shall be performed.
 - c. Clinical Chemistry: Blood chemistry determinations shall be done at the end of the testing period. Appropriate tests that assess electrolyte balance, carbohydrate metabolism, liver function, and kidney function shall be performed.
11. Gross Necropsy
 - a. All test animals shall be subjected to a complete gross necropsy.
 - b. All tissues listed under Histopathological Examination shall be saved from all animals in the study.
 - c. No more than 10% of a tissue of any group shall be lost to autolysis, cannibalism or management problems.
12. Organ Weights: Organs where present that shall be weighed include the liver, kidneys, thyroid (dog), adrenals, and testes.
13. Histopathological Examination
 - a. For rodents, all gross lesions from all animals shall be examined microscopically. The tissues listed below for all animals in the control and high-dose group as well as

in all animals from other dose groups that died during the study shall be examined microscopically. In addition, if changes are seen in any of the examined tissues, then the same tissues from all animals in the lower dose groups shall be examined.

b. For non-rodents, all gross lesions and tissues listed below shall be examined microscopically for all animals in the study.

c. The following principal tissues shall be examined:

Adrenals	Pituitary
Aorta	Prostate
Bone Marrow	Representative Lymph Node
Brain (2 levels)	Salivary Gland
Cortical	Sciatic Nerve with Skeletal Muscle
Cerebellum with brain stem	Small and Large Intestine
Esophagus	Spinal Cord (2 levels)
Eye	Thoracic
Gall Bladder (if present)	Lumbar
Gonads	Spleen
Heart	Stomach
Kidneys	Thymus
Liver	Thyroid/Parathyroid
Lungs (with mainstem bronchi)	Trachea
Mammary Gland	Urinary Bladder
Pancreas	Uterus

The compound being tested may necessitate the examination of other tissues.

Current Standard for Chronic Oral Exposure Studies, Excluding
Carcinogenicity Assessment

1. Test Duration: Animals shall be exposed to the test substance for at least 365 consecutive days.
2. Species: The rat, the mouse or the dog shall be the species tested. The rat and the dog are the species of choice. Species other than the three mentioned above may be used if adequate justification is available to demonstrate the appropriateness of that species. The strain of test animals used shall be identified.
3. Age: Dosing of rodents shall begin as soon as possible after weaning and acclimation and in any case before the animals are 6 weeks old. Dogs shall not be less than 3 months and not more than 6 months of age at initiation of dosing.
4. Number of Animals: For an acceptable study, data from at least 20 animals/sex for rodents and 4 animals/sex for non-rodents for the control, lowest, and intermediate dose groups shall be available for evaluation at terminal sacrifice.
5. Control Group(s): A concurrent control group is required. When a carrier vehicle is used, it shall be added to the diet at a concentration similar to the maximum given in any dosage group. A vehicle of unknown toxicological properties shall not be used. If a vehicle is employed, then a vehicle control group shall be incorporated into the study. In all other respects, the control group shall be handled and maintained in a manner identical to that used with the test groups. If more than 5% of the diet is being replaced, then a control diet of equivalent nutritional value is needed.
6. Dose Group(s): At least three dose levels shall be used. The highest treatment level shall result in toxicological changes unless prohibited by the physical or chemical characteristics of the test substance. The lowest dosage level shall be one in which there is no evidence of toxicity. Administration of the test substance to animals at all dose levels shall be done concurrently.
7. Diet: A diet known to provide adequate nutrition for the species tested shall be used.

8. Route of Administration: The test substance shall be administered by the oral route. The test substance shall be administered to the animals in the diet, in drinking water, by stomach tube, or in capsules, provided that all animals are treated by the same method. The doses shall be calculated on the basis of mg of test substance per kg of body weight and adjusted weekly.
9. Observations of Animals: Toxicological signs shall be recorded daily. Where possible, these shall include time of onset, intensity, and duration. Estimates shall be made of food consumption (or water consumption when the test substance is administered in the water) every week during the test, and the animals shall be weighed every week.
10. Clinical Testing: The following determinations shall be made at the times indicated below for each type of testing. For rodents, these determinations shall be made on at least 10 animals of each sex in each group. For dogs, the measurements shall be made on all animals in the study.
 - a. Ophthalmological Examination: If changes in the eyes are detected, detailed examinations shall be conducted on all animals. In dogs, pretest observations shall be conducted.
 - b. Hematology: The following determinations shall be made at termination and at least once during the study after 90 days of exposure and at least 6 months prior to termination: Hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts and a measure of clotting potential such as clotting time, prothrombin time, thromboplastin time, or platelet count.
 - c. Clinical Chemistry: Blood chemistry determinations shall be done at termination and at least once during the study after 90 days of exposure and at least 6 months prior to termination and at termination. Appropriate tests that assess electrolyte balance, carbohydrate metabolism, liver function, and kidney function shall be performed.
12. Gross Necropsy
 - a. All test animals shall be subjected to a complete gross necropsy.
 - b. All tissues listed under Histopathological Examination shall be saved from all animals in the study.

- c. No more than 10% of the tissues of any group shall be lost to autolysis, cannibalism or management problems.

13. Organ Weights: Organs that shall be weighed include liver, kidneys, thyroid (non-rodent), adrenals and testes.

14. Histopathological Examination

- a. For rodents, all gross lesions from all animals shall be examined microscopically. The tissues listed below shall be examined microscopically for all animals in the control and high-dose group as well as in all animals from other dose groups that died during the study. In addition, if changes are seen in any of the examined tissues, then the same tissues from animals in the lower dose groups shall be examined.
- b. For non-rodents, all gross lesions and the tissues listed below shall be examined microscopically for all animals in the study.
- c. The following principal tissues shall be examined:

Adrenals	Pituitary
Aorta	Prostate
Bone	Salivary Gland
Bone Marrow	Sciatic Nerve with Skeletal Muscle
Brain (at least 3 levels)	Seminal vesicles
Eye	Small and Large Intestine
Esophagus	Spinal Cord
Gall Bladder (if present)	Thoracic
Heart	Lumbar
Kidneys	Spleen
Liver	Stomach & Fore Stomach
Lungs (with mainstem bronchi)	Testes
Lymph Node	Thymus
Mammary Gland	Thyroid/Parathyroid
Ovaries	Trachea
Pancreas	Urinary Bladder
	Uterus

The compound being tested may necessitate the examination of other tissues.

Current Standard for Oral Carcinogenicity Studies in the Rodent

1. Study Duration: Animals shall be exposed to the test substance 7 days per week for at least 104 consecutive weeks. Exposure periods longer than 130 weeks are not recommended.
2. Species: The rat and mouse are the species of choice, although other species may be used if adequate justification is available to demonstrate the appropriateness of that species. The strain of test animals used shall be identified.
3. Age: Dosing of rodents shall begin as soon as possible after weaning and acclimation and in any case before the animals are 6 weeks old.
4. Number of Animals
 - a. Number at risk: At least 50 animals/sex/group shall be started in the study for an acceptable negative study.
 - b. Survival: Data from at least 25 animals/sex/group exposed for 2 years shall be available for evaluation unless compound-related toxicity occurs in the high-dose group. In that event, only 10 animals/sex in the high dose group are required to have 18-month data available.
5. Control Group(s): A concurrent control group is required. The control group shall be given only the carrier vehicle used in administering the test substance. A carrier of unknown carcinogenic potential shall not be used. In all other respects, the control group shall be handled and maintained in a manner identical to that used with the test groups. If, when the test substance is given, more than 5% of the diet is being replaced, then a control diet of equivalent nutritional value is needed.
6. Dose Group(s): At least 3 doses shall be included in the study. Under most circumstances, the high dose shall elicit signs of minimal toxicity without substantially altering the normal life span of the test species due to effects other than tumor formation. The low dose used in the study shall not induce evidence of compound-related toxicity other than tumors. No dose level of the test substance shall exceed 5% of the total diet for non-nutritive additives. Nutritive additives may be fed up to 20% of the diet provided that it does not cause a significant nutritional deficit.

7. Diet: A diet known to provide adequate nutrition for the species tested shall be used.
8. Route of Administration: The test substance shall be administered by the oral route. The test substance shall be administered to the animals in the diet, in drinking water, or by stomach tube provided that all animals are treated by the same method. The doses shall be calculated on the basis of mg of test substance per kg of body weight and adjusted weekly.
9. Observations of Animals: Throughout the test period, each animal shall be observed at least once daily. Animal weight, food consumption and mortality observations shall be reported at least once per week. All signs of behavioral abnormalities or clinical signs of toxicity or pharmacological effects, morbidity, and mortality, shall be recorded.
10. Hematology: The following determinations shall be made at 12, 18, and 24 months on at least 25 animals/sex/dose group: erythrocyte count, total and differential leukocyte counts. Where possible the same animal should be sampled at each time. If changes are seen at each subsequent time period, all animals shall be studied.
11. Gross Necropsy
 - (a) All test animals shall be subjected to complete gross necropsy, which shall include examination of the external surface, orifices, tongue, teeth, cranial cavity, the external and cut surfaces of the brain, spinal cord, and the abdominal, thoracic, and cervical viscera.
 - (b) All tissues listed under Histopathological Examination (sec. 12) shall be saved from all animals in the study.
 - (c) No more than 10% of the tissues of any group shall be lost to autolysis, cannibalism, or management problems.
12. Histopathological Examination
 - a) All gross lesions from all animals shall be examined microscopically.
 - b) The liver, kidney, and lungs with mainstem bronchi of all animals shall be subjected to microscopic examination.

- c) In addition, the following tissues shall be subjected to microscopic examination in all high-dose and control animals.

Adrenals	Pituitary
Aorta	Prostate
Bone	Rectum
Bone Marrow	Representative Lymph Nodes
Brain (a least 3 levels)	Salivary Gland
Cecum	Seminal Vesicles
Colon	Skeletal Muscle
Corpus and Cervix Uteri	Spinal Cord (2 levels)
Duodenum	Spleen
Esophagus	Stomach and Fore Stomach
Eye and Contiguous Harderian Gland	Testes
Exorbital Lacrimal Glands	Thymus (if present)
Gall Bladder (if present)	Thyroid/Parathyroid
Heart	Trachea
Ileum	Urinary Bladder
Jejunum	Vagina
Mammary Gland	Zymbals Gland (if present)
Nasal Turbinates	
Ovaries and Fallopian tubes	
Pancreas	
Peripheral Nerve (sciatic)	

If changes are seen in any of these tissues, then those tissues from all animals in the other dose groups shall be examined. Likewise, if changes are observed in any tissue of an organ system, then the other tissues of that organ system shall be subjected to microscopic examination in all animals.

Current Standard for Combined Chronic Toxicity and Carcinogenicity
Studies in the Rodent

1. Study Duration: Animals shall be exposed to the test substance 7 days per week for at least 104 consecutive weeks. Exposure periods longer than 130 weeks are not recommended.
2. Species: The rat and mouse are the species of choice, although other species may be used if adequate justification is available to demonstrate the appropriateness of that species. The strain and source of the test animals used shall be identified.
3. Age: Dosing of rodents shall begin as soon as possible after weaning and acclimation and in any case before the animals are 6 weeks old.
4. Number of Animals
 - a. Number at risk: At least 50 animals/sex/group shall be started in the study for an acceptable negative study.
 - b. Survival: Data from at least 25 animals/sex/group exposed for 2 years shall be available for evaluation unless compound-related toxicity occurs in the high-dose group. In that event, only 10 animals/sex in the high-dose group are required to have 18-month data available.
 - c. For an acceptable chronic toxicity study, data from at least 10 animals/sex for the satellite (1 yr interim sacrifice) control, low and intermediate dose groups shall be available for evaluation.
5. Control Group(s): A concurrent control group is required. The control group shall be given only the carrier vehicle used in administering the test substance. A vehicle of unknown toxicological/carcinogenic potential shall not be used. In all other respects, the control group shall be handled and maintained in a manner identical to that used with the test groups. If, a test substance is given, which replaces more than 5% of the diet, then a control diet of equivalent nutritional value is needed.
6. Dose group(s):
 - a. Carcinogenicity phase: At least 3 doses shall be included in the study. Under most circumstances, the high dose shall elicit signs of minimal toxicity without substantially altering the normal life span of the test species due to effects other than tumors. The low dose used in the study shall not induce evidence of

induce evidence of compound-related toxicity. Normally no dose level of the test substance shall exceed 5% of the total diet for non-nutritive additives. Nutritive additives may be fed up to 20% of the diet provided they do not cause significant nutritional deficit.

- b. Chronic toxicity satellite phase: At least 3 doses shall be included in the satellite study. Under most circumstances, the high dose shall result in toxicological changes unless prohibited by the physical or chemical characteristics of the test substance. The lowest dosage level shall be one in which there is no evidence of toxicity.
- 7. Diet: A diet known to provide adequate nutrition for the species tested shall be used.
 - 8. Route of Administration: The test substance shall be administered by the oral route. The test substance shall be administered to the animals in the diet, in drinking water, by stomach tube, or in capsules provided that all animals are treated by the same method. The doses shall be calculated on the basis of mg of test substance per kg of body weight and adjusted weekly.
 - 9. Observations of Animals: Toxicological signs shall be recorded daily. These shall include time of onset, intensity, and duration. Estimates shall be made of food consumption (or water consumption when the test substance is administered in the water) every week during the test, and the animals shall be weighed every week.
 - 10. Clinical Testing: The following determinations shall be made at the times indicated below for each type of testing. For rodents, these determinations shall be made on at least 10 animals of each sex in each group.
 - a. Ophthalmological Examination: If changes in the eyes are detected, detailed examinations shall be conducted on all animals.
 - b. Hematology: The following determinations shall be made at termination and at least once during the study after 90 days of exposure and at least 6 months prior to termination: Hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte count, and a measure of clotting potential such as clotting time, prothrombin time, partial thromboplastin time, or platelet count.
 - c. Clinical Chemistry: Blood chemistry determinations shall be done at termination and at least once during the study after 90 days of exposure and at least 6 months prior to termination. Appropriate tests that

assess electrolyte balance, carbohydrate metabolism, liver function, and kidney function shall be performed.

11. Gross Necropsy

- a. All test animals shall be subjected to a complete gross necropsy.
- b. All tissues listed under Histopathological Examination (sec. 13) shall be saved from all animals in the study.
- c. No more than 10% of any group of tissue shall be lost to autolysis cannibalism, or management problems.

12. Organ Weights: Organs that shall be weighed include the brain, liver, kidneys, adrenals and gonads.

13. Histopathological Examination

- a. All gross lesions from all animals shall be examined microscopically.
- b. The liver, kidney, lungs (with mainstem bronchi) of all animals shall be subjected to microscopic examination.
- c. In addition the following tissues shall be subjected to microscopic examination in all high-dose and control animals.

Adrenals
Aorta
Bone
Bone Marrow
Brain (a least 3 levels)
Cecum
Colon
Corpus and Cervix Uteri
Duodenum
Esophagus
Eye and Contiguous Harderian Gland
Exorbital Lacrimal Glands
Gall Bladder (if present)
Heart
Ileum
Jejunum
Mammary Gland
Nasal Turbinates
Ovaries and Fallopian tubes

Pancreas
Peripheral Nerve (sciatic)
Pituitary
Prostate
Rectum
Representative Lymph Nodes
Salivary Gland
Seminal Vesicles
Skeletal Muscle
Spinal Cord (2 levels)
Spleen
Stomach and Fore Stomach
Testes
Thymus (if present)
Thyroid/Parathyroid
Trachea
Urinary Bladder
Vagina
Zymbals Gland (if present)

If changes are seen in any of these tissues, then tissues from animals in the other dose groups shall be examined. Likewise, if changes are observed in any tissue of an organ system, then the other tissues of that organ system shall be subjected to microscopic examination in all animals.

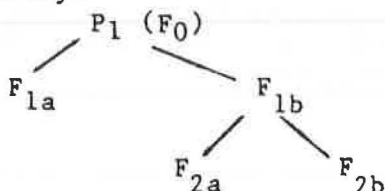
Current Standard for Teratology Studies (including teratology phases of reproduction studies)

1. Duration: Dosing shall begin soon after implantation and continue through the period of organogenesis. All dams surviving until the day prior to normal delivery shall be sacrificed.
2. Species: Mammalian species shall be used. Strains with low fecundity are not recommended. The strain and source of test animals used shall be identified.
3. Age and Parity: All test and control animals shall be young, mature, prima gravida females. Untreated adult males shall be used to induce the pregnancies.
4. Number of Animals: Each group shall consist of at least 20 pregnant rodents or at least 12 pregnant rabbits.
5. Control Group(s): A concurrent control group shall be used. When the test substance is administered in a vehicle, only the vehicle shall be administered to the controls. A vehicle of unknown teratogenic potential shall not be used. If no vehicle is used, then the controls shall be sham treated.
6. Dose Group(s): At least three dose groups shall be used unless limited by the physical or chemical nature or the biological effects of the compound. The highest dose level shall either induce overt maternal toxicity or affect fetal development. The lowest dose shall produce no fetal toxicity or abnormalities.
7. Route of Administration: For teratology phase of reproduction studies the test substance shall be administered in the diet or drinking water unless the physical characteristics or pattern of human exposure suggests a more appropriate method of oral administration such as by stomach tube. For separate teratology studies the test substance shall be orally administered by a stomach tube unless the physical characteristics or pattern of human exposure suggests a more appropriate method.
8. Diet: A diet known to provide adequate nutrition for the species tested shall be used.
9. Observation: Throughout the test period, each animal shall be observed at least once daily. Pertinent behavioral changes and all signs of toxicity, including mortality, shall be recorded. Any female showing signs of abortion or premature delivery shall be sacrificed on the day such signs are observed. Females shall be weighed at least at the start and termination of substance administration, and at the time of sacrifice.

10. Necropsy: Immediately after a female is sacrificed, the ovaries and uterus shall be excised and examined for corpora lutea, embryonic or fetal deaths, and the number of live fetuses, and these data shall be recorded. The fetuses shall be examined externally, weighed individually, and weights recorded. For rodents all fetuses shall be examined either for soft tissue or skeletal abnormalities. For non-rodents all fetuses shall be examined for both soft tissue and skeletal abnormalities.

Current Standard for Reproduction Studies

The following is a schematic diagram of the sequence of mating for a two-generation reproduction study.



1. Duration: Exposure to the test substance shall be continued until weaning of the F₂ generation. Dosing of the P₁ males and females shall begin as soon as possible after weaning and acclimation: Dosing of females shall be initiated at least 2 weeks prior to mating. Dosing of males shall be initiated at least 10 weeks prior to mating. At initiation The P₁ females shall be nulliparous.
2. Species: The rat is the species of choice. Other species may be used if adequate justification is available to demonstrate the appropriateness of the selected species. The strain of test animal used shall be identified.
3. Number of Animals: For each mating, one female shall be placed daily with the same male from the same dose group until pregnancy occurs or 3 weeks have elapsed. The matings of each test and control group animals shall result in approximately 20 pregnant dams per group. At least one male and one female from each litter and no more than two males and two females from a litter shall be used to produce the next generation. Sibling matings shall be avoided.
4. Control Group(s): A concurrent control group is required. If a vehicle is used in administering the test substance, the control group shall receive the vehicle. A vehicle that is known to produced no effects on reproduction shall be used. The control group shall be handled and maintained in a manner identical to that used in the treatment groups.
5. Dose Group(s): A least three dosage levels shall be tested in addition to the control. Unless limited by the physical/chemical nature or biological effects of the test substance, the highest dose level shall ideally induce toxicity, but not mortality, in the P₁ animals. In actual practice, mortality is often encountered and attainment of this ideal may be difficult. The low dose shall not induce any adverse effects.
6. Diet: A diet known to provide adequate nutrition for the species tested shall be used.

7. Route of Administration: The test substance shall be administered by the oral route. The test substance shall be administered in the diet, drinking water, or by gavage. The method of administration shall be the same for the control and test groups.
8. Number of Litters: When two litters are produced the second mating shall be at least 10 days after weaning of the previous litter.
9. Disposition of each generation and litter
 - a. P₁ sacrifice and gross necropsy after weaning of the F_{1b};
 - b. F_{1a} sacrifice and gross necropsy after weaning;
 - c. F_{1b} mated after 70 days of age to produce the F_{2a&b}. The F_{1b} parents are sacrificed on the day prior to normal delivery of the F_{2b} litter. The handling of the F_{1b} females and the F_{2b} litter follow the standard set forth under teratology.
 - d. ~~At weaning the F_{2a} litter shall be sacrificed, and gross necropsied.~~
10. Culling: At day 4 after birth each litter, where possible, shall be randomly culled to 10 animals. No culling is also acceptable.
11. Observation: Throughout the test period, each animal shall be observed at least once daily. Pertinent behavioral changes, food consumption, and all signs of toxicity, including mortality, shall be recorded. These observations shall be reported individually for each animal.

Information Requirements:

- a. P₁ males and females selected shall be weighed on the first day of dosing and weekly thereafter.
 - b. F_{1a} and b males and females shall be weighed at birth and on day 4, (before and after culling) and 21 and weekly thereafter.
 - c. F_{2a} and b males and females shall be weighed at birth and on day 4 (before and after culling) and 21 after birth.
 - d. The sex of each (all) pups shall be determined and recorded at birth, day 4 (before and after culling) and at weaning.
12. Gross Necropsy: All animals shall be subjected to a complete gross necropsy.

13. Histopathology: The vagina, uterus, ovaries, testes, epididymes seminal vesicles, prostate, and target organs of all P and F₁ animals selected for mating shall be preserved for microscopic examination. All tissues in these animals showing gross pathological changes shall be examined. If these organs have not been examined microscopically in other multiple dose studies, they shall be examined in all high dose and control animals in this study. If changes are seen in any of the examined tissues, then the same tissues from the other P and F₁ animals selected for mating in the other dose groups shall be examined.

CURRENT STANDARDS FOR TEST DATA REPORTING

- A. Identification of Responsible Parties: Each test report shall be signed by the person responsible for the test and identify:
1. The laboratory where the test was performed by name and address;
 2. The inclusive dates of the test; and
 3. Each person primarily responsible for separate components of the test and the component for which the person is responsible including (a) the conduct of the test, (b) analysis of the data, (c) the writing of the report, and (d) any written or other matter contained in the report.

B. Body of Report

The test report shall include all information necessary to provide a complete and accurate description and evaluation of the test procedures and results. Each report must include the following sections:

1. Summary and Conclusions: This section of the test report should contain a summary of the data, an analysis of the data, and a statement of the conclusions drawn from the analysis. The summary must highlight all positive data or observations and any deviations from control data which may be indicative of toxic effects.
2. Materials: This section of the report shall include, but not be limited to, the following information:
 - (a) Identification of the test substance, including:
 - i. chemical name, molecular structure, and a qualitative and quantitative determination of its chemical composition, including names and quantities of known contaminants and impurities, so far as is practical. The determination shall also include a listing of materials as unknowns, if any, so that the entire test sample is accounted for;
 - ii. manufacturer and lot number and physical properties of the substance tested, and such information as physical state, pH, stability, and purity; and
 - iii. exact identification of diluents, suspending agents, emulsifiers, or excipients, or other materials used in administering the test substance.

(b) Animal data, including:

- i. species and strain used and rationale for selection of the strain if other than a common laboratory strain;
- ii. source of supply of the animals, diet (lot number, composition, etc.), and water;
- iii. description of any pre-test conditioning;
- iv. a description of the method used in randomization of animals to test or control groups; and
- v. numbers of animals of each sex in each test and control group.

(c) Data on facilities should include a description of the caging conditions, including: number of animals per cage, bedding material, ambient temperature, humidity, and lighting conditions.

3. Methods

- (a) Deviation from guidelines: This section should indicate all ways in which the test procedure deviates from these guidelines and state the rationale for such deviation.
- (b) Specification of test methods: This section should include a full description of the experimental design and procedures, the length of the study, and dates the study began and ended.
- (c) Statistical analysis: All statistical methods used should be fully described or identified by reference.
- (d) Data on dosage administration, including:
 - i. all dose levels administered, expressed as mg/kg of body weight and
 - ii. the method and frequency of administration.
- (e) Data on observation methods, including:
 - i. duration; and
 - ii. the method and frequency of observation of the animals.

4. Results

The tabulation of data and individual results must accompany each report in sufficient detail to permit independent evaluation of results, including summaries and tables that show, when appropriate, relationship of effects to time of dosing, sex, etc.

- (a) Data presented for each animal shall include: hematology, clinical chemistry, and other tests performed, a description of all toxicological or pharmacological effects and abnormalities, accompanied by the animal's identification number, test group (dose level and sex), and days of study when the signs appeared and disappeared. When numerical averages are presented, they should be accompanied by an appropriate measure of variability, such as the standard error. All animals placed on study must be accounted for.
- (b) Findings from all clinical observations, necropsy, and histopathological examinations: Special attention should be given to an attempt to correlate clinical observations made during the course of a study to post mortem findings.
- (c) Evaluation of data: an evaluation of test results, including their statistical analysis, shall be made and supplied, based on the clinical findings, the gross necropsy findings, and the histopathological results. This should include an evaluation of the relationship, or lack thereof, between the animal's exposure to the test substance and the incidence and severity of all abnormalities, tumors and other lesions, organ weight effects, effects on mortality, and any other general or specific toxic effects.

5. References

This section of the test report should include the following information:

- (a) Availability and location of all original data, specimens, and samples of the test substances which are retained in accordance with the testing requirements.
- (b) Literature or references, including, where appropriate, those references for (1) test procedures, (2) statistical and other methods used to analyze the data, (3) compilation and evaluation of results, and (4) the basis upon which conclusions were reached.

IV. Suggested Reading

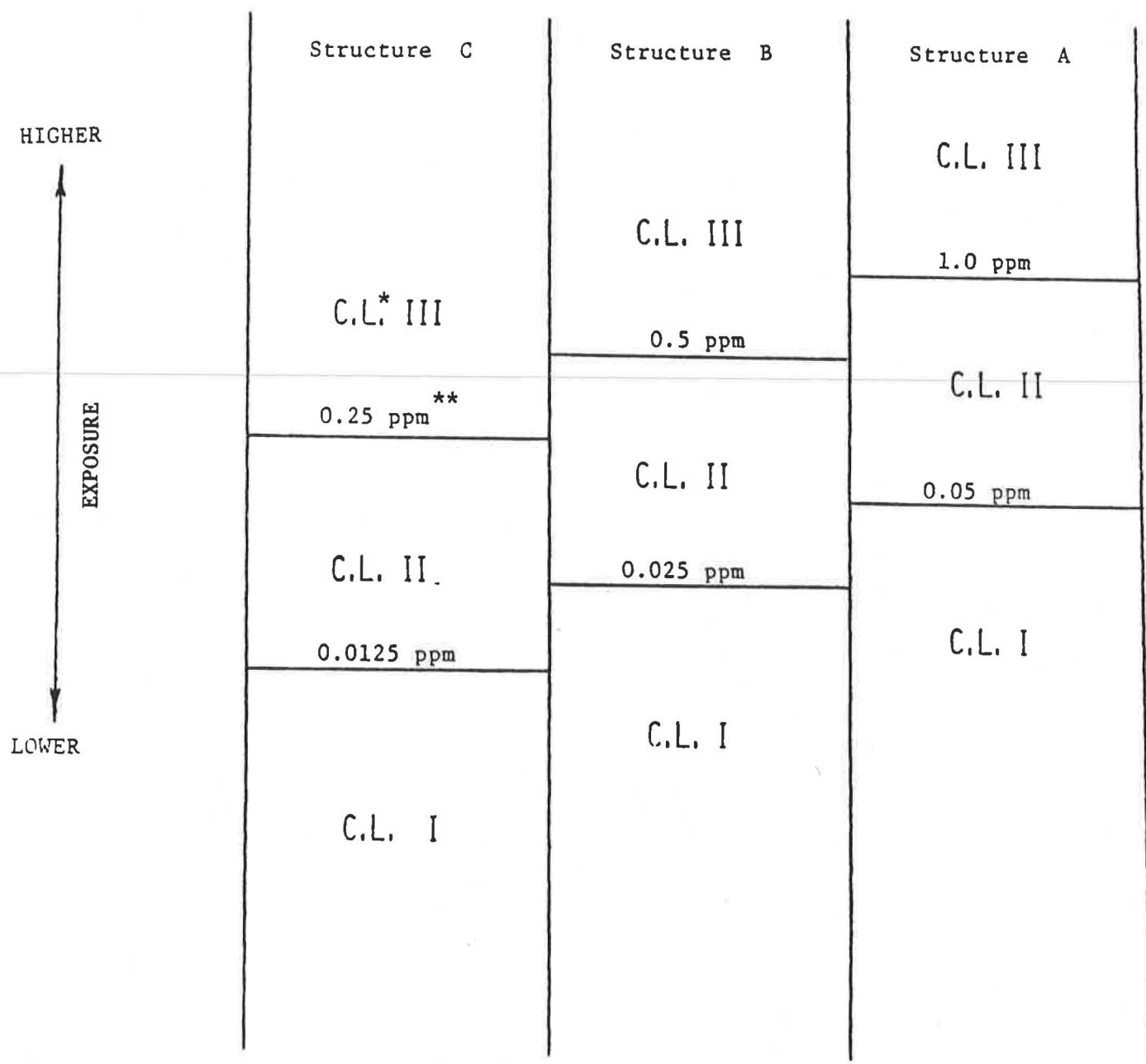
1. Brodie, B. B. 1964. Of Mice, Microsomes, and Man. The Pharmacologist 6:12-26.
2. Committee for the Revision of NAS publication 1138, Committee on Tox., Nat'l. Res. Counc., Nat'l. Acad. Sci. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. pp. 1-22, 74-85, and 130. Prepared for the Consumer Prod. Safety Comm. Nat'l. Acad. Sci.: Washington, D. C.
3. Cornfield, J. 1954. Measurement and Comparison of Toxicities: The quantal response. In Statistics and Mathematics in Biology pp 327-334. Ed. by O. Kempthorne, T. A. Barieroft, J.W. Gowen, and J.L. Lush. Iowa State College Press: Ames.
4. FAO/WHO Expert Committee on Food Additives. 1958. Procedures for the testing of intentional food additives to establish their safety for use. Wld. Hlth. Org. Tech. Rep. Ser. No. 114, pp. 11-17.

Appendix IV

Figures

FIGURE 1

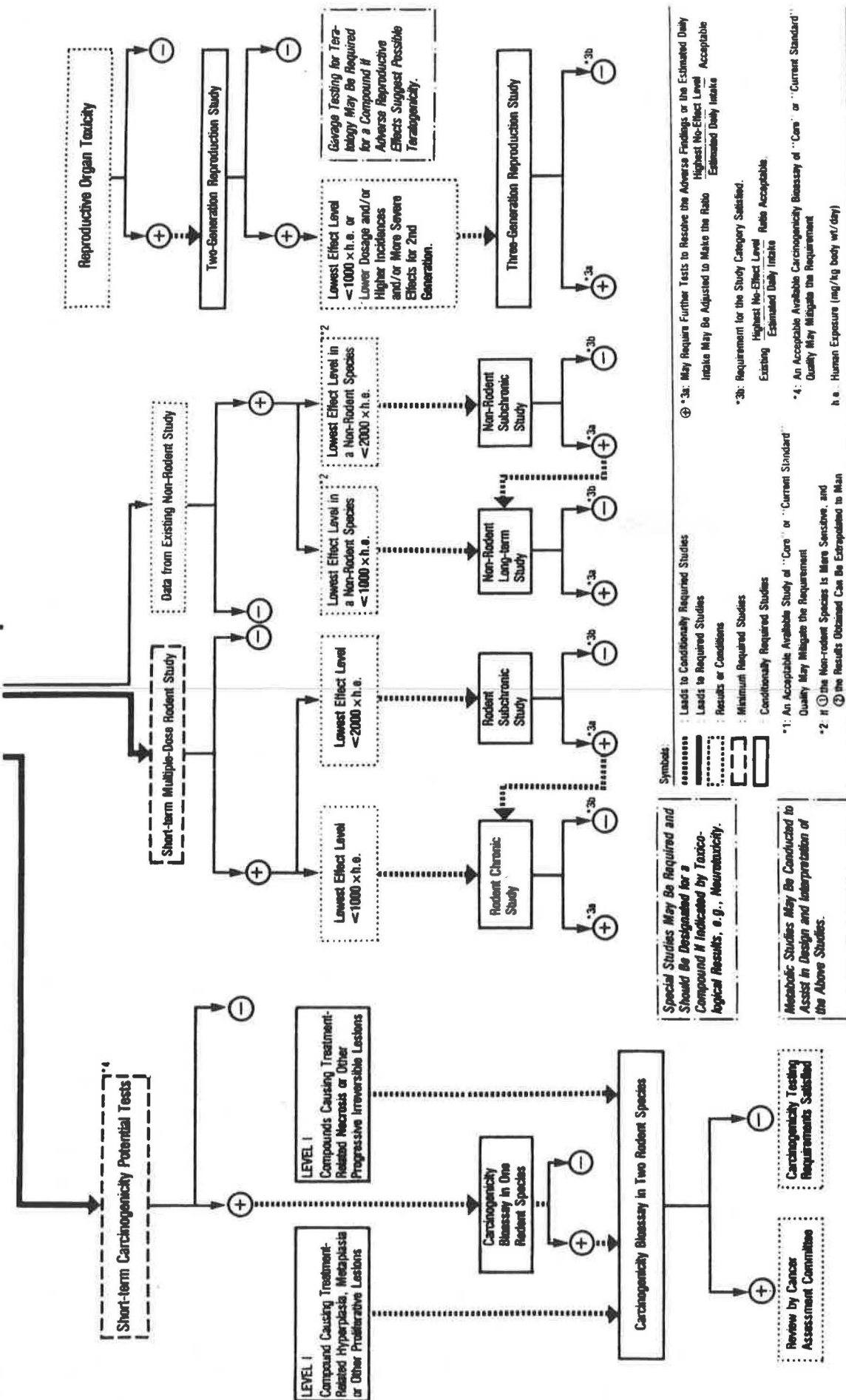
CONCERN LEVEL from EXPOSURE AND STRUCTURE



* C.L. = Concern Level

** ppm = parts per million dietary exposure to the additive

Concern Level 1 Compound

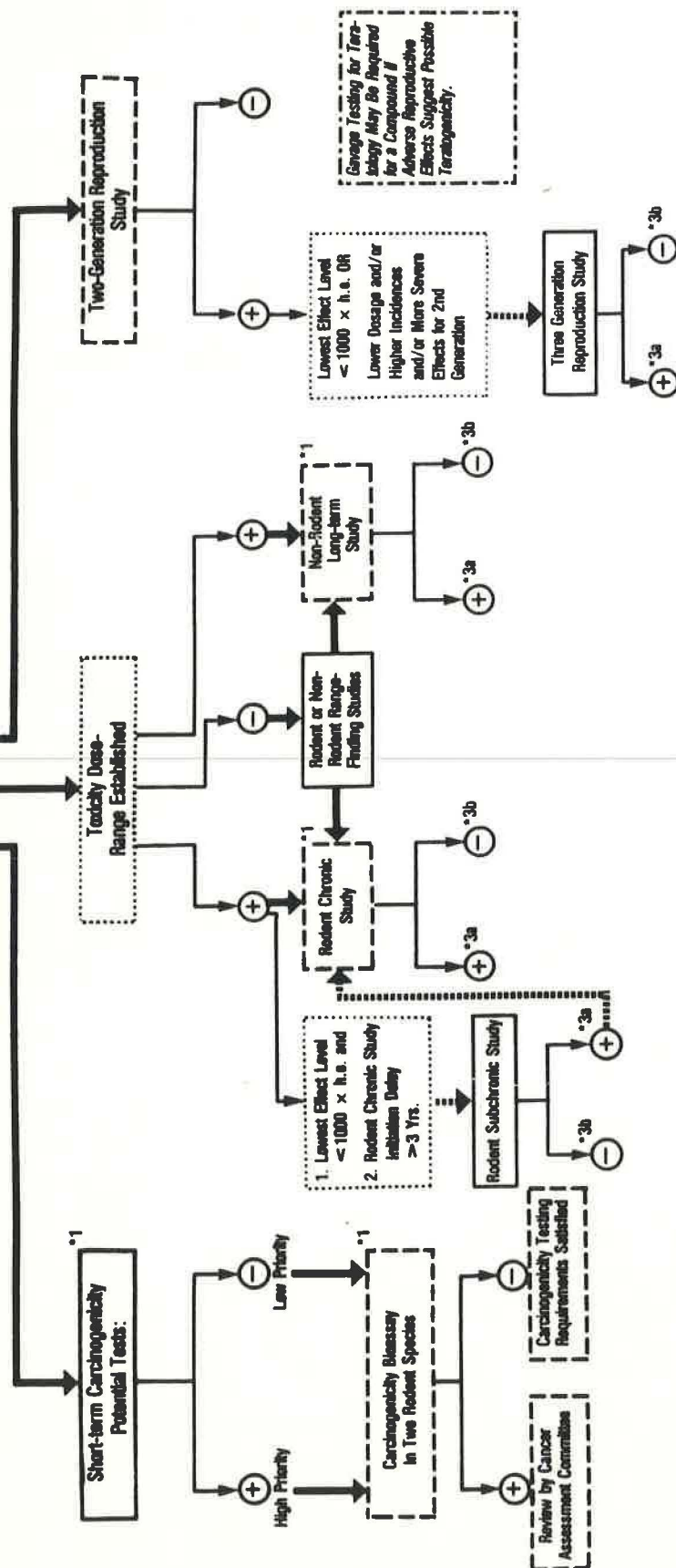


Concern Level II Compounds



FIGURE 4

Concern Level III Compounds



Synonyms:

Special Studies May Be Required and Should Be Designated for a Compound if Indicated by Toxicological Results, e.g., Neurotoxicity.

Metabolic Studies May Be Conducted to Assist in Design and Interpretation of the Above Studies.

LEARNING OBJECTIVES

rapidly changing of speed

RESULTS OF COMPARISONS

Minimum Required Studies

☐ : Conditionally Required Studies

1. An Acceptable Available Study of "Care" or "Current Standard"

Quality May Minimize the Requirement

Quantity may influence the performance

④ the Results Obtained Can Be Extrapolated to Man

Ⓢ 39 New Benjamin Further Tests to Resolve the Adverse Findings of the Estimated Daily

Highest No-Effect Level **Acceptable**

Estimated Daily Intake

a 2b. Determined for the Study: Category C: 1 had

- 30: Modification for the study sampling system.

Existing	Highest No-Effect Level	Ratio Acceptable
Estimated Daily Intake	Estimated Daily Intake	

Estimated yearly income

"4: An Acceptable Available Carcinogen"

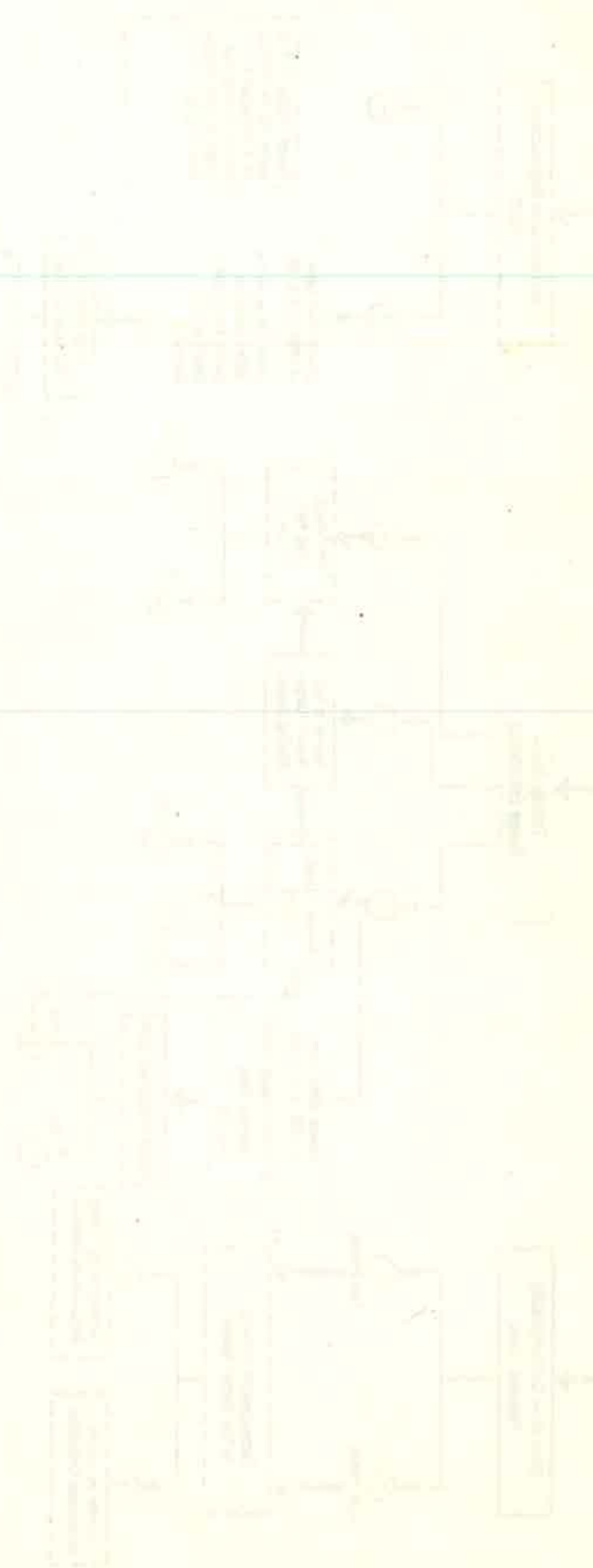
Quality May Match the Requirement

h e : Human Exposure (mg/kg body wt/day)

1. The first step in the process is to identify the problem. This involves gathering information about the situation and determining what needs to be solved.

2. The second step is to analyze the problem. This involves breaking the problem down into smaller, more manageable parts and identifying the underlying causes.

3. The third step is to develop a solution. This involves brainstorming ideas and selecting the most effective one.



CONCLUSION: The process of problem-solving is a continuous cycle that involves identifying the problem, analyzing it, developing a solution, implementing it, and evaluating the results. Feedback loops are essential for improving the process and ensuring that the solution is effective.

11/11/11