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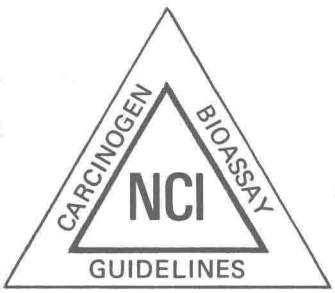

CARCINOGENESIS PROGRAM
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PREFACE

The National Cancer Act of 1971 provides legislative authority for the National Cancer Institute to plan and develop an expanded, intensified, and coordinated cancer research program. An urgent and essential part of this program is the need to protect the public from chemical and physical carcinogenic hazards and their effects. In an effort to meet this need, the Carcinogenesis Program of the Division of Cancer Cause and Prevention has undertaken as one of its major activities the identification of carcinogens in the environment and workplace. The responsibility to develop and maintain this activity has been assigned to the Bioassay Operations Segment in conjunction with the Carcinogen Bioassay and Program Resources Branch.

It is the purpose of this document to provide guidelines for the bioassay of chemicals for carcinogenic potential in small rodents. The initial draft of this document was written following a workshop at which the Carcinogenesis Bioassay Program's protocols were reviewed by experts in the various scientific disciplines applicable to carcinogen bioassay. The recommendations made at the workshop were reviewed by investigators active in the NCI Bioassay Collaborative Program. Later draft versions were reviewed by members of the scientific community at large. Included among the latter were workers affiliated with universities, other Federal agencies, commercial organizations, and trade associations, as well as ones from institutes and laboratories located abroad. An attempt was made to incorporate as part of this document as many of the reviewers' comments as were compatible with its intent. As a result of this extensive review by scientists representing diverse interests in our society, it is hoped that these guidelines will serve as the basis to standardize those aspects of a large-scale carcinogen bioassay which are essential to its scientific acceptability.

Appreciation is expressed to the individuals who participated in the Workshop on Carcinogen Bioassay Protocols held November, 1973, at the National Cancer Institute, Bethesda, Maryland. They were:

Richard R. Bates, National Cancer Institute
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Elizabeth K. Weisburger, National Cancer Institute

*Deceased

We also express our gratitude to the many individuals who have reviewed the material contained in this document and provided us with their comments. These comments have added greatly to the overall quality of the guidelines. In addition, we thank the many NCI staff members who have labored not only over difficult concepts but also over the sometimes more difficult task of putting them into clear and meaningful statements. In this regard, special recognition is given to Dr. Cipriano Cueto.

Questions or comments regarding the material contained in this document may be addressed to: Dr. James M. Sontag, Manager, Bioassay Operations Segment, Landow Building, Room A-306, National Cancer Institute, Bethesda, Maryland, 20014.

TABLE OF CONTENTS

I. INTRODUCTION

Most human cancers are believed to be caused by exposure to extrinsic factors, among which chemical agents are thought to be a major contributor. These agents must be identified, evaluated, and controlled if the incidence of human cancer is to be reduced. For this reason and the fact that many of these chemicals may have great social and economic impact, it is essential that the procedures used to determine their carcinogenicity be established on the best scientific bases as are practically possible. However, differences in scientific approaches and endpoints, as well as economic considerations, exclude the use of any single set of procedures to meet the objectives of all carcinogen bioassay studies. Notwithstanding these differences, certain features are common to all well designed and properly conducted long-term animal studies.

The guidelines contained herein are used by the *NCI CARCINOGENESIS BIOASSAY PROGRAM (CBP)* to screen environmental and occupational chemicals for carcinogenicity by their oral administration to small rodents. However, they also may be applicable, in part or *in toto*, to other long-term animal studies that have different procedures, objectives, or endpoints. It is not the intent of this document to address these variations. Although there are no substitutes for good animal care practices, deviations from these guidelines may sometimes be necessary for those laboratories not specially equipped to conduct large-scale bioassay studies.

II. GENERAL

The recommendations outlined in the *Guide for the Care and Use of Laboratory Animals*[1] should be adhered to throughout the bioassay study. The general areas covered are: 1. Laboratory Animal Management, 2. Laboratory Animal Quality and Health, 3. Personnel, 4. Use of Laboratory Animals, and 5. Physical Plant. When more rigid or specific standards should be applied they are noted in the appropriate sections of this document.

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1. Prepared by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council, DHEW Pub. No. (NIH) 74-23, Superintendent of Documents, U.S. G.P.O., Washington, D.C., 20402.

III. ANIMAL HUSBANDRY

III.A. *Source* Although it is ideal to have an animal production colony associated with a large-scale bioassay operation, animals supplied from commercial stocks are acceptable, providing they are high quality, disease free, genetically stable,[2] and adequately identified as to colony source. Animals should be shipped in sturdy, compartmented, and filtered containers. Arrangements should be made with the carrier to assure rapid and optimal shipping conditions that minimize trauma and extremes in temperature. Prompt notification of the animals' arrival should be given by the carrier to the receiving laboratory. Animals should be picked up immediately and placed into quarantine.

The animal strain/species used to study a test agent and its appropriate controls should be supplied from the same source. When animals from different sources are used to study different test agents, they should not be mingled and, optimally, should be maintained in separate rooms.

III.B. *Distribution* During the quarantine period and acute toxicity and repeated-dose studies, animals may be caged together according to the weight-space specifications (Appendix A) recommended in the *Guide for the Care and Use of Laboratory Animals*. However, for the subchronic and chronic studies, animals should be distributed from the outset of the studies as if they were in the upper weight range. This will obviate the need to later redistribute them to remain within the weight-space specifications. Even though the space to weight specification may be exceeded during the subchronic and chronic studies, no cage should contain more than five animals. As animals die or are sacrificed, surviving animals should not be combined or redistributed among the cages.

III.C. *Quarantine* The quarantine area should be physically separated from the testing area. Newly arrived animals should be taken, in their unopened shipping containers, directly to the quarantine area. A separate quarantine area should be provided for each species. Animals that are unsuitable by reason of size, health, or other criterion should be immediately discarded. Animals should be quarantined a minimum of seven days, after which they

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2. Most mice and rats now used come from production colonies developed and monitored by the CBP. These are periodically restarted with breeders obtained from the NIH Division of Research Services.

should be reexamined and those unsuitable should be discarded. A small, randomly selected number of animals from each shipment should be sacrificed and examined for parasites, enteric pathogens, and other diseased conditions.[3] When an epizootic disease is found among the animals, the entire shipment from which they came should be discarded. The quarantine area should be disinfected prior to the receipt of additional animals.

III.D. *Strain/Species* Both sexes of at least two species should be used for the bioassay of each test agent.[4] Considerations in selecting the proper species and strains should include the spontaneous tumor incidence, sensitivity to tumor induction, availability, genetic stability, hardiness, and longevity.

III.E. *Animal Facilities* The physical design and maintenance of the animal facilities are of great importance in assuring high standards of animal care, chemical and biological hazard control, and the proper conduct of the bioassay studies.

III.E.1. *Physical Plant* The animal facilities should be designed and operated to minimize the introduction of external biological and chemical agents into the building, as well as between individual animal rooms. The animal facilities should be effectively separated from the offices, laboratories, and other rooms that are not essential to the maintenance or treatment of the animals. The "clean-dirty" corridor flow should be used to minimize the inadvertent transfer of contaminants into the animal rooms. Service areas should be located to effectively support the animal facilities.

III.E.2. *Construction and Materials* The construction of the building and the materials used should facilitate the sanitation of the bioassay operation. Utilities such as water and electric lines and ventilator ducts should be located outside of the animal rooms. Components that interrupt the surface integrity

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3. See *Guide to Infectious Diseases of Mice and Rats*. A report of the Committee on Laboratory Diseases, Inst. Lab. Animal Resources, NRC, NAS, 2101 Constitution Ave., Washington, D. C., 20418; Pub. ISBN 0-309-01914-1.

4. The CBP uses primarily the B6C3F_1 hybrid (C57BL/6 x C3H/f) mouse and either the Fischer strain 344 or Osborne-Mendel rat.

of a room, e.g., electrical outlets and light fixtures, should be recessed into the surface and sealed. Seamless materials that are durable, waterproof, and fire-resistant should be used for interior surfaces. Paint and glazes should be highly resistant to chemical solvents, scrubbing, high-pressure sprays, and impact. Floor drains and windows are not necessary in facilities housing small rodents. Where they already exist, they should be blocked and sealed.

III.E.3. *Animal Rooms* The size and number of animal rooms should be sufficient to assure a separate room for each species. Ideally, a separate room also should be used for each test agent under study. Cages with solid sides and bottoms and covered by filter tops must always be used when studies cannot be physically separated.

III.E.4. *Ventilation, Temperature, and Humidity* Special attention should be given to ensure that the animal facilities are properly ventilated. Each animal room should undergo 10-15 fresh-air changes per hour. The air pressure should be adjusted so that the animal rooms are slightly positive to the "dirty" corridor and negative to the "clean" one. All air must be adequately filtered before it enters or leaves the animal facilities. The temperature and humidity should be maintained at those settings that have been reported to be optimal for the animal species being used.[5] An automatic recording and alert system should be used to monitor the ambient conditions in each animal room.

III.E.5. *Emergency Power* An emergency power source should be available as a backup to the primary system. Its generating capacity should be sufficient to power the animal facilities' air-condition and light systems.

III.F. *Animal Identification* Whenever individual animal data are to be routinely recorded, each animal should be marked at the outset of the study by a standard method of identification; e.g., ear notching, toe clipping, or tagging.

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5. The CBP recommends a temperature of 74°F ±2°F (23.3°C ±1.1°C) and a relative humidity of 40% ±5% be maintained in rat and mouse rooms.

III.G. *Cages/Racks* Plastic or stainless-steel cages with solid sides and bottoms should be used, especially for studies in which the test agent may be scattered or excreted. Wire-mesh cages may be required for certain types of studies; e.g., inhalation. Animals housed in cages with solid sides and bottoms should be changed to a sanitized cage[6] with fresh bedding as frequently as necessary, but not less than once weekly. Wire-mesh cages should be sanitized no less than once every two weeks.

Racks may be of either the shelf or suspended-drawer type and should be sanitized either in place or moved to a wash area for cleaning. If a rack washer is not available, they should be scrubbed with a suitable detergent and hosed down under high pressure. Racks should be sanitized at least once every other week.

III.H. *Filters* When cages with solid sides and bottoms are used, nonwoven polyester fiber filters should be placed over them as a disease control and chemical containment measure. Bonnets or filter sheets should be replaced by sanitized ones at least once every two weeks.

III.I. *Feeders* Feeders that are adequately designed to prevent soiling, bridging, and scattering of the feed are acceptable when pellet-type rations are used. Although no feeder is completely satisfactory for meal feed, a hopper-type feeder that is firmly attached to the cage appears to cause the least problems. However, this type of feeder may still require daily "bumping" to dislodge bridged meal. An open, unfixed feed cup should not be used nor should the feed be placed directly onto the cage floor. A sanitized feeder[6] should be supplied at least once weekly.

III.J. *Water/Water Bottles* An adequate supply of fresh and suitably treated water should be provided *ad libitum*. Potential pathogens carried in the water should be killed or removed through appropriate treatment; e.g., sterilization, pasteurization, or filtration. Each cage should be supplied at least twice weekly with a sanitized water bottle, stopper, and sipper tube.[6] The bottles should be filled and the stoppers and sipper tubes inserted into them only outside of the animal rooms. Empty or partially full

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6. To assure destruction of pathogens, water at 180°F (82°C) should be used for a period in the rinse cycle. Sanitization of bottle stoppers and sipper tubes also may be done by either germicide treatment before washing or by boiling after washing.

water bottles should be replaced rather than refilled. When an automatic watering system is used, the valve-end should be located in such a manner as to prevent accidental flooding of the cage.[7]

III.K. *Rations* A nutritionally balanced standard laboratory feed which supports normal growth and maintenance should be used.[8] Other factors to consider in choosing a feed should include the constancy of its major ingredients and their sources, its moisture content, freshness, storage characteristics, and timely delivery. Non-nutritional intentional additives, such as antibiotics and estrogens, should be avoided. Whenever a change in diet manufacture occurs during the course of a study, both the test and associated control groups should be switched at the same time to the new feed. Periodic analyses of the feed for pesticide (chlorinated hydrocarbons, etc.), mycotoxin (aflatoxins, etc.), and industrial (polychlorinated biphenyls, etc.) contaminants are recommended. The data from such analyses should be retained and included in the final report on each test agent. Whenever practical and consistent with the disease control program, the feed should be sterilized. Care should be taken that the nutrients are not degradated or the palatability of the feed altered. Feed should be provided as often as necessary, but not less than once weekly, to assure an adequate supply of fresh rations. When a test agent is given in the diet, its stability also must be considered as a factor in determining how often the feed should be renewed (see Section V.C.). Contaminated or spoiled feed should be replaced within the same working day in which it is found.

III.L. *Bedding* Although mycotoxin-free ground corncob may be used, heat-treated hardwood chips are considered the most desirable type of bedding. Softwood chips or creosoted wood should not be used. The bedding should be sterilized. In studies in which open wire-mesh cages are used, an absorbent material that effectively collects and holds waste matter should be placed under them.

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7. A break in the primary containment barrier is created by the aperature in the cage which allows the animals to reach the valve end. Thus, before use of an automatic watering system, consideration should be given to the nature of the compounds to be tested and safety requirements.

8. Consideration should be given to the use of the *NIH Open Formula Rat and Mouse Ration* (Appendix B).

IV. SAFETY and HEALTH

IV.A. *Personnel Protection* Each test agent should be treated as a potential carcinogen. Thus, every precaution should be taken to prevent inadvertent exposure of personnel and the environment to the test agent. Each aspect of the bioassay study, from receipt of the test agent through histopathology, must be monitored and controlled. Personnel whose medical condition, e.g., depressed immune response, pregnancy, and steroid or cytotoxic drug treatment, may make them unusually susceptible to the possible harmful effects of a test agent should be excluded from any area where accidental exposure might occur. Individuals who are allergic to laboratory animals should not be exposed to them unless adequately protected and approval has been given by the medical or safety officer. Each laboratory should develop a safety and health plan for the handling of potential carcinogens. Applicable federal, state, and local regulations must be adhered to, as well as the National Cancer Institute's safety standards for research involving chemical carcinogens (Appendix C).

IV.B. *Animal Protection* Strict hygienic and disease prevention measures should be routinely practiced. Access to the animal facilities should be restricted to only those individuals essential to their operation. Personnel should receive adequate animal care and personal hygiene training and instruction as to the proper operating procedures. Personnel with respiratory infections or other disease conditions which may affect the animals' health should be excluded from the animal rooms.

IV.C. *Protective Clothing and Equipment* All personnel who may be exposed to either the animals or test agents must be adequately protected. A complete change of clean clothing should be provided daily and should include a fully fastened laboratory suit, gloves, boots, and head cover. The protective clothing should not be worn outside of the work area. An appropriate face mask or respirator should be worn as protection against dust, mists, or fumes.

V. CHEMICAL QUALITY and TREATMENT MIXTURE CONTROL

V.A. *Storage/Shelf Life* The proper method(s) for the long-term storage of each bulk test agent should be determined prior to its bioassay. A sample of the bulk test agent should be analyzed periodically during the study to ensure that it has retained its original characteristics.

V.B. *Purity* The purity of each test agent should be determined prior to its bioassay. In some instances, identification and determination of the percent of each impurity, as well as the purification of the test agent, may be desirable.

V.C. *Stability* The stability of each test agent should be determined under the same conditions in which it will be administered, e.g., in the feed and/or vehicle, and temperature and/or pH, prior to the start of the bioassay study. This analysis should be done on samples taken from newly prepared treatment mixtures and from the last of usable ones; e.g., diet mixes. The frequency at which fresh treatment mixtures are prepared may depend on the stability of the test agent under its conditions of storage and/or administration.

V.D. *Homogeneity/Concentration* When the test agent is incorporated into the feed, its homogeneity and concentration in the diet mix should be determined before the start of the bioassay study. Random samples from freshly mixed batches should be analyzed periodically during the study to ensure that the proper mixing and formulation procedures are being used.

V.E. *Treatment Mixtures* Treatment mixtures should be stored in inert, shatter-proof containers with air-tight covers. Each container should be clearly marked so that its contents can be easily and accurately identified. The storage containers should be located in a secure area that is free of congestion and with restricted access.

An inventory of each treatment mixture should be maintained on a current basis. A record also should be kept of the preparation and usage of each treatment mixture, including the dates and quantities prepared or used and the names of the responsible individuals.