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FEDERAL COMMUNICATIONS COMMISSION

May 17, 1984.

The Federal Communications Commission will hold a Closed Meeting on the subjects listed below on Thursday, May 24, 1984 following the Open Meeting, which is scheduled to commence at 9:30 A.M. in Room 856, at 1919 M Street, NW., Washington, D.C.

Agenda, Item No., and Subject

Hearing—1—Application for review in the GAF Broadcasting Company, Inc., New York, New York, FM radio comparative renewal proceeding (BC Docket Nos. 82-371-72).

Hearing—2—Petitions for Reconsideration and stay of Commission Orders rejecting claims of attorney-client privilege and the attorney-work-product doctrine in the A.S.D. Answer Service, Inc., DPLMRS proceeding (Docket Nos. 82-587-90).

These items are closed to the public because they concern Adjudicatory Matters (See 47 CFR 0.603(j)).

The following persons are expected to attend:

Commissioners and their assistants
Managing Director and members of his staff
General Counsel and members of his staff
Chief, Office of Public Affairs and members of his staff

Action by the Commission May 16, 1984. Commissioners Fowler, Chairman; Quello, Dawson, Rivera and Patrick voting to consider these items in Closed Session.

This meeting may be continued the following work day to allow the Commission to complete appropriate action.

Additional information concerning this meeting may be obtained from Sally Lawrence, FCC Public Affairs Office, telephone number (202) 254-7674.

William J. Tricarico,
Secretary, Federal Communications Commission.

[FR Doc. 84-13758 Filed 5-18-84; 9:57 am]
BILLING CODE 6712-01-M

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FEDERAL MINE SAFETY AND HEALTH REVIEW COMMISSION

May 17, 1984.

TIME AND DATE: 10:00 a.m., Wednesday, May 23, 1984.

PLACE: Room 600, 1730 K Street, NW., Washington, D.C.

STATUS: Closed (Pursuant to 5 U.S.C. 552b(c)(10)).

MATTERS TO BE CONSIDERED: In addition to the previously announced items to be discussed after oral argument, the Commission will consider the following:

2. Secretary of Labor, MSHA v. Pontiki Coal Corp., Docket Nos. KENT 83-181-R, etc.
3. Secretary of Labor, MSHA v. Belcher Mine, Inc., Docket No. SE 84-4-M.

It was determined by a unanimous vote of Commissioners that Commission business required that a meeting be held on these items, and a majority vote of Commissioners voted that the meeting be closed. No earlier announcement of the addition was possible. 5 U.S.C. § 552b(e)(1)

CONTACT PERSON FOR MORE INFORMATION: Jean Ellen, (202) 653-5632.

Jean Ellen,
Agenda Clerk.

[FR Doc. 84-13769 Filed 5-18-84; 10:30 am]
BILLING CODE 6735-01-M

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FEDERAL RESERVE SYSTEM

TIME AND DATE: 11:00 a.m., Tuesday, May 29, 1984.

PLACE: 20th Street and Constitution Avenue, NW., Washington, D.C. 20551.

STATUS: Closed.

MATTERS TO BE CONSIDERED:

1. Personnel actions (appointments, promotions, assignments, reassignments, and salary actions) involving individual Federal Reserve System employees.
2. Any items carried forward from a previously announced meeting.

CONTACT PERSON FOR MORE INFORMATION: Mr. Joseph R. Coyne, Assistant to the Board; (202) 452-3204.

Dated: May 18, 1984.

James McAfee,
Associate Secretary of the Board.

[FR Doc. 84-13832 Filed 5-18-84; 3:45 pm]
BILLING CODE 6210-01-M

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FEDERAL TRADE COMMISSION

TIME AND DATE: 2:00 p.m., Tuesday, June 19, 1984.

PLACE: Room 432, Federal Trade Commission Building, 6th Street and Pennsylvania Avenue, NW., Washington, D.C. 20580.

STATUS: Open.

MATTER TO BE CONSIDERED: Consideration of recommendation of Bureau of Consumer Protection and Bureau of Economics that the Commission publish the attached notice of proposed rulemakings in the "Eyeglasses II" investigation.

CONTACT PERSON FOR MORE INFORMATION: Susan B. Ticknor, Office of Public Affairs: (202) 532-1892
Recorded Message: (202) 523-3806

Benjamin I. Berman,
Acting Secretary.

[FR Doc. 84-13793 Filed 5-18-84; 3:58 pm]
BILLING CODE 6750-01-M

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FEDERAL TRADE COMMISSION

"FEDERAL REGISTER" CITATION OF PREVIOUS ANNOUNCEMENT: FR 49, May 9, 1984, page No. 19766.

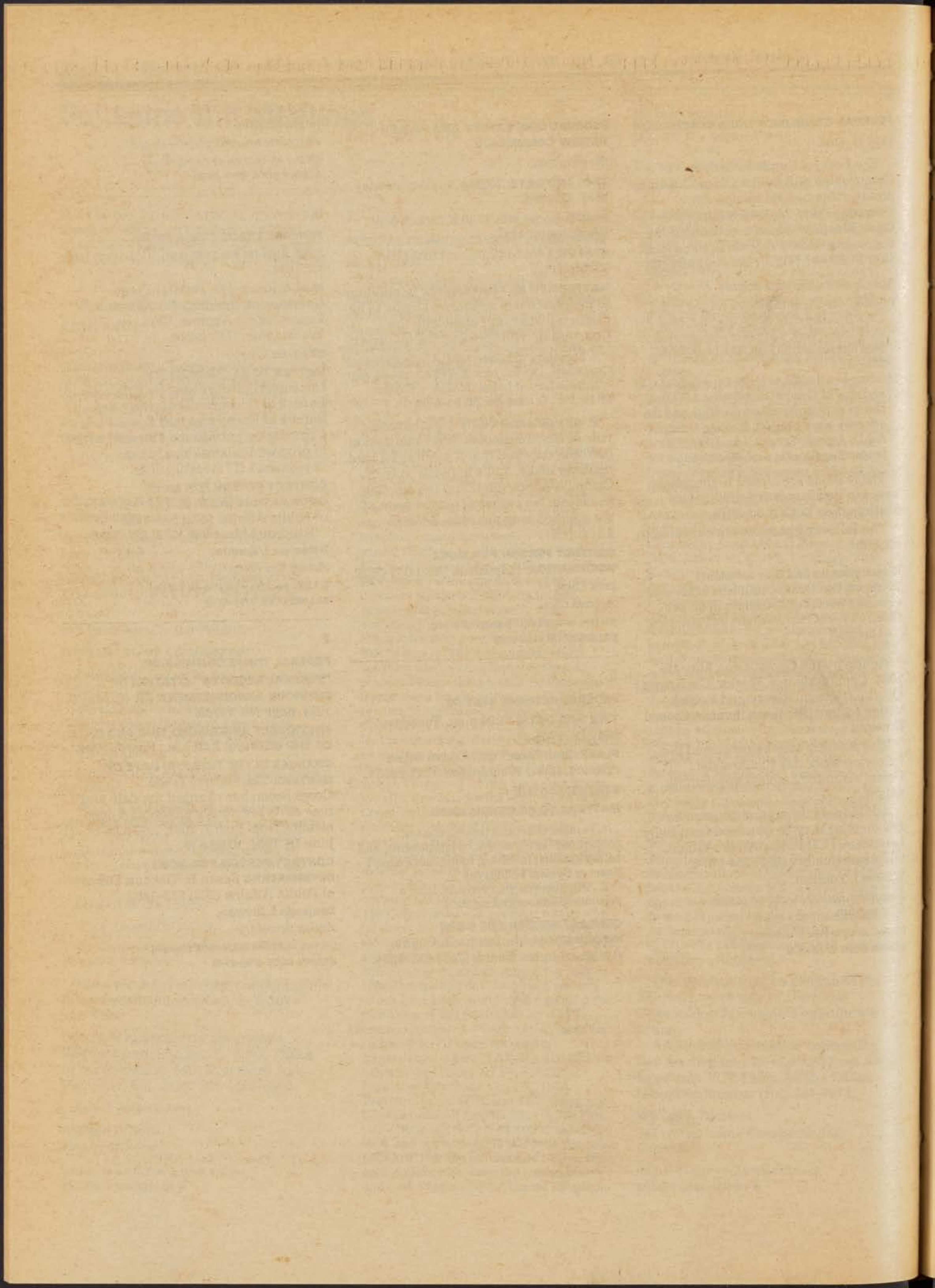
PREVIOUSLY ANNOUNCED TIME AND DATE OF THE MEETING: 2:00 p.m., June 7, 1984.

CHANGES IN THE TIME AND DATE OF MEETING: The Federal Trade Commission has changed the date and time of its previously announced open meeting from June 7, 1984, 2:00 p.m. to June 19, 1984, 10:30 a.m.

CONTACT PERSON FOR MORE INFORMATION: Susan B. Ticknor, Office of Public Affairs: (202) 532-1892

Benjamin I. Berman,
Acting Secretary.

[FR Doc. 84-13794 Filed 5-18-84; 12:45 pm]
BILLING CODE 6750-01-M



federal register

Tuesday
May 22, 1984

Part II

Office of Science and Technology Policy

Chemical Carcinogens; Notice of Review of the Science and Its Associated Principles

OFFICE OF SCIENCE AND TECHNOLOGY POLICY

Chemical Carcinogens; Review of the Science and Its Associated Principles, May, 1984

AGENCY: Executive Office of the President, Office of Science and Technology Policy.

ACTION: Notice.

SUMMARY: The Office of Science and Technology Policy has prepared this draft document to be used as a framework for regulatory agencies in assessing cancer risks from chemicals. The document does not attempt to formulate policy or develop standardized methods of risk assessment. It tries to define principles, based upon current scientific information, to serve as guidelines in certain matters when considering carcinogens. The present document is the culmination of drafts extensively reviewed by governmental and non-governmental scientists. The public is now invited to review and comment on this document prior to final publication.

DATE: Comments must be received on or before August 1, 1984.

ADDRESS: Send comments to: Ronald W. Hart, Ph.D., Director, NCTR, Chairman, OSTP Interagency Staff Group, National Center for Toxicological Research, Jefferson, AR 72079; Telephone: 501-541-4000.

Dated: May 10, 1984

Jerry D. Jennings,

Executive Director, Office of Science and Technology Policy.

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INTRODUCTION

I. Background

Over the years, agencies and programs have been established to deal with the problems of cancer. Congress has enacted legislation and appropriated funds to address various aspects of hazardous substances (Table 1). A detailed analysis of the legislative acts concerning toxic substances reveals an evolution of thought in that each reflects the scientific views that existed at the time of enactment. Consequently, these acts are not uniform in their view of the disease, the role chemical substances might play in its incidence, and what ought to be done about potential toxic substances and potential carcinogens.

TABLE 1.—FEDERAL LAWS RELATED TO EXPOSURES TO TOXIC SUBSTANCES

Legislation	Agency	Area of concern
Food, Drug and Cosmetics Act (1906, 1938, amended 1958, 1960, 1962, 1968, 1976).	FDA	Food, drugs, cosmetics, food additives, color additives, new drugs, animal and feed additives, and medical devices.
Federal Insecticide, Fungicide and Rodenticide Act (1948, amended 1972, 1975, 1978).	EPA	Pesticides.
Dangerous Cargo Act (1952)	DOT, USCG	Water shipment of toxic materials.
Atomic Energy Act (1954)	NRC	Radioactive substances.
Federal Hazardous Substances Act (1960, amended 1981)	CPSC	Toxic household products.
Federal Meat Inspection Act (1967)		
Poultry Products Inspection Act (1968)	USDA	Food, feed, color additives, and pesticide residues.
Egg Products Inspection Act (1970)		
Occupational Safety and Health Act (1970)	OSHA, NIOSH	Workplace toxic chemicals.
Poison Prevention Packaging Act (1970, amended 1981)	CPSC	Packaging of hazardous household products.
Clean Air Act (1970, amended 1974, 1977)	EPA	Air pollutants.
Hazardous Materials Transportation Act (1972)	DOT	Transport of hazardous materials.
Clean Water Act (formerly Federal Water Control Act) (1972, amended 1977, 1978)	EPA	Water pollutants.
Marine Protection, Research and Sanctuaries Act (1972)	EPA	Ocean dumping.
Consumer Product Safety Act (1972, amended 1981)	CPSC	Hazardous consumer products.
Lead-Based Paint Poison Prevention Act (1973, amended 1976)	CPSC, HEW (HHS), HUD.	Use of lead paint in federally assisted housing.
Safe Drinking Water Act (1974, amended 1977)	EPA	Drinking water contaminants.
Resource Conservation and Recovery Act (1976)	EPA	Solid waste, including hazardous wastes.
Toxic Substances Control Act (1976)	EPA	Hazardous chemicals not covered by other laws, includes pre-market review
Federal Mine Safety and Health Act (1977)	DOL, NIOSH	Toxic substances in coal and other mines.
Comprehensive Environmental Response, Compensation, and Liability Act (1981)	EPA	Hazardous substances, pollutants and contaminants.

Most abbreviations are same as below. Additional ones are:

DOL—Department of Labor;
DOT—Department of Transportation;
HEW (HHS)—Department of Health, Education and Welfare (many functions assumed by HHS—Department of Health and Human Services);
HUD—Department of Housing and Urban Development;
NIOSH—National Institute of Occupational Safety and Health;
NRC—Nuclear Regulatory Commission;
USCG—United States Coast Guard.

II. The Purpose of This Report

The purpose of this document is to: (1) Articulate a view of carcinogenesis that scientists generally hold in common today (Part II); and (2) draw upon this understanding to compose, as was done here by senior scientists from a number of Federal agencies, a series of general principles that can be used to establish specific guidelines for assessing carcinogenic risk (Part I). Because of present gaps in understanding, the principles contain statements of what is generally accepted as fact as well as judgmental (science policy) decisions on unresolved issues, and there has been, however, an attempt to clearly distinguish between the different types of information presented. These principles also provide to all interested parties specific reference to the analysis upon which they are based. They can serve as the basis for consistent regulatory cancer guidelines that the Federal agencies can tailor to meet the requirements of the legislative acts they are charged to implement. Similar documents, with varying degrees of specificity, have been produced in the past, *e.g.*, in the late 1970s and early 1980s several agencies of the Federal government produced statements directed toward providing a consistent basis for a general Federal cancer policy. This document should be seen, in the broad view, as part of an ongoing process, on behalf of the Federal government, that strives to periodically update and review current understanding of carcinogenesis and the scientific process of how this understanding is utilized.

This document is the result of the combined efforts of senior scientists from the following Federal health-related units, operating in the Interagency Staff Group, under the direction of the Office of Science and Technology Policy (OSTP):

Bureau of Foods (B.F.), Food and Drug Administration (FDA)
Consumer Product Safety Commission (CPSC)
Environmental Protection Agency (EPA)
Office of the Commissioner (OC), FDA
Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA)

National Cancer Institute (NCI), National Institutes of Health (NIH)
National Center for Toxicological Research (NCTR), FDA
National Institute of Environmental Health Sciences (NIEHS), NIH
National Toxicology Program (NTP)
Occupational Safety and Health Administration (OSHA)

III. The Context of This Report

This document is written in light of a decision-making process used by many of the regulatory agencies, which involves the assessment of carcinogenic risks posed by chemical substances. The scientific inputs to these evaluations can be best appreciated by examining the decision-making process in some detail.

Risk can be conceived of as being composed of two aspects, each of which can be addressed by science: Hazard* and exposure. Although other definitions have been used historically, this document conforms to present usage and "hazard" generally refers to the inherent toxicity of the substance (for some toxic endpoint) and is deduced from a wide array of data including: epidemiological evaluations, long-term animal studies, short-term tests, information on mechanisms, and the results of structure/activity relationships. "Exposure" generally refers to the amount of the substance that people come in contact with. The "risk", in a quantitative risk assessment, is estimated by coupling the results of the exposure and hazard assessments. As either the hazard or exposure approaches zero, the risk also approaches zero.

As a first step in assessing the cancer risk associated with the use of a particular chemical substance, the qualitative evidence that a given chemical substance is likely to be a human carcinogen must be evaluated. In this step, as in the whole process, a number of assumptions and approximations must be made in order to deal with inherent limitations found in the existing data bases. Then, estimates of human exposure, and distribution of exposures likely to be encountered in the population are made. Next, one or more methods for estimating the dose-response relationship at doses below those generally used experimentally must also be evaluated. Finally, the exposure assessment is combined with the dose-response relationship to generate an estimate of risk. The various ways in which these steps are conducted and combined and their attendant uncertainties treated constitute what is generally referred to as "cancer risk assessment", although an assessment can be terminated at different stages to estimate risk for different purposes.

Some legislation calls for action in the presence of any risk. Other forms of legislation use the concept of unreasonable risk, defined, in some acts, as a condition in which the risks outweight the benefits; *e.g.*, the Toxic Substances Control Act (TSCA) and the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). A spectrum of approved responses, from simply informing the public of a risk through restricted use to a complete ban, may be available to bring the risks and benefits into appropriate balance.

This document does not perform a risk assessment nor does it suggest that one method of cancer risk assessment is better than another; but, rather, it attempts to review the science of chemical carcinogenesis and develops from this review a set of general principles. It is not a comprehensive treatise; nor a document written for the lay public but is rather a semi-technical review which tries to evaluate the impact of scientific findings of the last decade on general assumptions or principles important to risk assessment. This is based upon the inherent belief of the group that elucidation of the basic mechanisms underlying cancer and the identification of cancer-causing agents and conditions, when coupled to research aimed at identifying and evaluating the problems created by such agents, should provide the optimal adminstrative bases for making sound and reasonable judgments.These overlapping approaches to evaluating the problems of cancer, we believe, should form one of the strongest foundations upon which a technologically based society could base its decisions.

IV. The Content of This Report

This document is composed of two parts. Part I presents a set of general principles that may be used by regulatory agencies as they review their own specific guidelines for performing cancer risk assessments. Part II addresses the current state-of-the-science concerning carcinogenesis and cancer risk assessment and forms the underlying scientific basis of Part I.

The general principles in Part I, as has been suggested for similar guidelines in the National Academy of Sciences report on Risk Assessment in the Federal government**, can be useful despite an inherent tendency toward over simplification and the mixing of scientific knowledge with risk assessment science policy. Many components of the risk assessment process lack definitive scientific bases. Often a choice must be made among several different scientifically plausible options. The above cited report identified over 50 components where the uncertainty in the science requires, in part, scientific judgements and science policy decisions to go from one component to another to reach a decision. This document attempts to leave the majority of these necessary scientific inferences to the scientific regulatory agencies. However, some are so important and fundamental to all cancer risk assessment procedures, regardless of the source of hazard or the different statutory provisions of the agencies, that they are included in some of the principles. Where this has occurred, we have attempted to indicate, both in Part II and the wording of the principle itself, that a public health science policy decision is embodied in it. For example, Principle 8 makes the point that an animal carcinogen should be considered a suspect human carcinogen. Many scientists would agree that, while there is a significant amount of evidence to support qualitative animal to human extrapolation for carcinogenesis, the evidence falls short of establishing this proposition as a scientific fact (when determining the response of different species to chemcials, many chemicals appear to be carcinogenic in one species or strain and not in another, even when only rodents are being compared). Nonetheless, this principle has been accepted by all health and regulatory agencies and is regarded widely by scientists in industry and academia as a justifiable and necessary inference. It is not possible to draw a sharp distinction in every instance between principles which are based solely on science and those which embody a choice based also on science policy. However, several of the principles clearly reflect certain aspects of science policy, *e.g.* Principles 8, 11 and 26.

Part II of this document is divided into six separate chapters, each discussing the current information in an area of science used in the cancer risk assessment process.

Chapter 1 highlights some of the recent advances in our understanding of

*The European community uses the term "risk" where we have used the term "hazard" and vice versa.

** Managing the Process: Risk Assessment in the Federal Government, National Academy of Sciences, Washington, D.C., 1983.

the mechanisms of carcinogenesis. A general consensus has evolved which describes cancer as a multi-stage process involving a variety of events which can include metabolic conversion, initiation, promotion, and proliferation. The chapter highlights some advances in our understanding that have implications in the way we assess cancer risk. At the same time, the chapter also identifies some of the vast amount of information that remains to be collected and analyzed.

Chapter 2 examines short-term testing and the relationship between genetic toxicity and carcinogenicity. Tests for gene mutation, chromosomal aberration, DNA damage and repair, and cellular (morphologic) transformation have provided valuable information on the potential for chemical substances to cause cancer. This field is currently characterized by a great deal of activity, and the data generated must be carefully analyzed and verified before use in cancer risk assessment.

The third chapter contains an evaluation of long-term animal tests, which have been a major factor in past assessments of cancer risk. A review of the usefulness in cancer risk assessment of the data developed via this approach to chemical evaluation is made, and potential limitations and pitfalls of such data relative to extrapolation to human populations are discussed.

The fourth chapter examines the current state of epidemiological knowledge relating to human cancer. Data collected during the past 50 years permit the analysis of trends in cancer incidence/mortality and provide information related to the various etiologies of cancer. Recent developments in cancer epidemiology are highlighted and their potential impact on risk assessment pointed out. Much of the discussion concentrates on the methods to be used in such studies in attempting to overcome past, often cited, limitations of this approach.

In addition to these chapters which examine factors associated with hazard assessment, part II also contains a discussion of exposure assessment. As Chapter 5 points out, the field of exposure assessment has been marked by significant progress in the areas of monitoring data, computer modeling, transformation and transport of chemicals, and laboratory approaches to determining the behavior of chemicals in the environment. At the same time uncertainties remain, and any risk assessment will necessarily be affected by these limitations.

Chapter 6 concludes Part II of the document and discusses how the hazard and exposure elements of the preceding five chapters are used as inputs into the qualitative and quantitative cancer risk assessment processes. This chapter depends heavily on all the other chapters of this volume and attempts to make clear the various approaches that are used in risk assessments, noting both the limitations and advantages of each. Common themes within each chapter are the uncertainty, the gaps in data, and the questions of interpretation associated with some aspects of the scientific information used in the risk assessment process. A critical evaluation of this information comprises Part II of the document and led to the principles listed in Part I. These general principles were developed to provide interim guidance in areas of uncertainty until such time that additional scientific experimentation provides the required information needed to improve estimations of risk in human populations. As a note of caution, it should be recognized that the chapters of Part II, taken *in toto*, provide the underlying support for the principles articulated in Part I. To read Part I without a full appreciation of the material in Part II is to invite oversimplification and misinterpretation.

Finally, a note is needed, chiefly addressed to specialists on the various topics that are briefly discussed in Part II. If reports of a broad nature are to be written at all, and general reports are often necessary to present organized thought on subjects of vital interest, it is inevitable that those who write them must spend less time on a topic than someone who concentrates almost exclusively on one specific interest. Therefore, there are certain to be areas which some authorities think are not discussed enough, others which some feel go on *ad infinitum*. Also, since the chapters in Part II of this document is an attempt to provide a balanced account of what is generally accepted, derived from a vast amount of information involving the work of thousands of individuals, citing relatively few, some must be slighted. Both problems are inevitable in a work of this type. Hopefully, deficiencies in the choice of topic for emphasis or failures to acknowledge contributions have not been egregious.

PART I—PRINCIPLES

Preface

The principles contained within this segment of the OSTP carcinogen document were derived by the authors of the various chapters, from the information detailed in Part II, in cooperation with all members of the Interagency Staff Group. This section attempts to provide, in a non-technical form, some important general statements relevant to the evaluation of the role of chemicals in carcinogenesis. These statements are intended to serve as a bridge connecting the basic science as expounded in Part II and the multifaceted process of risk assessment.

Since there are gaps in the information available, differences in evaluations and in scientific opinion may exist about certain of the points highlighted as prinicples. However, these principles derive from a *weltanschauung* utilizing a balanced approach with an appreciation of all elements of the problem, from hazard identification and estimation, through exposure and risk assessment. It is clearly understood that new information and newly emerging concepts may modify some of these statements, however, for the time being, as a result of an ardous cooperative effort these statements, we believe, represent an up-to-date summary on a number of important topics.

I. Principles Derived From the Mechanisms of Carcinogenesis

1. Carcinogenesis is a multistage phenomenon that may involve the genome both directly and indirectly. These stages of carcinogenesis may be, to varying degrees, influenced by a number of variables such as age at exposure, diet, hormonal status, intra- and inter-species variability, which should be considered when trying to predict human response to potentially carcinogenic agents (Chapter 1, Section VI).

2. Appropriate *in vitro* and *in vivo* tests can indicate that an agent has a certain action such as genetic toxicity or promotion. Such information may be useful in evaluating mechanism(s) of cancer induction. However, in evaluation of human risk, the attribution of observed findings of carcinogenicity to a particular biological effect must rest upon sound evidence that the effect is responsible for the cancer induction. It must be kept in mind that a chemical may contribute to carcinogenesis in multiple ways (Chapter 1, Section VI).

3. At the present stage of knowledge, mechanistic considerations, such as DNA repair and other biological responses, in general do not prove the existence of, the lack of existence of, or the location of, a threshold for carcinogenesis. The presence or absence of a threshold for one step of the carcinogenic process does not necessarily determine the presence or absence of a threshold for the whole

process. For example, there is strong evidence that the existence of DNA repair will not result in a threshold for the accumulation of DNA damage (Chapter 1, Section V, Part A); however, the consequences of this for a threshold for carcinogenesis is unclear.

4. The carcinogenic effects of agents may be influenced by non-physiological responses induced in the model systems. Testing regimens inducing these responses, such as extensive organ damage, saturation of metabolic pathways, saturation of DNA repair with functional loss of the system, should be evaluated for their relevance to the human response to the agent (Chapter 1, Section V, Part B) and evidence from such a study, whether positive or negative, must be carefully reviewed.

II. Principles for Tests of Cancer Induction

A. Short-Term Tests

5. Short-term tests, such as assays for point mutations, chromosomal aberrations, DNA damage, and *in vitro* transformation are useful in: (1) Screening for potential carcinogens; (2) supporting a judgment on the carcinogenicity of a chemical; and (3) providing information on carcinogenic mechanisms (Chapter 2, Section IV, Part B).

6. At the present, short-term tests are limited in their ability to predict carcinogenicity and cannot supplant data from epidemiological investigations or long-term animal studies since the tests do not necessarily screen for all potential means of cancer induction and do not necessarily mimic all reactions that would occur *in vivo*. Additional research is required to improve existing tests and develop ones that identify chemicals which act by genetic mechanisms not yet detected or which act by other, non-genetic, mechanisms (Chapter 2, Section IV, Part C).

7. Short-term tests should be carefully selected to ensure they have been adequately validated. Several tests with different endpoints may be required to characterize a chemical's spectrum of response (Chapter 2, Section IV, Parts A and B).

B. Long-Term Animal Tests

8. In the context of the result from a long-term test, the term carcinogen should be used in a broad sense, *i.e.* a substance or process capable of increasing the incidence of neoplasms (combining benign and malignant when scientifically defensible) or decreasing the time it takes for them to develop. Agents found carcinogenic in animal studies, subject to the considerations discussed in Principles 4 and 14, are considered suspect human carginogens (Chapter 3, Section II, Part A).

9. Some experimental animal models normally have high incidences of certain tumors. The evaluation of tumor data from such animals can pose special problems. For example, the interpretation of cancer incidence in some strains of rats with testicular or mammary tumors, or in some strains of mice with lung or liver tumors must be approached carefully in the light of other biological evidence bearing on potential carcinogenicity (Chapter 3, Section II, Part C).

10. Protocols for long-term tests should be designed to achieve an appropriate balance between the two essential characteristics of a biological assay: Adequate biological and statistical sensitivity (a low false negative rate) and adequate biological and statistical specificity (a low false positive rate) (Chapter 3, Part II, Section D, Number 2). The absence of biases in selection and allocation of animals between control and treatment groups as regards diet, husbandry, necropsy and pathology is crucial (Chapter 3, Part I, Section B, Number 2).

11. It is appropriate to use test doses that generally exceed human exposure levels in order to overcome the inherent insensitivity of the traditional design of the long-term animal test. The highest dose should be selected after an adequate prechronic study and after evaluating other relevant information, *e.g.* pharmacokinetic data, as necessary to determine the highest dose consistent with predicted minimal target organ toxicity and normal lifespan, except as a consequence of the possible induction of cancer (Chapter 3, Section I, Part B, Number 4 and Section II, Part B, Number 1).

12. The diagnosis of pathologic lesions is complicated and requires judgment and appropriate experience. Diagnoses can differ depending on the tissue and species involved and can change with time as techniques improve and data on bioassays accumulate. Accurate interpretation of tumor data is contingent upon careful attention to gross observation, tissue sampling, slide preparation and histologic examination. Diagnosis of tumors should be guided by evidence of their histogenic origin and stage of progression (Chapter 3, Section II, Part C).

13. Appropriate statistical analysis should be performed on data from long-term studies to help determine whether the effects are treatment related or possibly due to chance. These should include a statistical test for trend and a test based on pairwise comparisons, including appropriate correction for differences in survival. The weight to be given to the level of statistical significance (the p-value) and to other available pieces of information is a matter of overall scientific judgment (Chapter 3, Section II, Part D).

14. Decisions on the carcinogenicity of chemicals in animals should be based on consideration of relevant biological and biochemical data (Chapter 3, Section II, Part B, Number 3), including the following (Chapter 3, Part II):

(a) Use of background or recent historical control incidence of tissue specific tumors can be an aid in the evaluation of tumor data. Care should be exercised when combining different control groups to avoid inappropriate combinations of such groups.

(b) Evidence of probable reproducibility is important. This evidence can consist of independent confirmation of the original findings or may be derived from intergroup comparisons of tumor incidence data, between dose groups, sexes, strains or species.

(c) Evidence of dose response increases confidence that the effect is treatment related; similarly the lack of an observed dose-response may reduce the likelihood that the effect is associated with the treatment.

(d) Confidence is increased when: (1) The incidence of tumors is markedly elevated in the treated groups compared to controls, particularly when the tumors in controls are infrequent; (2) tumor incidence is significantly increased at multiple anatomical sites; and (3) tumor latency is reduced.

(e) In addition to tumor incidence at specific tumor sites, the stage in the development of neoplasia should be evaluated. For example, the finding that the majority of neoplastic lesions at a specific tissue site is more advanced in a treated group compared to its control may provide additional evidence of a treatment related effect. Conversely, the finding that the control group lesions are more advanced might argue that a marginal elevation of tumor incidence is not treatment related.

(f) The incidence of preneoplastic lesions in treatment or control groups may, in certain instances, provide evidence for the biological plausibility of a neoplastic response and contribute to the interpretation of a bioassay.

(g) Identification from prechronic studies, or other toxicity studies of effects in the target organ(s), can assist in evaluating whether or not differences in tumor incidences are treatment related.

(h) Information on the activity of chemicals at the physiological, cellular and molecular level may be important to the evaluation of carcinogenicity data on a case-by-case basis.

III. Principles for Epidemiology

15. The strength of the epidemiological method is that it is the only means of assessing directly the carcinogenic risk of environmental agents in humans; however, because of the limitations of the available information, *e.g.*, the elements discussed in Principle 18 as well as the paucity of data, primary reliance is often placed on animal testing (Chapter 4, Section II, Part A).

16. Descriptive epidemiological studies (based on the measurement of disease rates for various populations), including correlational studies (in which the rate of disease in a population is compared with the spatial or temporal distribution of suspected risk factors), are useful to generate and refine hypotheses, or provide supporting evidence in evaluating relationships detected by other means, but rarely, if ever, provide information allowing a practical causal inference (Chapter 4, Section IV, Part A).

17. Well designed, conducted and evaluated analytic epidemiological investigations of either case-control or cohort variety can provide the basis for practical casual inferences especially useful for public health decisions (Chapter 4, Section IV, Part B).

18. Elements in interpreting the likely practical causality of epidemiological observations include the magnitude of the risk estimates (strength of the associations); the possibility of their being due to chance (statistical significance); the rigor of the study design to avoid various kinds of bias, including those related to selection, confounding, classification and measurement; dose-response relationships; the temporal relationships between exposure and disease; the specificity of the associations; their biological plausibility; and the reproducibility of the findings (Chapter 4, Section III).

19. A high quality negative epidemiological study, while useful, cannot prove the absence of an association between chemical exposure and human cancer. Within the scope of the study, specifically for the populations studied (including concomitant exposures), for the levels and durations of exposure to the agent evaluated and for the time assessed following exposure, likely upper bounds on the estimates of risk can be made and the statistical likelihood of the study to detect an effect can be assessed (Chapter 4, Section VI).

IV. Principles for Exposure Assessment

20. It is desirable that exposure routes employed in animal health effects studies are comparable to human exposure routes both for the simplification of risk assessment and because there may be important route-dependent differences in molecular, biochemical and physical parameters in organs (Chapter 1, Sections II and III).

21. At present, a single generally applicable procedure for a complete exposure assessment does not exist. Therefore, in the near term, it is expected that integrated exposure assessments (utilizing monitoring data, results from physical and chemical models, and consideration of all routes of exposure through all media) will be conducted on a case-by-case basis (Chapter 5, Section II).

22. The depth and accuracy of an exposure assessment should be tailored to provide the degree of knowledge required to support analytical needs. A preliminary assessment using available crude data can often shed light on the upper or lower bounds of potential risks (Chapter 5, Section II, Part D).

23. An exposure assessment should describe the strengths, limitations and uncertainties of the available data and should indicate the assumptions made to derive the exposure estimates (Chapter 5, Section III, Part F).

24. In general, an array or range of exposure values is preferable to a single numerical estimate (Chapter 5, Section III, Part F).

V. Principles for Risk Assessment

25. Decisions on the carcinogenicity of chemicals in humans should be based on considerations of relevant data, whether they are indicative of a positive or negative response and should use sound biological and statistical principles. This weight of evidence approach can include consideration of the following factors and should give appropriate weight to each on a case-by-case basis (Chapter 6, Section II, Part A):

(a) Findings from long-term animal studies (See Principle 14);

(b) Results from epidemiological studies (see Principles 16–18);

(c) *In vivo* and *In vitro* short-term tests;

(d) Structure-activity relationships of chemicals.

(e) Known metabolic differences between animals and humans.

26. No single mathematical procedure is recognized as the most appropriate for low dose extrapolation in carcinogenesis. When relevant biological evidence on mechanism of action (*e.g.*, pharmacokinetics) exists, the models or procedures employed should be consistent with the evidence. However, when data and information are limited, as is the usual case, and when much uncertainty exists regarding the mechanism of carcinogenic action, models or procedures which incoporate low-dose linearity are preferred (Chapter 6, Section II, Part C).

27. The quantification of the various sources of uncertainty involved in cancer risk assessment can be as important as the projection of the risk estimate itself. The sources that might be addressed include:

(a) The statistical uncertainty associated with the given risk estimate;

(b) The variability introduced by the selection of a particular low-dose extrapolation procedure;

(c) When risk estimation is based on laboratory generated data, the biological variability associated with the use of a particular test organism and its scaling or extrapolation to man (Chapter 6, Section II, Part D).

28. An estimate of cancer risk for humans exposed to an agent can be no more accurate than an exposure assessment that it utilizes. Lack of adequate exposure data is frequently a major limiting factor in evaluation of carcinogenic risks for humans (chapter 6, Section II, Part B).

29. While several considerations often enter the risk assessment process, it is important to try to maintain a clear distinction among facts (statements supported by data), consensus (statements generally held in the scientific community), assumptions (statements made to fill data gaps), and science policy decisions (statements made to resolve points of current controversy) (Chapter 6, Section II, Part D).

30. Differences in human susceptibility, and variable and extreme exposures to chemicals suggest the likelihood that there are subpopulations that are at greater than average risk. (Chapter 1, Section III, Part C, and Chapter 5, Section II, Part C). Increased consideration should be given to the identification of high risk populations.

31. Because of the uncertainties associated with risk assessment, a full evaluation of risk to humans should include a qualitative consideration of the basic strengths and weaknesses of the available hazard and exposure data in addition to any numerical estimations that are made (Chapter 6, Section II, Part D).

PART II—STATE-OF-THE-SCIENCE

Preface

This segment of the OSTP carcinogen document contains the details about the topics emphasized in Part I, and, as such, is a summary of the current science in a number of fields important to adequate risk assessment. Special emphasis has been placed on those areas considered to be particularly relevant to estimation of hazard and risk.

This section of the document is composed of six chapters. A chapter on the mechanisms of cancer induction is followed by two on testing, one each for short-term and long-term testing. These methods of hazard evaluation are complemented by a chapter summarizing a number of aspects of epidemiology, which estimates human hazard. There is a chapter on exposure assessment, a critical problem in assessing human risk, followed by a chapter, that draws on the others, which discusses risk assessment as a whole. It is clear that the limited space available in this review does not allow a totally comprehensive treatise on the various subjects involved, and specialists in the various fields may disagree with the importance of certain aspects of the discussion. The focus has been on a balanced presentation of what is consensus in the various fields discussed as they intersect with the need to understand the effects of chemical agents.

Chapter 1—Current Views on the Mechanisms of Carcinogenesis

I. INTRODUCTION

A. Philosophical Perspectives

Underlying the study of mechanisms is the search for causality in biological phenomena. Since science is continually advancing, our understanding of such relationships must be periodically re-evaluated in light of new, generally agreed upon findings. Such a review is especially important in rapidly advancing, highly specialized and interdisciplinary areas that are of significance to public health. The mechanisms underlying induction of cancer represent such an area and, therefore, are re-examined here in light of the findings of the last few years.

This disussion first places our understanding of the mechanisms of cancer induction in a historical perspective and provides a synopsis of some current commonly held beliefs. The bulk of the discussion in the text focuses on the interaction of cancer-related agents with factors both external and internal to an organism and the consequences of such interactions at the molecular, cellular, tissue and organismic levels relative to the development of cancer. The sequence of events presented is not written to advance any specific mechanism of cancer induction, but to indicate the response of various levels of biological organization to cancer-related agents. It should also be appreciated that the data presented have been obtained from studies using various research model systems (e.g. cell-free, cellular, whole animal) to isolate various components of the process derived from various strains and species treated under a great variety of experimental conditions. This approach to the study of cancer is generally accepted for many reasons, primarily the belief that, while biological systems vary, the degree of similarity at the level of their basic processes is greater than at higher levels of integration.

B. Historical Perspectives

Studies in paleopathology have indicated that while cancers occurred in pre-historic times (*1*), their incidence may be increasing concurrent with an increase in mean life span and the development of complex societies (*2*). The study of carcinogenesis is at least 300 years old. During the first 200 years, the studies were primarily descriptive. The involvement of life-styles in cancer occurrence was first observed by Ramazzini (1700) who noted that nuns exhibited a higher frequency of breast cancer than other women (*3*). Subsequently, Hill (1761) associated the use of tobacco snuff with cancer of the nasal passage (*4*) and Pott (1775) noted the occurrence of scrotal cancer with occupational exposure to soot in chimney sweeps (*5*). In the latter half of the nineteenth century, Rehn (1895) published evidence that aromatic amines were associated with bladder cancer (*6*), Unna (1894) and Dubreuilh (1896) associated sunlight exposure with skin cancer (*7, 8*), and Weismann (1881) suggested an association between the "germplasm" (the reproductive or life principle) and cancer (*9*). Over the next 50 years, with the development of appropriate models, numerous agents were found to induce cancer in experimental animals. Over the last 30 years, there has been an increased emphasis on understanding the mechanisms of carcinogenesis and, recently, on the role of factors such as personal habits in the enhancement or inhibition of tumor formation (*10, 11, 12*). Consequently, in the last decade, more progress has been made in understanding the overall mechanisms of carcinogenesis than at any other time in history.

C. Current Beliefs

From the mass of data on carcinogenesis, a consensus has emerged on a number of important scientific issues:

First, cancer can be induced by radiation, biological, "physical" and/or chemical agents.

Second, on a biochemical and molecular level, there are important similarities among mammalian species.

Third, an estimate of the potency of carcinogens may never be exact and may vary with life style, habits, age, sex, individual genetic differences, ethnic background, test strain and/or species, diet, dose rate, route of administration, vehicle or solvent used (if any) as well as the presence or absence of other agents, and the environmental conditions prior to, during, or after exposure.

Fourth, cancer development is a multi-stage process that may involve the genome, both indirectly (frequently termed epigenetic events) and directly, which may include the participation of chemicals or viruses, and which may be modulated by higher order functions, *i.e.* at the organ and organismic level.

Fifth, numerous factors may alter the frequency of cancer induction by altering one or more of these stages.

Sixth, the genesis of a cancer appears to require an alteration in the ability of a cell to elaborate its appropriate genetic program *i.e.* in its information processing capacity, with the subsequent fixation and propagation of that alteration.

Seventh, we still lack an in-depth understanding of the mechanisms and stages of cancer induction and expression.

Eighth, only by understanding the stages of tumorigenesis and carcinogenesis, the substances and processes which modulate them, and how these may differ among cells, organs, individuals, strains and species will we ultimately understand the role of substances, radiations, viruses and/or life-style factors in human cancer.

II. PREABSORPTION MODIFICATION OF CARCINOGENS

Natural exposure to carcinogens occurs by dermal contact, inhalation, ingestion, or a combination of these routes of exposure. Alterations of an agent can occur as a result of interaction with factors that are in association with, but not actually part of, the organism. Examples of these factors are solar radiation interacting with an agent on the surface of the skin, (*13*), interaction with cellular secretions, as in the case of certain inhaled carcinogens (*14*), or metabolic conversions by gut microflora, as may occur after ingestion (*15–24*). An illustration of the impact that these factors can have is exemplified by the role of the gut microflora in the

metabolism of xenobiotics. It has been suggested that the potential for metabolism in the gastrointestinal tract is similar to that of the liver (*20*). However, hepatic metabolism of non-nutrients is predominantly oxidative and synthetic (conjugations), while reactions by intestinal microflora are mostly of a hydrolytic and reductive nature (*20*). It also appears that intestinal microbial metabolism is most important for polar compounds that are not well absorbed from the gut and for those that are excreted, free or conjugated, in the bile (*20*). Examples of this include the metabolism of cycasin to a carcinogenic aglycone (*19*) and enzymatic reduction of a number of azo dyes by numerous genera of anaerobic gut bacteria (*24*). Intestinal microfloral metabolism can give rise to competition or cooperation between mammalian and microbial enzyme systems in sequential reactions in the cells and in the enterohepatic circulation.

Intra- and interspecies comparisons of microfloral metabolism of xenobiotics have been rare. In one study, rat, monkey and human intestinal microflora exhibited the capacity to reduce several benzidine and benzidine-congener based dyes to potentially carcinogenic aromatic amines under test conditions (*15*). Since there is a similarity between the biological effects of these dyes and the effects of benzidine on humans (*25*), this study suggested that, for these dyes at least, gut microflora may play a significant role in the etiology of urinary bladder cancer. Diet composition has also been shown to effect microbial metabolism. Diets with high pectin content can alter the metabolic activities of bacteria in the gut, as well as directly effect hepatic covalent binding of carcinogens (*26*). Further studies in this area, especially in regard to the relation between gut microflora in animals with different feeding habits, *e.g.* carnivore, herbivore and omnivore, and their susceptibility to certain classes of carcinogens would be of interest. Species and individual differences in microflora composition could quantitatively and qualitatively alter the nature of the agent to which the animal is ultimately exposed and, thus, the biological response.

III. ORGANISMIC AND CELLULAR METABOLISM

A. Activation, Detoxification and Reactivation

Carcinogens include biological agents, such as some viruses (*27*), radiations, such as X-rays and ultraviolet light (*28*), "physical agents", such as plastic surfaces and wounding (*29*), chemical agents, both organic and inorganic (*30*), and combinations thereof. Chemical carcinogens occur in a number of chemical classes including polycyclic aromatic hydrocarbons and their derivatives, aromatic amines, azo dyes, nitrosamines, nitrosamides, halogenated hydrocarbons, alkylating agents and metals, as well as in plant and microbial products (*30*). Ordinarily, not all chemicals belonging to any class are carcinogenic, nor are all those compounds within a class which exhibit carcinogenicity equally potent. Carcinogens may be naturally occurring (*31, 32*) (*e.g.* in food (*33*)), result from industrial processes (*32, 34, 35*), or represent the consequences of social activities (*36*). With the exception of a few direct acting agents, the organic carcinogens (*37*) and, possibly, some of the inorganic metal complexes (*38*) require metabolic activation in order to exert their cancer-inducing properties. In general, by whatever route chemicals enter the body, the enzymes involved in biotranformation of a chemical to carcinogenically active (as well as to a number of carcinogenically inactive) metabolites as part of the same mechanisms responsible for detoxication of drugs. Included in this interpretation of the detoxication mechanism is the partitioning of a compound and its metabolites into various body compartments and the effects of differing physiology (blood pH, blood flow to various organs, etc.) on the distribution of the compound and its metabolites. For example, the prolonged retention of acidic urine in dog and man, compared to the rat, can result in very different profile or aromatic amine metabolites than in the rat, due to the effect of a lower urine pH over a longer time on N-hydroxyarylamine conjugates (*39*). Also included in this approach is the total metabolic potential of the organism. For instance, nasal epithelium can activate chemical carcinogens (*40*), a consideration especially important to understanding the effects of exposure by inhalation.

The enzyme systems involved in biotransformation can be divided for convenience into two groups that convert a compound into either a phase 1 or phase 2 metabolite. Phase 1 metabolites are primary oxidation or reduction products, which are usually more water soluble than the parent compound and thus may generally be more easily eliminated from the body. These metabolites may be then conjugated with various soluble intracellular constitutents, forming glucuronides or sulfates, to become even more water soluble and available for excretion from the body. These conjugated metabolites constitute the phase 2 metabolites (*41*), which are usually excreted. However, deconjugation prior to excretion may release an active carcinogen at a site distant from the site of conjugation (*42*).

The reactions that occur in the formation of either phase 1 or phase 2 metabolites require specific enzymes for their conversion (some of the better characterized of which are listed in Table 2). Interactions between a chemical carcinogen and these metabolizing systems may form a large spectrum of phase 1 and phase 2 metabolites. A generally accepted mechanism for the reaction of metabolically activated agents with the cell has been proposed by Miller and Miller (*37*) namely that the ultimate carcinogenic forms of organic chemical carcinogens are electrophilic (electron deficient) reactants that bind with target intracellular nucleophilic (electron-rich) macromolecules such as DNA and proteins. The reactive metabolites formed may also react with other nucleophiles such as glutathione or water (*43, 44*). Through these latter processes, the effects of agents can be neutralized by forming less biologically reactive metabolites that are very polar and may be more easily excreted. The efficiency of this is an important factor in tumor induction. For example, if detoxification by glutathione is efficient, the effective local concentration of the active metabolites of the agent is reduced, which results in decreased binding to macromolecules. However, the electrophilic properties of some chemicals may be enhanced by glutathione conjugation (*45*) although this is usually not the case. Thus, determining the details of chemical biotransformation is desirable when assessing the impact of a chemical.

Table 2—Some Enzymes Needed for Phase 1 and Phase 2 Reactions

Phase 1

(1) Enzyme systems containing cytochrome P-450 and the FAD-containing mono-oxygenase catalyze epoxidation of aromatic rings or olefinic double bonds, produce hydroxylation of aromatic rings or alkly chains, perform oxidative dealkylation, and N-oxidation (44). This system occurs mainly in the endoplasmic reticulum of liver, Kidney, lung and intestine. The enxymes may occur in multiple forms and exhibit different or overlapping substrate-specificity (44).

(2) Epoxide hydrolase catalyzes the hydrolysis of arene oxides and alkene oxides into *trans*-dihydrodiols.

(3) Dehydrogenases, flavin-containing cytochrome-450 reductases and xanthine oxidase catalyze the reduction of nitroaromatics to nitrosoarenes, N-

hydroxyarylamines, and arylamines, and the reduction of azo compounds to amines.

Phase 2

(1) Glutathione S-transferases catalyze the conjugation reaction between glutathione and a variety of electrophilic compounds such as arene oxides. These enzymes exist in multiple forms (44).

(2) UDP-glucuronyltransferases catalyze the conjugation reactions between glucuronic acid with substrates such as phenol and bilirubin. These enzymes are also found in multiple forms (44).

(3) Sulfotransferases catalyzes sulfate ester formation.

(4) N-Acyltransferases and N,O-acyltranferases catalyze N-acetylation and N,O-acyltransfer of aromatic amines and arylhydroxamic acids.

Understanding the metabolism of chemical carciongens is often complicated by the diversity of the enzymatic reactions. For example, for aromatic amines and related compounds, the N-hydroxy derivatives may be the only phase 1 metabolites needed to generate the nitrenium ion-species that bind with target intracellular macromolecules (*39, 46*). Alternatively, the common metabolic activation of nitrosamines and nitrosamides occurs via the formation of diazo intermediates, which subsequently decompose to molecular nitrogen and active alkyl carbonium ions (*30*). To illustrate the complexity of metabolism of a chemical carcinogen, most of the known metabolic pathways and the phase 1 metabolites formed from benzo(a)pyrene, one of the most ubiquitous of environmental compounds known to be a carcinogen in animals, are shown in Figure 1. In this system, many of these metabolites can be converted to different conjugates by phase 2 enzymes. The enzymes cytochrome p-450 and epoxide hydrolase exhibit high selectivity for a particular molecular region and its local conformation (*47*). This results in arene oxides, *trans*-dihydrodiols and *trans*-dihydrodiol-epoxides being formed as specific optically active isomers (enantiomers). These enantiomers exhibit markedly different biological activities (*48*).

Simply knowing the identity of the major matobolites of a compound may not always allow prediction of its hazard. For example, metabolism of polycyclic aromatic hydrocarbons to *trans*-dihydrodiols was long known, but thought to be a pure detoxification process (*49*). However, identification of the *anti*-isomer of the dihydrodiol-epoxide of benzo (a)pyrene, which is derived from the *trans*-7,8-dihydrodiol (Figure 1), as the electrophilic metabolite of benzo(a)pyrene which binds DNA (ultimate carcinogen) has proven that some *trans*-dihydrodiol metabolites can be enzymatically activated (*50, 51*). Additionally, detoxified metabolites formed in the liver may be reactivated in other organs, *e.g.* the bladder (*39*). The metabolic fate of a compound is a function of three processes occurring together (activation, deactivation, and reactivation), whose dynamic interaction results in the local concentration and life time of the active metabolite(s). The combination of whole animal, organ and cellular processes results in a local variable dose-rate of active species with time. These changes may be significant factors in producing the biological effects seen with a particular agent.

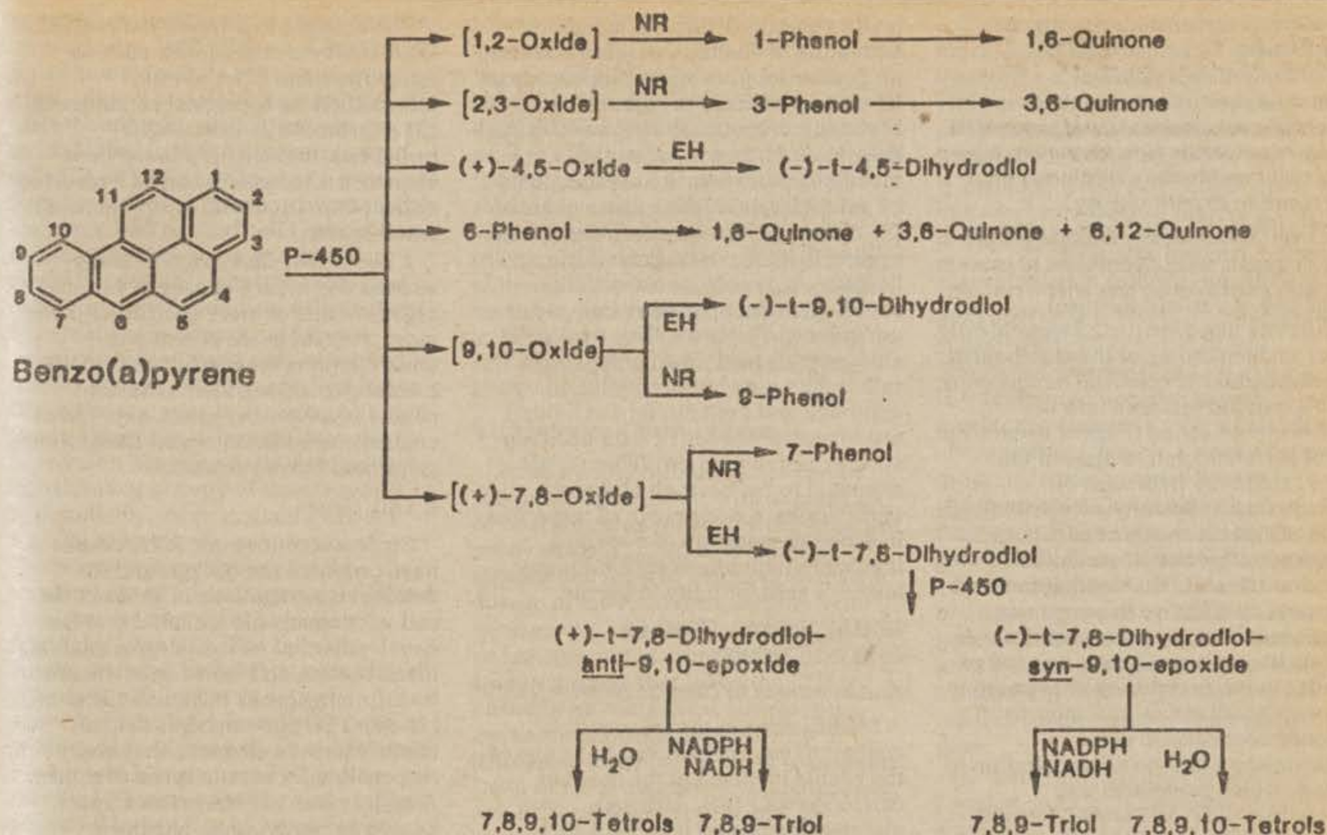

Figure 1. Metabolic pathways and metabolites of benzo(a)pyrene obtained from metabolism by rat liver microsomes. Abbreviations: P-450, Cytochrome P-450 containing enzymes together with cofactors; EH, Epoxide hydrolase; NR, Non-enzymatic rearrangement; t, *trans*.

B. Modulation of Metabolism

Compound metabolism is a function of the three processes in tissues described above, in combination with the cellular, organ and whole animal distribution and excretory processes. This dynamic interaction can be modulated by a number of factors. Such factors include enzyme inhibitors, enzyme inducers, metabolite scavengers (see below), starvation, age, sex, stress,, tissue ablations, nutritional factors, individual genetic differences, and hormonal status (*44, 52*). Various synthetic chemical agents have been shown to act as inhibitors, inducers, or scavengers (*53*) and this diversity of effects has been suggested as well for some commonly eaten foods, which contain naturally-occurring inhibitors or inducers, and which also appear to modulate metabolism (*11, 33*). Briefly, there are three general mechanisms accounting for the modulation of metabolism by inhibitors or inducers (*53*): (1) Stimulation of cellular metabolism which results in an increased deactivation and/or activation. For example, a wide range of compounds including polycyclic aromatic hydrocarbons, phenobarbital, flavones, halogenated hydrocarbons, and indoles induce an increase in mono-oxygenase activity and changes in the relative proportions of the different isozymes. Other enzymes, such as glutathione S-transferases may simultaneously be enhanced. Affinity constants may be altered. As a consequence of any and all these changes, the relative proportions of various metabolites may change, thereby influencing the ratio of metabolic activation to deactivation (*54*). (2) Interference with enzymatic activation of chemical carcinogens to form the ultimate carcinogenic metabolite(s). For example, disulfiram is thought to inhibit dimethylhydrazine-induced neoplasia of the large bowel by directly reducing the rate of conversion of the agent into a reactive metabolite (*53*). (3) Alteration of scavenging (neutralization) of the reactive metabolites by nucleophiles, such as glutathione, or other defense systems, such as superoxide dismutase. For example, starvation, other nutritional imbalances, or compounds such as butylated hydroxyanisole may drastically alter glutathione levels (*55,56*). Decreases in glutathione levels may lead to a decreased proportion of the reactive metabolite being detoxified. Conversely, the increased levels of glutathione and glutathione transferase produced by butylated hydroxyanisole may indicate greater detoxification of a reactive metabolite, provided that the glutathione conjugate products are not reactivated.

However, it is the balance between the activation and detoxification systems for a chemical carcinogen, and their interaction over time, rather than the change in one of these processes *per se*, which is important. If mono-oxygenase activity has been stimulated, resulting in increased reactive metabolite formation, a greater proportion of biologically available carcinogen will result only if the

detoxification mechanisms can not compensate. As an example of compensation, low doses of acetaminophen, whose reactive metabolite reacts with glutathione, will not be hepatotoxic (*57*). However, larger does will deplete the glutathione pool and result in hepatotoxicity. Alternatively, if glutathione levels are decreased, concentrations of electrophilic metabolites may increase.

The use of various chemical modulators has contributed significantly to our understanding of the metabolism and disposition of chemical carcinogens. With extended research into the mechanism of action of these agents, the use of such modulators may, in the future, aid in the treatment of individuals accidentally exposed to high doses of certain chemical carcinogens. However, at present, it would be premature to state that modulators will be generally effective in preventing cancer induction by some chemicals or that such agents could ever be used as an alternative to reducing or preventing exposure to identified carcinogens. An indication for caution is the demonstration that co-administration of aspirin, which modulates the metabolism of N-[4-(5 nitro-2-furyl)-2-thiazolyl]formamide (FANFT), a potent rat urinary bladder carcinogen, will decrease the number of bladder tumors induced by FANFT, at the time however increasing the number of forestomach tumors produced (*58*).

C. Organ and Species Differences

Because the total enzyme level and substrate specificity of the phase 1 and phase 2 metabolizing enzymes exhibit organ, individual, and species differences (*52*), the metabolic patterns of both activation and deactivation of a particular agent could also differ markedly. An example of this is the differences in the monooxygenase activities in nasal epithelium in different species (*59*). Also, there exist multiple forms of cytochrome P-450 enzymes, each of which exhibits its own specificity toward the substrate metabolized (*44*). Since glutathione S-transferases and UDP-glucuronyltransferases also occur in multiple forms (*44*), their particular form in different tissues, strains, individuals or species may also vary. Moreover, the activity of each metabolizing enzyme in a specific tissue of the same strain could vary widely depending on age, sex, nutritional factors, hormonal status, or other factors (*32*). for these reasons, unless one knows the rates of the activation-deactivation processes and the participating isozymes responsible for these reactions, it is impossible to predict quantitatively the metabolic activation of chemical carcinogens from an *in vitro* to an *in vivo* system, or from laboratory animals to humans. Metabolic comparisons between animal species and humans, when the metabolic pathways are similar, could be useful for qualitative asessments of the relevance of a specific animal test system to human risk. Presently, however, a paucity of comparative metabolism data on either induced or noninduced systems exists since most studies are based on an ontogenetic rather than a molecular-evolutionary approach and are therefore of limited use for extrapolation of data between species and organs (*60*). There is also expected to be, because of these variabilities, a wide range of responses in different members of a species, leading to individuals with different levels of susceptibility to agents.

IV. MACROMOLECULAR INTERACTIONS

A. Alterations in Genetic Information

Modification of the information content of cells is believed to be one of the events involved in the onset of carcinogenesis (*61*). Although macromolecules such as RNA (*37*), protein (*62*), and lipid-containing membranes (*63*) will be altered by many carcinogens, and may be important at different stages in carcinogenesis, it is believed that the primary initial site for cancer induction is the DNA. The evidence for this hypothesis rests on a number of observations: (1) The great majority of biological carcinogens that have been examined interact with the DNA of the host cell (*64*); (2) many, but not all, radiations and chemical carcinogens are mutagens and mutations result from alterations in the DNA (*65*); (3) individuals with certain genetic diseases which are characterized by defective DNA repair, such as xeroderma pigmentosum, are prone to cancer at a number of sites and cells from these individuals have increased sensitivity to several radiations and chemical mutagens (*66–68*); (4) ultraviolet (254 nm) light-induced tumor formation in the gynogenetic fish, *Poecilia formosa*, is directly related to the amount of DNA damage remaining after different levels of photoreactivation repair of the DNA (*69*). Agents which by themselves do not induce cancer but influence the expression of cancer cells may or may not act on the DNA. These agents will be referred to throughout the text, but especially in the subsequent sections on promotion, progression and multiple agents.

The changes in information content of DNA can take many forms such as integration into DNA of viral information for biological carcinogens, chromosomal alterations with radiations, and changes in the base sequence after replication as a result of adduct formation with many chemical carcinogens.

1. Biological Carcinogens. Many viruses will induce cancer in various organs of one or more species (*70*). The most progress made recently in understanding the mechanisms of viral carcinogenesis relevant to human cancer involves *onc* genes, expression control elements, dominant transforming genes and human viruses.

a. Onc Genes

Studies on oncogenic RNA viruses have provided the background for detailed investigations of genes in the cell with oncogenic potential and for development of methodologies enabling identification and isolation of dominant transforming genes in human tumor cells (*71–84*). This approach has helped identify genetic elements that may be responsible for certain types of cancer. A major class of RNA tumor viruses, known as retroviruses, has been particularly useful in providing new insight into the cancer process. Retroviruses are unique among animal viruses in their mode of transmission and their intimate assocation with a wide variety of vertebrate species (*85*), factors which may have allowed them to become oncogenic in a number of species. Certain members of this group, the acute transforming retroviruses, appear to have arisen by recombination of the viral genome with cellular genes. The acquisition by these viruses of cellular genes, termed *onc* (for oncogenic) genes (*86*), is associated with the ability of these viruses to induce rapid neoplastic disease in newborn animals and to transform cells, an *in vitro* model for carcinogenesis, in culture. More than two dozen such viruses have been isolated and characterized, and more than 18 *onc* genes have been identified in both avian and mamalian species (*87, 88*). These RNA tumor viruses can be thought of as vectors that carry isolated cellular genes critical for malignant neoplastic disease. These isolated *onc* genes can then be used to make probes for examining human cancer cells for active oncogenic sequences.

The *onc* genes appear to have been derived from the normal genome of a cell which the virus had once infected. These genes are representatives of a gene family that is highly conserved in

vertebrates (and, in some cases, even in invertebrates (*87*)). There are human genes homologous to retrovirus *onc* genes (*88–93*) and RNA transcripts corresponding to specific *onc* genes can be identified in certain human cancer cells (*94, 95*). Most cellular *onc* genes are found in low or single copy number and are expressed in most cell types at low levels (*93*). Also, some *onc* genes are rarely expressed, some are expressed chiefly in specific kinds of cells derived from malignant tumors, and some appear to be expresed only in cells of certain lineage or differentiation states. When the *onc* gene is expressed at high levels, the increase in gene dosage or expression has been correlated with the transforming activity of the virus (*89*). Alternatively, there is some evidence that the removal of a suppressor or regulatory gene, with the possible involvement of an oncogene as a second step, may be important in human retinoblastoma (*96*). The cellular functions coded by most *onc* genes are unknown, although a variety of activities have been found. The gene product of one family of oncogenes, *src*, is a protein kinase, usually tyrosine specific (*97*), similar to the kinase often activated by binding of a number of growth factors to their receptors. Sometimes, only pieces of a receptor are coded, *e.g., erb-b* codes for the integral membrane and large interior portions of the epidermal growth factor receptor (*98*) and *fgr* seems to produce a hybrid protein resulting from the recombination of actin and part of a tyrosine specific gene kinase (*99*). Interestingly, phorbal tetradeconate acetate (TPA) (an important model compound in tumor promotion), (see below), also activates a tyrosine specific protein kinase (*100*), which phosphorylates insulin and somatomedin receptors (*101*). The *onc* gene of simian saroma virus was probably derived from genetic material encoding platelet-derived growth factor (PDGF), which is a growth factor in the type of tissue which bones becomes cancerous as a result of this virus (*102*). Beyond the receptor level, an early effect of PDGF is to stimulate *myc* (*103*), an oncogene that codes for a nuclear protein (*104*). This is especially interesting in that it has been shown that at least two oncogenes working together, *ras* and *myc*, are necessary to transform primary fibroblasts in culture (*105*). Altogether, these data suggest that there is a two part model for oncogene involvement in transformation, one part centering around the stimulation of some growth factor, and the other, around an alteration in the cell nucleus. The first part, as suggested by the data concerning PDGF (*102*) may involve the interaction of a cell with other cells, and the second part may be an intrinsic change in the cell itself. Although this is a speculation, and there are important gaps to information, a general picture of the mechanism of viral carcinogenesis is starting to emerge which also may be very important in understanding chemical carcinogenesis. An indication of this is the evidence that each of nine mammary carcinomas induced by a single injection of nitroso-methylurea in rats contained an activated *ras* gene (*106*).

b. Expression Control Elements

Some normal cell *onc* genes can be activated when linked with expression control elements (ECE) derived from the retroviruses (*71, 107, 108*). The ECE regulate the level of expression or gene dosage of the retrovirus genome in an infected cell and can be used to "switch on" expression of other genes. Recent studies suggest that when the ECE is linked to an *onc* gene it activates the gene's transforming potential by increasing the gene's level of expression (*107*). *In vivo*, the same two factors, *onc* and ECE, which appear to link by chance, have been shown to be responsible for oncogenesis in animals infected with certain retroviruses (*107*).

The viruses from which the acute transforming retroviruses seem to have originated, the leukemia retroviruses, usually cause leukemias or lymphomas, but can also cause a variety of other neoplastic diseases in animals after long latent periods (*85*). When these viruses infect a large population of cells, viral genetic information can integrate at many loci in the host genome. The stable integrated form of the virus (called the provirus) is bracketed by the same ECE described above. These ECE are virus specific and are generated during the integration process (*109*). During the past two years, it has been demonstrated that, in some instances, these viruses will integrate at loci in the host chromosome adjacent to cellular *onc* genes and will cause neoplastic disease (*87, 88*). Similar to the situation described above for the acute transforming retroviruses, this specific integration results in elevated levels of expression of a specific cellular *onc* gene which becomes regulated by the viral ECE. The long latent period required for neoplastic disease induced by these viruses may be associated with the frequency of or time to specific integration in the host chromosome adjacent to a cellular *onc* gene of the viral ECE.

Some radiations and chemical carcinogens might enhance either the frequency of initial integration of the virus or subsequent rearrangement of viral material by inducing chromosomal translocations. For example, in Burkitt's lymphoma, a characteristic portion of human chromosome 8 (carrying c-*myc*) is translocated to chromosome 14, and the site of immunoglobulin heavy chains (*109–111*). This *onc* gene is the same one activated by a retrovirus transcription control element in avian bursal lymphoma disease (*112,113*). Also, c-*abl* is translocated in patients with chronic myelocytic leukemia with a Philadelphia chromosome which is a translocation of the gene from chromosome 9 to chromosome 22 adjacent to an immunoglobulin light chain cluster of genes (*114*). Such translocations might conceivably result in the juxtaposition of an *onc* gene and an ECE, altering *onc* gene expression. The same result might arise from direct genetic insult (via some carcinogen) to the ECE which normally regulates expression of the cellular *onc* gene.

Considerable study is now being focused on ECE. It has been known for some time that certain nucleotide sequences which bracket structural genes are associated with the initiation and termination of gene expression (*115*). However, it was first shown with transforming genes that a region termed "enhancer" must be present in order for these start and stop signals to be recognized (*71,107,116,117*). Although it is not yet known how enhancers function, apparently they can be located in a number of different positions surrounding the structural gene. Moreover, even though the few that have been examined bear no sequence homology, the enhancer sequences from retroviruses have been shown to function and allow expression of a DNA tumor virus, SV40 (*117*). Also, the sequences seem to be utilized in normal differentiation, as shown by the evidence that immunoglobulin heavy-chain class switching in a pre-B cell line is accompanied by gene rearrangement (*118*), and, in the development of Burkitt's lymphoma, the activation of the translocated c-*myc* gene seems to occur by an enhancer in the heavy-chain immunoglobulin locus (*119*). It is possible that translocation into a region controlled by an enhancer or mutations in gene enhancer or activator sequences of cellular *onc* genes could participate in expression of a transforming phenotype.

c. Dominant Transforming Genes. Another area of considerable study during the past two years has been the direct isolation of dominant tranforming genes from human tumor cell lines (*82–84*). This technology is a direct result of studies of viruses and cancer. A biological assay referred to as transfection was used early in tumor virology to demonstrate that host genomic DNA containing dominant viral *onc* genes could be transferred to normal cells (*85*). The early studies were developed and refined using cells transformed with acute transforming retroviruses; they demonstrated clearly that the integrated provirus could be transferred as part of the cellular genomic DNA into specific cell lines capable of assimilating foreign DNA into their chromosomes. By using this technology it has been possible to transfer dominant transforming genes from a variety of tumor cell lines from different species, including human. These include genes from human lung cancer, colon cancer, bladder cancer, and breast cancer cell lines (*82–84–120–122*). Utilizing techniques of recombinant DNA, it has also been possible to identify and isolate (in certain instances) these dominant transforming genes from the transformed recipient cell and to show that these isolated genes are of human origin (*82–84*). In the molecular cloned state, the genes have high oncogenic potential. It is of particular importance that some of these genes (for example, genes associated with human bladder, lung, and colon cancers) are related to a specific family of one of the acute transforming retroviral *onc* genes (*123–125*). In the case of a human bladder cancer gene, it has recenty been shown by three investigative teams that a single base change in a normal human gene results in expression of the oncogenic potential (*126–127*). Also, in the case of chemically-induced tumors, there is suggestive evidence that the activation of an oncogene occurs by a single base change (*106*). The study of RNA tumor viruses has provided an initial understanding of how viral *onc* genes cause cellular transformation. The correlation with human *onc* enes provides an enormous leap in our understanding of causal factors in human neoplastic disease.

d. Human Viruses

Recently, retroviruses of the kind that cause leukemia in animals have been isolated from humans. These human leukemia viruses (HTLV) are known to be associated with several types of T-cell leukemias in man (*129–130*). When they are transmitted to normal human T-cells, they induce a morphology, behavior, and phenotype similar to those naturally occurring in transformed cells isolated from individuals with T-cell leukemia and lymphomas (*131*). This virus, the presence of which correlates with clusters of the disease, thus may represent a true human cancer virus (*132–133*). However, other viruses, such as herpes viruses, especially Epstein-Barr virus (EBV), hepatitis B virus, and papilloma viruses, have also been associated with human cancers (*134*). Although these viruses do not fit all the presently recognized criteria for a true human tumor virus, they may nonetheless influence the development of certain human cancers. Thus, EBV appears to be a stimulus for proliferation of B-cells which may be an early event in the development of African Burkitt lymphoma (*135*). Herpes simplex, a DNA virus, is associated with cervical cancer (*136*), and hepatitis B virus to liver cancer (*137*). Papilloma viruses cause bening skin growths (warts) and some strains have recently been shown to be associated with squamous cell carcinoma (*138*). Much current work is focused on a possible similar role for papilloma viruses in cancers of the genital tract and accessory organs (*139–140*). How, and even whether, these and other viruses play a rule in various types of cancer or interact with various "physical" and chemical carcinogens is poorly understood; but the belief that DNA is a critical target for the biological carcinogens is strongly supported by current studies. New methodology to prepare antibody—using chemically synthesized peptides from nucleic acid sequences—make it possible to generate antibodies against several different *onc* gene products (*141*) and to characterize the molecular properties of the transforming proteins in transformed cells. This method should aid in developing a better understanding of how chemicals and viruses interact with one another.

2. Radiation. Radiations, both ionizing (the corpuscular radiations and X and gamma-ray photons) and non-ionizing (ultraviolet light [UV]), have been shown to act as carcinogens (*28*). Ionizing radiations are ubiquitous, with cosmic rays and naturally occuring radioisotope decay being examples of major sources of background radiation. UV is also ubiquitous, being a component of sunlight.

Carcinogenesis by ionizing radiations is generally related to the special distributions within cells of the ionizations they produce (*28*) as well as total dose, dose rate and fractionation, and target tissue (*142*). The deposition of energy which results from the interaction of radiation with biological media creates ions, free radicals and excited molecules (*142*). The free radicals are thought to be most important product since: (1) Hydroxyl free radicals are involved in some of the biological effects of radiation (*143*); (2) chemicals that are cellular free radical scavengers inhibit the ability of ionizing radiations to transform cells in vitro (*144*); and (3) chemicals involved in protection from free radicals, such as selenium which is important in the action of glutathione peroxidase (an endogenous scavenger), inhibit radiation induced transformation (*142*). Presumably, these data also indicate a role for modulators of metabolism in radiation induced carcinogenesis. Free radicals will react with many cellular macromolecules, but the production of single-strand breaks, double-strand breaks, chromosomal aberrations, and the production of mutations by ionizing radiation, argue that the DNA is the critical cellular target for the biological effects (although the relationship of any of these phenomena with carcinogenesis is not definitive) (*142*). Although experimental dosimetry is much better defined for radiation compared to most chemical agents, the molecular lesion(s) significant for carcinogenesis is (are) unknown and may be different for different radiations. The absence of "radiolabelled adducts," common for many chemical agents, has hampered analysis. One example of a possibly significant lesion is the reaction of the hydroxyl radical with the deoxyribose in DNA, which results in DNA strand breakage (*125*), although many other lesions are formed.

UV photocarcinogenesis is wavelength dependent with UVB (photons with a wavelength of 280–320 nm) being most effective on mouse skin (*146*). Although the photoproducts produced by UV irradiation in cells are myriad (*147*), reaction with the DNA has been shown to be the critical factor in carcinogenesis. There are a number of lines of evidence which suggest this: (1) In patients with actinic keratoses (a premalignant condition) the DNA repair capacity for UV induced damage (UV excision repair) in lymphocytes was found to be less than in matched controls (*148*); (2) individuals with xeroderma pigementosum, who exhibit a predisposition for sunlight-related cutaneous malignancies, although having seven genetic complementation groups, were all found to be deficient in the ability to repair damage induced by UV(*67*); (3) by use of antibodies, DNA

has been shown to be altered immediately and directly by UV irradiation (*149*); (4) the persistence of UV photoproducts after photoreactivation, a repair mechanism which specifically corrects pyrimidine dimers in the DNA induced by UV, is directly correlated with the capacity of UV irradiated tissue to develop into tumors (*69*); (5) the action spectrum (relationship of intensity of a biological response to frequency) of UVB photons is the same for their carcinogenic action and the production pyrimidine dimers in mouse skin (*150*).

It is important when making estimations of risk for chemical agents that we realize what are the origins of many of the tenets upon which these estimations are made. For example, the hypothesized mechanisms of the action of radiation, while not the primary concern of this document, have served, and continue to serve, as the basis for some of the primary models for chemical carcinogenesis (*151, 152*). Much evidence that damage to the cellular DNA is a step in the induction of tumors comes from studies using radiation. Similarly, various models for high-dose to low-dose extrapolation have their origins in studies using radiation and the best data for these models have been obtained from such studies.

3. Chemical Carcinogens. The interaction of chemical electrophiles with DNA, as discussed in the section on metabolism, may lead to covalently bound adducts, intercalations, strand breaks, phosphotriesters, crosslinks, apurinic sites, apyrimidinic sites, deaminations, and/or hydrations, all of which are forms of DNA damage that may alter genetic information (*53, 153–165*). Studies of chemical carcinogenesis have focussed on covalent adducts. Examples of this are the observations that: (1) Simple alkylating agents can methylate or ethylate any of the nitrogens or oxygens in either of the four bases in the DNA as well as the sugar residues and phosphate backbone of the DNA (166); (2) the epoxide derivatives of aflatoxin can attack the N–7 position of guanine (167) (as well as other sites); (3) activated aromatic amines attack the C–8 and N–2 position of guanine (*168, 169*); and (4) the dihydrodiol-epoxide derivatives of benzo(a)pyrene and certain other polynuclear aromatic hydrocarbons attack the N–2 position of guanine and, to a lesser extent, form adducts with adenine and cytosine (*170, 171*). Activated derivatives of these and other carcinogens form similar lesions in the DNA of a number of diverse tissues and species (*171, 172*).

The biological significance of these alterations in the DNA is not always clear, and different alterations may have very different biological effects. Some alterations may cause mutations (*173, 174*). For example, modification of the 0–6 position of guanine will cause this base to be misread, during DNA replication, as adenine (*175*). Also, the major guanine adduct of benzo(a)pyrene, through a transversion, may be misread as a cytosine or thymine in replication (*176, 17*). Alterations in the control of DNA are possible. The amount of methylation of DNA, which may be used in the control of genetic expression (178), can be altered by some agents. Some of the carcinogenic metals may work through alteration of the enzyme that replicates DNA (DNA polymerase) to cause alterations in the genome through miscoding of DNA bases after replication (this will be discussed in detail in the section on fidelity/infidelity of replication).

4. "Physical" Carcinogens. This class of carcinogens encompasses a number of very diverse phenomena including skin abrasion, scar formation, implantation of non-reactive materials (foreign bodies), schistosomiasis, etc. (the term physical carcinogen is also sometimes used to describe radiation, *i.e.* a physical agent). Common factors of this class seem to be: (1) The need for the development of a chronic fibrosis, or at least an inflammatory response; and (2) any chemical properties, *e.g.* of a foreign body, are not important (*29*). An interesting mechanism, with a number of steps, proposed for foreign body carcinogenesis may be relevant to the whole class. During tumor induction, there is an acute inflammatory response with an outgrowth of capillaries as cells adhere to the surface of the foreign body. This response is induced by a number of substances (including mitotic stimuli similar in some ways to growth factors), elaborated by cells in the vicinity of the foreign body. Fibroblasts increase in number, which leads to tissue deposition of collagen (fibrosis). After a latent period, uncontrolled proliferation begins (*179*). The initiation event seems to occur away from the foreign body surface, inducing an initiated cell through changing the environment of the cells in the vicinity of the foreign body (*29*). However, the inflammatory response results in an active cellular proliferation of a number of vascular-derived cells. This step can also occur in the inflammatory response generated by the other "physical" carcinogens. By its presence, the foreign body causes the macrophages to become quiescent and the reactive tissue to develop into a chronic fibrotic state, so that there is surface-in-dependent maturation of the initiated cell (*29*). Asbestos, which is frequently considered a member of this class, may induce cancer by a different pathway. Unlike other "foreign bodies" fragments of asbestos fiber are incorporated into and activate macrophages (*180*). However, the end result is a fibrosis, *i.e.* asbestosis, which, though more diffuse, is similar in many ways to fibrosis induced by the foreign bodies (*29*). The size of the asbestos fiber and the possibility of chemical effects because of fiber dissolution of adsorbed molecules make direct comparison with foreign body cancers difficult.

It is not clear how, if at all, this class of carcinogens interacts with the genome. The range of speculations include: (1) No interaction with the genome, with the recruitment of omnipresent previously initiated cells by a proliferative stimulus into neoplasia; (2) the environment of the inflammatory response, especially the production of free radicals by macrophages, causes chromosomal damage and rearrangement resulting in transformation; (3) a stimulation of proliferation may result in mutation as a result of infidelity of replication (see below); however there is little information in the area.

5. General Statements. The interaction of electrophiles, or the products of irradiation, with DNA can play a role in the induction of a tumorigenic phenotype through either of two mechanisms: (1) Directly by altering genetic material through a somatic mutation (*173*); or (2) indirectly by altering gene expression (*178*). For example, in the former case, somatic mutation can arise from the direct interaction of DNA with electrophiles, free radicals derived from carcinogens, or with activated oxygen produced by metabolism of the agent. In the latter case, the alteration of gene expression can arise from either: (1) A direct mutational event in a regulatory gene controlling expression (*e.g.* base transition) (*173, 181*); (2) changes in gene expression resulting from an increased rate of abnormal base incorporation due to enhanced DNA synthesis, or abnormal methylation of nucleic acid bases (*178*); or (3) induction of genetic transpositions or gaps in the DNA leading to an alteration in gene expression (higher order alterations) (*12*). Direct alterations in the informational content of cells seem to be common for many, but not all, carcinogens. The potential involvement

of an *onc* gene with either direct or indirect alteration of the genome has been discussed in a previous section.

Understanding the significance of these changes may now be possible with the recent development of new methods to determine the structural alterations caused in DNA by chemical carcinogens and radiation (*183*). This has occurred as a result of recent advances in spectroscopic and spectrometric instrumentation, especially in the areas of nuclear magnetic resonance spectroscopy and mass spectrometry. Significant progress has also been made in understanding the effect of carcinogens upon DNA's secondary structure (*184–186*). For example, recent studies have demonstrated that while methylating and ethylating agents do not induce major steric changes in DNA, aromatic amines induce "base displacement" (*187*). Additionally, 2-acetylaminofluorene appears to induce a flip from the conventional right-handed B-helix of DNA to the left-handed Z form (*170, 187*). Such changes, while not induced by all chemical carcinogens could, if they occur *in vivo*, suggest mechanisms by which chemicals induce major steric alterations in the structure of DNA. The biological consequences of such changes, however, are uncertain. Even newer techniques are being developed, such as radioimmunoassays using monoclonal antibodies against specific forms of DNA damage (*188*) and reversed labeling methods that are able to detect a single damage base per cell (*189*), that may eventually allow routine estimation of DNA in humans.

V. MODULATION OF ALTERED GENETIC INFORMATION

For many carcinogens, it is necessary for cellular proliferation to occur before the damage induced by a given agent is expressed. For instance, a cell replication must occur within 48 hours to express X-ray induced transformation in culture (*190*). Replication must also occur *in vivo* in liver (*191*). One way to think about the process is that a cell is first "primed" for alteration by interaction with a carcinogen; cell replication then results in at least one daughter initiated cell, *i.e.* a cell irreversibly altered so that it can interact with the proper stimulus to form a malignant cell.

The alteration of genetic information that produces the initiated cell is modulated by a number of factors. Two of the most important are: (1) The amount of DNA damage (adducts, strand-breaks, etc.) that is removed from the "primed" cell and its daughters (DNA repair); and (2) the accuracy of DNA synthesis (fidelity/infidelity of replication), both as a result of damage to the genome and as a result of changes in cellular parameters (ion concentrations, etc.).

A. DNA Repair.

The processes of DNA repair (Table 3) are believed to be some of the primary modulators of physical and chemical carcinogenesis. The bulk of evidence for this assertion comes from studies implicating UV and ionizing radiation as etiologic agents in cancer (*193*). For example, as discussed above, individuals with xeroderma pigmentosum who are defective in repair of UV-induced and some forms of chemically induced DNA damage (*67*) exhibit a high incidence of light-related cancers (*194*).

Table 3—Mammalian DNA Repair Mechanisms

1. Nucleotide excision repair—a mechanism that removes bulky, noncoding lesions from the DNA in a manner similar to, but not identical with, bacterial nucleotide excision repair (163, 197–203).

2. Base excision repair—a mechanism that permits reinsertion of the proper base into the gap left in the DNA by the action of enzymes that effect the excision of inappropriate bases from DNA as the free base (DNA glycosylases), avoiding scission of the DNA backbone (204–207) as well as a system similar to the bacterial one requiring strand scission (208–213). Included as part of this form of repair, following Setlow (214), are direct demethylation by transfer of a methyl group to an acceptor protein (215, 216) and AP site repair (direct repair of a removed base) 205–207, 217).

3. Strand break repair—the process by which single and double-strand breaks are rejoined with the addition of a few or no additional nucleotides through the action of a sealing enzyme (218–223).

4. Photoreactivation—a light activated enzymatic mechanism specific for the breakage of the UV induced covalent bond attaching two pyrimidines in a cyclobutane-type ring (224–230).

5. Recombination repair (post-replication repair)—a system once believed to occur in mammalian system, although now controversial, by which bulky, noncoding lesions were transferred to DNA synthesized after damage (231–234).

6. Replication bypass (post-replication repair)—a process that may function in mammalian cells as an alternative to recombination repair in which the bulky lesions are bypassed and the gap created filled (235–236).

7. Inducible DNA repair systems—the most recently described and still speculative (for mammalian systems) repair system in which DNA damage triggers the induction of enzyme systems to remove the damage (237–243).

More generally, the available data are equivocal as to whether there is an increased risk of development of cancer of the internal organs (*68, 195*). Problems with interpretation of these observations include: (1) Chemically-induced and UV-induced damage may induce cancer by different means; (2) organ specific differences exist for cancer induction; and (3) the time to tumor may differ significantly between organs and death of the individual due to complications arising from the skin tumor prior to the onset of cancer in an internal organ or tissue. It is also possible, however, that the data bases were too small to support any conclusion and that a larger study should be conducted.

Because of the importance of DNA damage, a number of short term bioassay systems that measure it, directly or indirectly, have been developed to screen for potential carcinogens (see Chapter 2, this volume). One modulator of the amount of DNA damage an agent causes, DNA repair, has been studied in numerous *in vitro* and *in vivo* biological systems (*196*).

From these studies, various pathways for repair of numerous forms of DNA damage have been identified (Table 3) (*197–243*). Many of the processes of repair in mammalian cells are similar to those described in bacteria and thus it is assumed that these systems have been conserved during the course of evolution. It has been speculated that the primary role of these systems in evolution was to protect the genetic information of the species from both endogenous and naturally-occurring exogenous DNA damaging agents, especially long enough for the organism to replicate (*244*). All species examined thus far possess at least one form of DNA repair (214).

Since the nature of DNA lesions formed by agents which are genetically toxic, and the methods of their repair, are so similar across species, the use of prokaryotes to screen for this particular form of genotoxicity seems reasonable. However, the greater complexity of the mammalian systems require that care must be exercised in the interpretation of bacterial data. For example, chromatin structure in mammalian cells appears to make some regions of the DNA more accessible, and others less accessible, to adduct formation and repair (*245–247*). Furthemore, significant differences in the repair efficencies between tissues (*248*) and species (*249-260*) have been observed. In terms of general understanding of the signficance of these differences, it is known that among the placetal mammals there is a good correlation between the maximum achievable lifespan of a species and the ability of primary fibroblast cell cultures

from these species to perform unscheduled DNA synthesis following UV exposure (*260*), though some details of the correlation are controversial (*257*). It is not known whether there is any such relationship for DNA damage induced by chemical electrophiles. Also, in animals, the organization of cells into tissues presents other complicating factors. One example, based on tissue interactions, involves the complex effects of xenobiotic liver metabolism on the activated agent concentration in other tissues. Another involves tissues that are less active mitotically than others. The longer cells are given to perform DNA repair prior to DNA replication, the less likely it is that a transformation event will occur (*194, 256*). Still another example may be the maintenance of many cells in stationary phase (G_0) in a tissue. Those cells could be triggered into DNA replication, and run the risk of mutagenesis, by an agent delivered in a dose suffcently high to induce cell killing (see below, section on hyperplasia) (*261*). Since the latter possibility has been used to explain the apparent threshold effects seen in various long term low-dose vs. high-dose carcinogensis experiments using nitrosamines, by presuming that the high dose induces cell killing and resultant division leading to effects that are only seen with a high dose (*261*), understanding factors such as those are important in assessing risk. Other complicating whole animal effects include diet, age, and hormonal status, all of which impact DNA repair (*262-264*).

Many biochemical mechanisms are involved in the repair of DNA damage, and many factors may modify their effectiveness. One mechanism is a nuclear enzyme system which polymerizes activated ADP-ribose residues to form poly(ADP-ribose) (*265*). The biological function of ADP-ribosylation is not clear, however there is a relationship between it, DNA damage and DNA repair, which may involve the capacity of the system, demonstrated *in vitro*, to utilized damaged DNA (*266*). This system also seems to influence the production of sister chromatid exchanges (*267*) and has some relationship to mutagenesis and carcinogenesis, although not a simple one (*266*). Another mechanism, recently brought to light by the identification of a series of enzymes, collectively referred to as the DNA glycosylases (*209-213,268-274*), active in base excision repair (Table 3), has indicated that there is much greater specificity for the various kinds of DNA damage than was previously thought. Over a dozen of these enzymes have been isolated from bacterial and mammalian systems, each involved in the repair of a specific form of DNA damage. The apparent lack of nonspecific DNA damage recognition enzymes in adult mammalian tissues and the high degree of specificity of those repair systems that have been studied suggest that a series of enzymes can recognize many forms of DNA damage, including those induced by man-made substances. Another mechanism recently discovered involves an inducible antimutagenesis repair system in bacteria (*275*). An inducible system, considered part of post-replication repair, was also found in mammalian cells (*238, but see 239*). Los doses of certain mutagenic agents appear to induce a response in the liver that enhances the repair of damage subsequently induced by those agents. For example, in rat liver, an inducible protein transfers the intact methyl group, added to the uanine base by the potent carcinogen dimethylnitrosamine, from the DNA to a cysteine residue. Pre-stimulation of the animal with small doses of the compound can enhance this inducible repair system by as much as 3-fold (*216*).

As an example of the relation between knowledge of mechanisms and resolution of policy questions, one can consider the relationship of DNA repair systems and a threshold phenomena in carcinogenesis. In simple terms, the threshold concept maintains that there is a level of exposure to an agent below which there is either no observable effect and/or *no* adverse biological effect, as a result of some reserve (*e.g. see 276*). The practical manifestation of this concept is a dose-response cure which does not appreciably differ from zero risk at some non-zero dose. The concept is used to evaluate many toxic phenomena, but has been a source of controversy in the evaluation of carcinogenicity.The existence of DNA repair systems has been used by some authors to support speculation that a threshold exists for the biological effects of chemical carcinogens. Such a speculation is not supported by present data, as is shown below. The following analysis will concentrate on the question of a threshold for DNA damage and it must clearly be recognized that DNA damage is probably only one part of a complex chain of events leading to carcinogenesis. A threshold, or lack of threshold, for DNA damage may be a different matter than a threshold, or lack of threshold, for carcinogenesis.

First, no DNA repair system reduces DNA damage to zero. Consistent with this observation is the observation that DNA damage accumulates in postmitotic tissue, as a function of age even in the absence of known chemical exposure (*277*). The repair proficient systems found in dividing *Escherichia coli*, when fully induced, can reduce mutagenesis by up to 5,000-fold, but the mutation rate in exposed cultures is still 10 times that which is produced spontaneously (*278*). The fragmentary data from mammalian cells suggests that there may be an inducible system (*278*), but it would be unreasonable to assume that replicating eukaryotic cells would be at less risk than prokaryotic cells from low doses of a mutagen. Second, not all DNA repair systems are error-free. While the data on inducible error-prone bypass mechanisms in mammalian systems are meager (*278*), virus reactivation, which is likened to a mutagenic effect, has been well established in eukaryotes (*240, 278*). This suggests that mammalian cells also possess an error-prone bypass system. Third, major variations in DNA repair efficiency occur between cells, tissues, strains and species exposed to similar doses of the same carcinogen (*249-264*). This heterogeneity limits generalizations about the effectiveness of repair systems, constitutive or inducible, relative to the completeness of repair or the notion of a "safe" threshold. Tissue differences in repair might result in a dose response relationship indicating a threshold for repair in one tissue, but a relationship indicating no threshold for repair in another, thereby providing little hope for uniform protection within an organism. In tissue or cell culture, repair appears to vary between cells. Thus, even if biochemical analyses, which normally measure the average repair capacity of a heterogenous population of cells, demonstrate that a tissue or cell culture is repair proficient, the possibility of repair deficient subpopulations cannot be disregarded.

It is clear that DNA repair will not produce a threshold for DNA damage. However, it is not clear what the significance is of the DNA damage which results from exposure to exogenus agents. Since some damage results from normal metabolism and, probably, exposure to chemicals in foodstuffs (*33*) over the life of the animal, the importance of the additional burden induced by a carcinogen above this backround is hard to determine. As noted above, since DNA damage is only a part of the complex process leading to the production of cancer, this determination probably can only be made when a clear understanding of the

mechanism(s) of carcinogenesis is achieved.

B. Fidelity/Infidelity of DNA Replication.

Another modulator of the alteration of genetic information is the fidelity of DNA replication. Although it is not proven that the role of DNA replication in initiation is to stabilize incorporation of the effects of DNA damage in the genome ("fixation") (*279*), it remains the most attractive possibility, since the fidelity of replication (*280*) will help determine the integrity of genetic information in the initiated cell.

Before one considers the ways fidelity can be altered, it is important to understand the consequences of error accumulation in DNA in nondividing somatic cells (*244*). In dividing cells, one is concerned with replicative error propagation; that is, the transmission of mistakes from one generation to the next. Mechanisms that enhance the frequency of such mistakes could involve alterations in the DNA synthetic apparatus and have been referred to as intrinsic mutagenesis (*281*). In non-dividing cells, one is concerned about the effect of accumulated unrepaired DNA damage on cell function. When considering mechanisms in carcinogenesis, attention is normally limited to either dividing cells or to resting cells destined to undergo at least one further division cycle. With our present understanding, a cell which fails to undergo further division is not usually considerd pivotal with respect to neoplasia. However if amitotic cells become, through some mechanism, able to divide, or if non-dividing cells are involved in the control of dividing cell populations, the accumulation of damage in them can be significant. For instance, non-dividing neurons control the level of thyroxine, and it has been shown that this hormone is an important factor in X-ray transformation of cells (*282*). Therefore, although usually not appreciated, the effects of agents on non-dividing cells should not be ignored.

In dividing cells, a repaired or inadequately repaired DNA lesion, such as an adduct, when present on the DNA template at the time of replication, could result in a genetic alteration, e.g. base substitution, such as replacing a guanine with a cytosine. However, damaged DNA need not always cause significant base substitutions during DNA replication. First, parental cells containing the damaged DNA may fail to divide and thus be progressively diluted during cellular proliferation. Second, due to the redundancy of the genetic code, as well as other factors, approximately 25% of incorrect base substitutions may not result in amino acid substitutions. However, a number of modification of the DNA template or of the DNA polymerases have been shown to cause incorrect substitutions during copying by DNA polymerases *in vitro* (*283, 284*). If these modifications are not corrected by subsequent DNA repair, it is a reasonable expectation that the unrepaired lesions will in time result in either mutations or cessation of cell reproduction (*283, 284*). If these modifications are not corrected by subsequent DNA repair, it is a reasonable expectation that the unrepaired lesions will in time result in either mutations or cessation of cell reproduction (*258*). Alkylating agents and carcinogens have been shown to induce mis-incorporation *in vitro* by modification of DNA templates and by removal of purines from the DNA (*285–290*). Also, the induction of an error-prone repair pathway (SOS) may cause problems since the underlying concept of this repair pathway is that it can alleviate potentially lethal damage only at the expense of inducing mutations (*275*).

Even if the templates is undamaged, other factors which can be altered by carcinogens both directly and/or indirectly, may cause the template to be copied incorrectly. Table 4 lists some factors that have been found to diminish the fidelity of DNA synthesis *in vitro* in two different systems, (*285*) and may have to be considered *in vivo*. During replication, the fidelity of DNA synthesis is altered by only a few types of changes in the reaction conditions. The error rate of mammalian DNA polymerases *in vitro* has been shown to be dependent on the ratio of correct to incorrect nucleotides with polynucleotide and natural DNA templates (*291, 292*). There is evidence that alterations in cellular nucleotide pool sizes and content are mutagenic (*293–295*), and the cellular homeostatic mechanisms for precise maintenance of deoxyribonucleotide concentrations may be altered by various chemical agents. Furthermore, agents may directly alter some of the nucleotides in the pools available for replication, leading to possible misincorporation (*296, 297*).

Table 4—Changing Reaction Conditions That Diminish the Fidelity of DNA Synthesis

1. Ratio of incorrect to correct nucleotide substrates (291–297).
2. Type of incorrect nucleotide substrates (293–297).
3. Different metal activators for DNA polymerase (285, 288, 292).
4. Nonactivating metal mutagens and/or carcinogens (285).
5. Depurination of template (290).

Lindahl and Nyberg (*298*) estimated that the *in vivo* rate constant for thermal depurination is approximately 3 x 10^{-11} sec $^{1-1}$ suggesting that as many as 10,00 purines may be lost from the genome of a mammalian cell per generation time (20 hours). Furthermore, this rate consfant may be significantly increased after alteration of bases by alkylating agents (*298*). The intensity of this damage formation suggests that during replication, the cellular mechanisms for correcting depurinated sites on DNA might not be able to repair every site before the polymerase complex involved in replication encounters a damage site. Thus, replication over a damaged site may be a secondary result of cellular interaction with a carcinogen. Lastly, mutagenic/carcinogenic metals, not reacting directly with DNA, have been shown to affect the fidelity of DNA synthesis *in vitro* (*285*). Thus, various agents that do not damage DNA directly or enhance the production of metabolites that can induce such damage may exert their effect by alltering the fidelity of DNA replication. Methods to assess or screen for such agents in mammalian cells are generally lacking. The end result of these processes is that a daughter cell can have an irreversible alteration in genetic information (*279*) even though the apparent cell which interacts directly with agent does not. This altered cell may be similar to normal cells in many respects, but may be capable of giving rise to a cancer, *i.e.* be initiated, and may lead to carcinogenesis if, during the lifespan of the animal, it encounters an appropriate stimulus, *e.g.* perhaps a "physical carcinogen".

As previously noted, it is not clear what role DNA synthesis has in intiation though it has been shown that manipulation of the rate of cellular proliferation under some conditions may modify tumor incidence. This has been demonstrated in : (1) Partial hepatectomy in animals treated with methylnitrosourea, dimethylnitrosamine (*279*), benzo(a)pyrene and radioactive particles that localize in the liver (*299*); (2) chemical treatment combined with diet changes, drugs or natural physiological agents such as hormones or bile acids (*279*), and (3) the stimulated hyperplasia resulting from the toxic effect of carbon tetrochloride (*300*); and (4) regeneration in skin (*301*). Much of these data are hard to evaluate however since some of the treatments induce a chronic inflammatory response, especially the skin regeneration work which utilizes skin abrasion, and it is not clear whether one is studying the effects of proliferation or the

inflammatory response, which is probably an important factor in carcinogenesis by "physical agents". On the other hand, retinoids, which stabilize the differentiated nondividing state of epithelial cells among their other biological effects, have been used to inhibit mammary and colon carcinogenesis (*302, 303*).

The doses of agent used to produce cancer experimentally may be much lower than those doses which cause frank cytotoxicity for a number of reasons. One is that a cytotoxicity could be a stimulus for cellular proliferation which could result in hyperplasia (*304*) (usually termed restorative or reactive hyperplasia). This replication may act to "fix" changes in cells previously "primed" by some unknown stimuls or it may result in replication errors through some of the mechanisms suggested above. An example of this hyperplasia is the effect of carbon tetrachloride in mice (*300, 305*). This agent has no or only minimally detectable genetic toxicity in most bacterial tests, but induces tumors while causing substantial necrosis in the target tissues of mice. Another example may be foreign body carcinogenesis, as discussed in Section IV.A.4., which may induce some necrosis at a site.

The possibility that hyperplasia can lead to carcinogenesis has led to speculation that a cytotoxic mechanism is important in the carcinogenic effect of all carcinogens which do not act directly on the DNA that are tested at a high dose (*306*). This hypothesis overlooks the obvious problem that although cytotoxicity can induce certain changes in cells, it is by no means the only way an agent can alter either replication or gene expression. For example, certain compounds can stimulate hyperplasia without significant cytotoxicity (*307*). Furthermore, it has been shown many times that neither the induced hyperplasia (*308*) nor the irritant properties resulting from promoters such as PMA (*309*) are sufficient to explain carcinogenic effect. Thus, a cytotoxic action should be considered simply as an additional possible mechanism of carcinogenesis and not equated to an explanation for carcinogenesis for all non-directly-genotoxic agents tested at high dose.

Whatever mechanism(s) is (are) important in the formation of initiated cells, it is clear that cells irreversibly altered in a heritable fashion are induced during this process and that these are the progenitors of further new cell populations during the carcinogenic process. The properties of these cell populations are discussed in the next section.

VI. CANCER FORMATION

How initiated cells with altered genetic information result in a cancer seems to be the least understood area of carcinogenesis. The process can be divided into two operational stages, promotion and progression. Promotion will be used to describe the process(es), usually initially reversible in test systems, but later irreversible (*310*), that results in cellular proliferation and terminates when one or more neoplastic cells, *i.e.* cells with the ability to grow autonomously, is (are) formed. Neoplastic progression will refer to the next step(s) in which cells become able to form tumors and become malignant with some potential to metastasize.

A. Promotion.

1. Promoter action. A promoter is experimentally defined as an agent which results in an increase in cancer induction after long-term application subsequent to treatment of an animal with an initiator. Under otherwise identical conditions, neither initiator nor promoter alone induces many tumors, nor does a regiment with reversed order (treatment with promoter followed by an initiator) (*308*). At a particular dose level and treatment schedule, a carcinogen that does not require supplemental promoter activity is termed complete. Many or all complete carcinogens act as both an initiator and promoter in their interaction with the organism and some act as initiator or promoter for different tissues in the same organism at the same time (*311, 312*). Also, the promoter action of a complete carcinogen may not act by the same mechanism as promoters used in initiator-promoter paradigms (*309*). Separation of these two aspects of carcinogenic action is frequently difficult. However the term remains a convenient description for one part of an experimentally isolated complex process. For the most part, the phenomenon of tumor promotion has been most carefully delineated in skin and liver carcinogenesis. It remains to be established more firmly with cancer at other sites though there is suggestive work in colon, breast, kidney, central nervous system (*279*) and bladder (*307,313*) carcinogenesis. At present, given the lack of information on mechanisms of carcinogenesis in humans and the myriad interactions of tissue and the biological effects of promoters demonstrated in experimental systems, it is premature to designate agents as acting through only a promotion-type mechanism although there are suggestions of promotion by a number of agents.

This experimental definition will not clearly differentiate between promotion and progression since is measures only the production of a malignant tumor. In practice, however, laboratory studies of promoter action are usually ended after a focal mass of cells (*e.g.* papilloma, nodule) is observed (*307*). The appearance of the first neoplastic cells after the formation of these focal masses has been proposed as a definition of the end of promotion (*279*). This definition is useful since it emphasizes the frequently seen reversible effects of promotion, *i.e.* regression of the direct results of a promoter (*e.g.* papilloma) with termination of its use, while clearly indicating that the role a promoter has in carcinogenesis is to induce a presumably irreversible event, a neoplastic cell. A similar definition is suggested by the separation of promotion into 2 stages, based on work in skin promotion in mouse (*310*). Stage one is partially reversible, while stage 2, which requires cell proliferation, is initially reversible and then becomes irreversible. Many of the focal masses induced by promotion are reversible, especially in skin and liver (*279*). How these non-neoplastic masses originate is not completely known, but they seem to arise through the differential stimulation of cells altered by the initiator to respond to the promoter (*279*). Of the persistent masses, in all systems studied, almost all do not become neoplastic *i.e.* acquire growth autonomy (*192*).

Promoters are usually thought of as exogenous compounds. Many of them are plant products (*307, 314*) such as certain of the phorbol esters (*e.g.* 12-0-tetradecanoylphorbol-13-acetate [TPA] or phorbal tetradeconate acetate [see Section IV.A.1], which is also known as phorbol myristate acetate [PMA]). However, there is also evidence that certain compounds normally present in the body (endogenous) may also be promoters (*315, 316*). Promotion by such endogenous compounds may be difficult to demonstrate since production of the compound in the body must usually be altered (which normally has many ramifications) to observe an effect, as opposed to exogenous compounds, whose levels are controlled by direct application to a tissue. Another type of interaction that seems to have promoter action occurs in foreign body tumorigenesis (*193*). Although it has been claimed that a single event determines the sarcoma which results from the implantation (*179*), it is clear that there is a step where pre-neoplastic

cells of probable vascular origin (produced in a surface-contact independent step) require a surface-contact dependent step to become neoplastic (*179*). Like other promoters, removal of a foreign body before the last step will, as a rule, prevent the sarcoma from developing. Thus some surfaces, as well as exogenous and endogenous compounds, seem to be able to act as promoters.

Despite recent progress, the mechanism of action of PMA, the model promoter, is not completely clear. There is good evidence PMA binds to a receptor (*317, 318*), that activates protein kinase C (*319*), which phosphorylates insulin, somatomedin (*99*) and a tyrosine-specific phosphorylation, similar to a number of growth factors. Other promoters interact with a cell receptor (*307, 320*). Also, estrogens, suggested to be promoters of mammary and liver tumorigenesis (*321*), are known to produce their main biological actions through receptors (*322*).

PMA produces profound biological effects *in vivo*, including edema, erythema, inflammation and cellular proliferation (a property which allows the promoter to participate in "fixation" if given within a short enough time after the initiator dose). But these same effects can be produced by agents that are not promoters (*307*). By stimulating specific separate effects of PMA in other ways, one can define at least two stages in promotion (*310, 323, 324*), supporting the hypotheses that cancer is a multistep process and that more than one aspect of PMA may be important in promotion. *In vitro*, promoters have been shown to induce an array of diverse morphological and biochemical changes (*307, 321, 325*). One of the most important seems to be the interaction of PMA and other promoters with cells to generate free radicals (*326–328*). A similar stimulation is seen with the use of either ionizing radiation or activated oxygen (*328*). The capacity to stimulate radicals seems to be correlated with promoter potency to generate tumors (*326*) and varies in a dose dependent manner for the strong promoter PMA (*329*). PMA can also: (1) Cause dedifferentiation or inhibit terminal differentiation (*330*); (2) stimulate cellular differentiation (*331*); (3) alter macrophages (*332*); (4) induce chromosomal alterations (*333*) and sister chromatid exchanges (SCE) (*334*); and (5) induce virus synthesis (*335*). It is not known which, if any, of these effects are central to the role of promoters in carcinogenesis since evidence for all models of promotion is indirect. However, the effects on chromosomes may be especially relevant.

PMA can induce chromosomal anomalies such as SCE, although the concentrations (100 ng/ml) required for this effect are quite high (*334*). The activated oxygen species produced by the promoters, perhaps through an interaction with membrane components, can induce chromosomal damage and a soluble substance which induces chromosomal abnormalities (a clastogen) in nearby cells (*333*). However, the promotional effects of PMA are reversible unless it is given for a fairly long time and a chromosomal change, such as an aberration, is almost certainly not reversible. This suggests that any chromosomal alteration would probably have to be induced by prolonged exposure to the promoter (*187, 308*). This analysis is consistent with the observation that papillomas, which emerge in a common response to promoter action in skin carcinogenesis during the stage when it is reversible, are primarily associated with a diploid karyotyed whereas malignant tumors usually have karyotypic alterations (*336, 337*). Although the selection process for cellular characteristics important to neoplasia may occur during the entire promotion process (indeed selection may be important even during initiation [*279*]), a certain type of chromosomal alteration might be the final event which results in a neoplastic cell. Also, the need for prolonged exposure, leading to a relatively large cumulated dose, may be why such high doses have to be given to observe the effect in short-term experiments *in vitro*.

Chromosomal changes, although not proven to be responsible for growth independence of neoplastic cells, are implicated in transformation (*338*). For example, SK–L7 human lymphoid cells markedly increase their cloning efficiency in soft agar and decrease their doubling time from 30 hours to 14 hours upon introduction of an additional chromosome 15 (*339–341*). More directly, a clastogenic effect is produced by cells from an individual with Bloom's syndrome, a clinical entity in which there is usually a high cancer incidence (*333*). Specific chromosomal transpositions, as seen in the case of the lymphoma, can be associated with changes in the regulation of gene expression and also could be associated with phenotypic changes such as differences in antigenic expression and unresponsiveness to regulator of cell growth.

The hypothesis that a chromosomal change is a causal factor in cancer induced by promoters is attractive, especialy when viewed with the evidence concerning the effects of transposition in oncogenes (see Section IV.A.1); however, few data directly support this contention (*195*). Although there is a lack of methods to measure such changes readily in vertebrates, this hypothesis is interesting since such genetic alterations: (1) Are much more drastic than localized changes in base sequences; (2) may be either site-specific or non-site-specific; (3) do occur at different frequencies; (4) appear to affect the stability of neighboring genes; and (5) are vitrually irreversible. In rapidly multiplying bacterial cells, conventional mutagens do not enhance the frequency of transposition (*342*); however, factors that trigger transpositions or govern their rate of movement are not yet understood well enough to determine whether mutagens enhance transposition in mammalian cells *in vivo*. Large-scale changes, such as rearrangements and deletions, rather than the small changes repairable by excision repair that can be detected in the usual tests for mutagenicity may be important components in humam cancer (*195*). While it appears that localized lesions in DNA can be carcinogenic (*54, 343*), it is not clear whether such lesions might either independently or through the direct or indirect induction of major genetic alterations (*344*). Furthermore, this analysis is limited since one is drawing general conclusions about malignancy from the pathogenesis of a rare disease in persons with a limited life span.

Evidence is starting to accumulate that both oncogene activation and treatment by some promoters have some aspects in common, *i.e.* the activation of some growth factor-like stimulus. For some cases, this stimulus seems to need some second factor, another oncogene involving a nuclear protein in the case of oncogenes, or Phase II promoters, involving replication, to cause transformation. In this light, chromosomal changes might be seen as merely a possible, but not obligatory, method to accomplish the same end, with the final, chromosomal change equivalent to the second oncogene activation or the effect of proliferation and other changes in the case of the PMA stimulation.

Cancers in humans from causes such as exposure to cigarette smoke or exposure to benzidine in the workplace have a very long latent period. It is not clear whether this long period is the time from exposure to the production of a neoplastic cell (promotion) or the time necessary to go from a neoplastic cell to a malignant tumor (progression).

Evidence that the carcinogenic risk of cigarette smoking declines with the length of time after smoking stops (*345*), suggests that most of the latent period is partially "reversible" and therefore probably occupied by a phenomenon similar to promotion.

This is also suggested by a recent model for the development of Burkitt's lymphoma, which seems to be a virally produced cancer (*346*). According to this model: (a) A lymphoma clone develops in one or more steps from B lymphocytes that have acquired neoplastic characteristics due to EBV transformation, and (b) the fully malignant phenotype occurs only when the lymphoblastoid cells acquire a specific chromosomal translocation that involves the immunoglobin locus. The long latency period seen in the disease has been suggested to arise from the need for a promoter-like stimulus that may cause proliferation and interfere with the differentiation of the long-lived initiated B cells (*337*).

Since neither initiator nor promoter alone is sufficient to cause tumors and since promotion appears to be involved in the carcinogenic process, especially in time to tumor induction, low-level exposure to certain carcinogens may only become significant after subsequent exposure to promoters. This bears on the question of a threshold for tumor promoters. Although it has been stated that promoters have a threshold (*306*), there is no good evidence for this. Studies of the dose-response curves for promoters (e.g. PMA [*347*]) have clearly been inadequate for the purpose of defining a threshold, as is usually acknowledged by the authors involved. There is a recent suggestion that promoters may be much more important for low doses of initiators than high doses and may expand the risk associated with the initiators (*348*). Important factors in tumor promotion such as dose, dosing schedule, tissue specificity, etc. create substantial uncertainty about any general statements regarding thresholds. Generally, animal bioassay studies, because of their design and the conditions under which they are conducted, fail to distinguish between initiating or promoting activity or between a direct or indirect action by the agent on the genome. Although it may be useful to group compounds with some criteria, *e.g.* mutagenic in a particular strain, or having a particular biological effect on a particular strain, one should be careful about implying that something is known about the mechanism of action. The grouping, since it is not based on knowledge of carcinogenic mechanism *in vivo*, may have no relationship to the multiple routes by which carcinogenesis could be induced in a particular instance. It is difficult to be clear about what is the target of a particular agent, when the mechanism of effect of any agent is still unknown. However, it is still important to define the kinds of promoters and to examine the patterns of exposure to them to help elucidate mechanism.

2. Specificity/Antipromotion. Tumor promoters often display tissue specificity, having primary effects on only one or a limited number of tissues in a particular species. For example, estrogens seem to be promoters in breast (*315*) and liver (*316*) in animal models. Mouse skin and bladder are also specifically responsive to certain promoters (*307*). An interesting sidelight to this aspect of promotion is that the PDGF-like material stimulated by the *sis* oncogene is active specifically for the tissue which develops the sarcoma produced by the virus (*102*). Since some promoters seem to be simulating growth factors, and many growth factors tend to be fairly tissue specific, this may be the source of the specificity seen.

The potential for any tissue to give rise to a tumor after initiation may be partly determined by inherent biological processes; treatment to prevent disease may be designed to interfere with those processes, especially with endogenous tumor promotion. Such inherent processes may be altered by diet, stress, or other conditions such as individual genetic differences or altering physiological function. For example, a number of experimental animal studies suggest that a high fat diet can promote carcinogenesis in breast and colon, while epidemiological studies show a positive relationship between dietary fat and breast cancer in some populations (*348*). However, to confirm that a low fat diet will decrease these types of cancer (*11*), additional studies need to be conducted in countries with a wide genetic diversity where a high proportion of the population's caloric intake comes from fats. It must be kept in mind that the total carcinogenic response should be considered. For instance, there are certain populations, with certain lifestyles and diets, such as the Japanese, which have a low incidence of one cancer. However, they frequently have a high incidence of some other type of cancer which has a low incidence in other populations (*349*).

Studies have also revealed several compounds, called antipromoters, that inhibit responses to PMA *in vitro* (*144*) and prevent tumor promotion *in vitro* (*350*). Antipromoters are effective during different phases of the multistep process of promotion. Although "pure" promotion has not been conclusively demonstrated in humans, the existence of antipromoters in other systems has been used to suggest that it may be possible to design cancer prevention methodology based on the interference of promotion with a putative antipromoter in humans. Clinical studies are currently underway to test the therapeutic efficacy of some compounds, including synthetic retinoids (*351*) which are known antipromoters.

While cell culture methods offer possibilities for screening for tumor promoters and antipromoters, it will first be necessary to determine which functions altered *in vitro* by promoters are critical to the promotion process and which are secondary. The species and tissue specificity of tumor promoters make it likely that a number of different cell types will be required for any screening procedure.

B. Neoplastic Progression

The progression of a neoplastic cell into a malignant tumor, which does not occur to every neoplastic cell, is the last major stage of cancer formation and is probably accomplished through a number of steps. Many cancers have more than the normal numbers of chromosomes and exhibit poorly controlled and sometimes higher rates of growth, invasiveness and anaerobic glycolysis, factors that may be important to their survival in the animal.

The route by which a neoplastic cell becomes a cancer may entail a sequence of interactions between tumor cells and both specific and nonspecific host defenses (*352*), as well as a series of events that result in the cell acquiring more of the characteristics of cells in a malignant neoplasm (*279*). These selection processes are non-exclusive of each other and may involve karyotypic changes (*353*). The factors that are important in neoplastic progression, especially in the pre-malignant stage after neoplastic cells emerge and before a malignant neoplasm is formed, may be similar to those in tumor dormancy, a phenomenon in which tumor cells reappear many years after apparently successful treatment. The major mechanisms of tumor dormancy seem to be: (1) Inhibition of cell division; and (2) cell division accompanied by cell lysis. Mitotic control can be exercised by macrophages (*354*), soluble factors such as lumphokines (*356*), nutrient depletion (*357*), or a hormonal requirement for growth (*358*). Continuing cell division in a tumor, the major characteristic of a neoplasm, can continue without much

effect if daughter cells are lysed about as fast as they are produced. This can result from immunological lysis, nutritional deprivation or very slow tumor cell production. Any or all these factors may lose their effectiveness during the selection processes for malignancy described above, perhaps in a number of steps.

An interesting model for the progression of a neoplasm to malignancy is the one proposed by Gersten, Fidler and Hart (354, 359). After a primary tumor is established, there is tumor vascularization, by an angiogenic factor (*357*), with invasion of surrounding tissue and blood vessels. This invasion seems to be accomplished throught tissue damage by a number of different enzymes, such as collagenase (*360*) and cathepsin B (*361*), and the role of tumor cell motility is not clear (*359*). The widespread dissemination of cancer cells occurs by the lymphatic (carcinomas) and hematogenous (mesenchymal tumors) routes. During lymphatic spread, the regional lymph node is involved in the host immune responses to neoplasia (*362*). During hematogenous spread, release of tumor emboli in the bloodstream results in most tumor cells being destroyed by interaction with a number of blood elements (*363*). However, the more that are released, the more that survive to form metastases. Arrest and subsequent growth of circulating tumor cells or implantation in another site seem to be a function of tissue and metastasis, and there are some data that the site is selected by the membrane properties of the metastatic cells (*364*). Metastatic cells then leave the blood vessel and form micrometastases which induce blood vessel proliferation leading to metastatic growth (*359*).

Progression seems to be modulated by a number of factors. Important among these is the immunological system. Many tumors induced by physical and chemical agents are antigenically distinct (*365–367*). To overcome the immunological defenses of an immunocompetent individual, the tumor could: (1) Simply not be immunogenic, *e.g.* contain only antigens expressed normally in the body; or (2) suppress the immunological response. Lack of immnogenicity seems to be very common in spontaneous tumors in animals (*367, 368*). This also seems to be the case for humans since, at present, only cells from presumably viral tumors, such as Burkitt's lymphoma (*369*), may be considered to be truly immunogenic, although there is evidence of some specific antigens associated with cells from human tumors grown in culture (*e.g.* colorectal tumor cells [*370*]) (*371*). A tumor could also induce immunodeficiency, either general or selective. Immunosuppression could be accomplished by a variety of mechanisms, including produciton of circulating immune complexes (*372*) (to inhibit antibody respnse) or a rise n suppressor T-cells (*373*) (to inhibit T-cell response). Production of an immunodeficiency seems to be important for highly immnogenic tumors, such as UV induced tumors (which are decisively affected by suppressor T-cells in the formation of primary tumors [*373*]) and viral tumors (which are profoundly influenced by immunocompetence [*135, 374*]).The immunodeficiency associated with UV-induced tumors is a selective one, perhaps because of a UV-induced deficit in antigen processing by a macrophagelike cell that subsequently produces the T-cell response (*373*).

Clinically, immunodeficiency usually refers to a more general type of immunodeficiency that can occur during the later stages of carcinogenesis (*375*). This may be of importance mainly in metastasis since it has been shown that immunosuppressive treatments and agents greatly increase the incidence of distant metastases in rats with highly immunogenic tumors (though the effect is reversed for tumors with low immunogenicity [*376*]). Its role may be wider, however, since it has been suggested, from evidence about lymphoma in immunosuppressed individuals, that the immune system may be important in the selection of a particular chromosomal alteration, *i.e.* be involved in promotion (*135*).

Implicit in this analysis is the fact that factors that can alter modulators of progression can also alter progression. For example, factors such as stress that result in a mild immunodeficiency, may also mimic the effects of immunosuppression in production of lymphoma. A cancer is a product of a long series of events and it is a reasonable assumption that modulators may interact in a positive or negative fashion with this sequence at any point.

VII. MULTIPLE AGENTS

Since people are exposed to many different agents at the different times in different sequences, the effect of multiple agents on carcinogenesis is of major concern. However there is little information of general import in the field. The great number of permutations of possible agents and doses makes understanding interaction of multiple agents very difficult.

In general, the action of two or more agents can be additive (if the agents are given in a dose range where the biological response is a linear function of dose) or multiplicative (if the response is a simple exponential response to dose), synergistic (greater than expected) or antagonistic (less than expected) (*377*).

One obvious example of synergy is the tumor initiator-promoter interaction described above. Tumor incidence is much less when either agent is given separately than when the two agents are given in the proper order. Another possible example is the relation between cigarette smoking and asbestos exposure in carcinogenesis. The effect of both carcinogens is much greater than a simple additive model would predict and is closer to multiplicative (*378*). Although the mechanism for this phenomenon is not known, possibilities include: (1) Asbestos increases the uptake of the carcinogenic substances in cigarette smoke (as shown when benzo(a)pyrene is used as a model compound [*379*]); (2) asbestos stimulates metabolism of carcinogen(s) in smoke (which has been shown for benzo(a)pyrene, one component of smoke) (*380*); (3) asbestos works as a promoter with cigarette smoking the initiator (*381*); or (4) some combination of the above mechanisms (*382*).

One example of antagonism is the interaction of 3'-methyl-4-dimethylaminoazobenzene (an azo dye) and certain polycyclic aromatic hydrocarbons (PAH), such as 3-methylcholanthrene (MCA) (*383, 384*). Some carcinogenic PAH, by stimulating metabolism, inhibit azo dye carcinogensis. Although the study and understanding of the interaction(s) of multiple agents is not easy, it becomes much more difficult (conceptually as well as experimentally) when it is not clear whether more than one agent is involved. An example is the interaction of immunosuppresive agents and EBV. Although immunosuppresive agents are presumed not to induce tumors, their use may allow the expression of EBV induced carcinogenesis, as has been suggested for renal transplant patients who have a substantially raised incidence of tumors, especially lymphomas (*135*). Since EBV is ubiquitous, exposure to an immunosuppressive might result in an increased number of tumors in a bioassay (*i.e.* be carcinogenic) although in fact it is simply allowing expression of tumors that arise from a viral infection. Other agents may alter the modulators at any stage in carcinogenesis and result in either more intense effects of a ubiquitous agent or the expression of a tumor derived from long-lived irreversibly initiated cells.

These examples illustrate just a few of the possibilities of interaction. Some agents may stimulate metabolism, interact chemically with other agents, or be so cytotoxic that tumors induced by another agent are killed. Other agents may alter modulators of different stages of carcinogenesis or stimulate the expression of tumors that would normally not be expressed in the lifetime of the animal. At present, in the absence of a well established and comprehensive theory of carcinogenesis, the complex interaction of agents is best understood by elucidating the action of each compound. Then the effects of each compound on the organism can be evaluated and their interaction estimated.

VIII. SUMMARY

Although the study of the phenomenology of cancer was initiated many years ago, analysis of the mechanisms involved in carcinogenesis was started much more recently, and the level of activity has risen significantly in the last decade. This review has attempted to place much of the data concerning cancer into a broad perspective. Exposure to a carcinogen begins at the interface of an animal with its environment, and an agent can be modified by factors associated with an organism at that interface. An example of this is the reduction of benzidine-based aso dyes to carcinogenic benzidine by gastrointestinal microflora. Since the microflora can vary with species as well as with food habits, this provides one source of variation in the activation of agents.

There is a wide variety of processes that are involved in the activition and detoxification of most agents. The complexity of these processes is illustrated by the major products of the metabolism of benzo(a)pyrene, a carcinogen which needs activation. Lifestyle, exogenous agents, diet, age, etc. can act on several levels to alter the balance of activation and deactivation. Examples of this are the stimulation of cellular metabolism producing more reactive species, the inhibition of a particular pathway resulting in a smaller amount of reactive moiety, and the alteration of cell scavengers that absorb reactive metabolites produced by metabolism, and lower the overall concentration of reactive moiety. Activation and deactivation are functions of species, tissue, and even individuals, providing another source of variation in response to a carcinogen.

The interactions of carcinogens with DNA, which seem to be important for the carcinogenic action of many initiators and complete carcinogens, vary across the different classes of carcinogens. Biological carcinogens, such as the acute transforming retroviruses, seem to insert genetic information which allows the expression of certain cellular *onc* genes whose products are important for transformation. Some of these products seem to have effects like certain growth factors. The discovery that the dominant transforming genes of certain human cancer-derived cells are similar to certain classes of *onc* genes derived from the acute transforming retroviruses has been a breakthrough in understanding certain causal factors in human neoplasia. Radiations damage DNA, but, with the exception of ultraviolet-light (UV) irradiation, the irradiation products are not very well characterized and the role of the damage is not clear. It has been shown that a particular DNA damage is involved in the carcinogenic action of UV light, but how this occurs is also not clear. The specific damage that many chemical carcinogens produce in DNA is much better characterized, but which lesions are important in carcinogenesis and how a lesion can interact to produce a cancer are not known. "Physical" carcinogens include a number of very different agents and processes which seem to have in common the production of a chronic fibrotic reaction.

Two modulators of DNA damage are DNA repair and fidelity of replication, which help to determine the effects of a carcinogenic stimulus that survives through replication in initiated cells. Recent work on DNA repair has determined that there is a set of enzymes, the DNA glycosylases, that confer specificity in base excision repair for different types of DNA damage. Also, post-replication repair has been found to be more complex than though previously, which may help explain how a specific lesion influences DNA. Since DNA repair is modified by many factors, and is tissue and species specific, this provides another source of variation in the final response of the organism to a carcinogen. An example of how knowledge about mechanisms impacts on risk assessment is the question of how DNA repair is related to biological thresholds for effects of carcinogens. Statements that DNA repair results in a threshold for carcinogenesis are naive and cannot be substantiated.

The fidelity of DNA replication can be altered by a damaged template or, more indirectly, through changes in the reaction conditions that result in miscoding. Any of the reaction conditions could be altered by a carcinogen or by a change in physiological state induced by a carcinogen, providing a route by which the DNA could be altered without the formation of direct binding between the DNA and a carcinogen derivative.

Cancer is a phenomenon with many steps. In an experimental paradigm, two major stages can be demonstrated: Initiation, the induction of an irreversible alteration of genetic information, and promotion, a reversible process terminated by the final irreversible alterarion of initiated cells into neoplastic cells. Each stage can be subdivided. The progression of neoplastic cells into a cancer, which has not been easy to separate experimentally from promotion, is the final stage in carcinogenesis.

Promotion can occur with both exogenous and endogenous compounds (and, perhaps, certain surfaces) and is usually characterized in a number of systems by a focal proliferation of cells of which a small fraction will persist after the promoter stimulus is removed. Few focal proliferations in any experimental system will become neoplastic. Although the aspect of tumor promotion which is critical for tumorigenesis is not entirely clear, a interesting possibility is the induction of chromosomal alterations. The relationship of this process to viral carcinogenesis and the mechanisms by which alternations may lead to a neoplasm are explored. However, a causal relationship of chromosomal changes to carcinogenesis has not been proven. Tumor promoters are tissue and species specific, perhaps providing some insights into how various tissues and species are differentially affected by carcinogens.

Neoplastic progression, the process in which a neoplastic cell becomes a malignant tumor, seems to involve several steps, none of which is yet well understood. In that complex process, the role of the immunological system as one of the important modulators of progression is discussed.

Evaluation of the effect of a number of agents acting concurrently has barely begun. Models for summation, potentiation, or inhibition of effects are poorly developed, especially when considering the range of effects that agents have on a number of metabolic and physiological endpoints. Variation with species, tissue and individual at every stage in carcinogenesis produces the added uncertainty if two or more compounds interact at many levels.

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Chapter 2—Short-Term Tests for Potential Carcinogens

I. INTRODUCTION

Cancer can be induced by a variety of radiations, biological, "physical" and chemcial agents, and is manifested in a variety of phenotypes. Notwithstanding our lack of understanding of the mechanisms by which cancer is induced and expressed (See Chapter 1 of this document) there is an urgent need to identify the agents that cause and promote these diseases. The rapid identification of potential chemical carcinogens is one of the major goals of short-term testing.

The hypothesis that conversion of normal cells to neoplastic cells results from heritable genetic changes—the somatic mutation theory of cancer—was proposed early in this century (*1*) and forms a basis for the development of many short-term tests. The relationship of mutation and carcinogenesis has been stressed for some time (*2*). However, early studies relating mutagenesis and carcinogenesis were generally unsuccessful due in part to the need for metabolic activation of most carcinogens. Pioneering studies by the Millers and associates (*3*) on metabolic activation of chemicals and the coupling of metabolic activation systems and mutable targets (*4, 5*) allowed further elucidation of the possible relationship between mutagenicity and carcinogenicity. During the past 15 years, many short-term systems that detect mutagenesis and other genetic effects have been developed and attempts have been made to correlate the results of these tests with carcinogenesis. The tremendous number of chemicals that remain to be evaluated for potential hazard and the mounting cost of currently acceptable rodent carcinogenesis assays amply justify the continued effort to develop and utilize short-term tests, and to establish a mode for their application in assessing the potential of chemicals to induce cancer.

The search for tests with the capabilty to identify potential chemical carcinogens has yielded a variety of assay systems with a diversity of endpoints. The existence of such a large number of assay systems (approximatley 100 have been described [*6, 7*]) reflects both limitations and differences between assays, as well as our uncertainty about the mechanisms of induction and progression of carcinogenesis. As described in Chapter 1, the induction of cancer may have many potential mechanisms (gene or chromosomal mutation, heritable changes in DNA transcription reflected in induction or cessation of gene expression, etc.), but no causal relationship has yet been established between and mechanism of induction and any specific neoplasia. In addition, a variety of factors or conditions such as tumor promoters, hormanal levels, rate of cell proliferation, cell type specificity, genetic predisposition, etc. are known to influence the expression of neoplasia, regardless of the nature of the inducing

agent. Because of the relatively small of known human carcinogens, the predictive capability of the short-term assays is usually assessed by validation with the many more chemicals which are carcinogenic in long-term animal tests.

This chapter describes the major categories of short-term assays for chemical carcinogens, attempts to evaluate the assay systems, and recommends further studies required to define their predictive ability.

The term "genetic toxicity" in used in this chapter to indicate any effect that can be attributed to an interaction between the test agent and the genetic material of the test organism. There have been attempts to classify carcinogens on the basis of whether they show evidence of direct interaction with DMA or act by some indirect effect ("epigenetic") (*8*). However, the assay systems conventionally used to make this distinction may not detect all types of chemical-DNA interactions, nor do they adequately accomodate the tissue or cell-specific metabolism or target specificity which many carcinogens demonstrate. So it is not possible to conclusively make a clear distinction between genetically toxic and "epigenetic" on the basis of currently available in vitro tests. However, the fact that certain factors or substances do appear to induce tumors without appearing to react directly with the DNA indicates a need for development of short-term tests complementary to those which detect direct effects on the DNA if all types of chemical carcinogens are to be detected.

Many of the tests for genetic toxicity are also useful in the identification of potential germ cell mutagens; however, this represents a distinct application of the tests, and the reader is referred to the recent report of the National Academy of Sciences (*9*) for further information on this subject. In addition, screening of chemicals for genetic activity can identify agents that may be involved in the aging process or atherosclerosis (*10, 11*).

II THE SHORT-TERM TEST SYSTEMS

There have been numerous descriptions of the assays that have been developed and evaluated for their capability to detect potential carcinogens, but the details of these systems will not be discussed herein. The reader is directed to the EPA Gene-Tox Evaluations (*12–40*), the International Collaborative Program (*41*) and the review by Hollstein *et al.* (*6*) for details and further references to the individual systems. The four principal biological endpoints represented by the available tests are gene mutation, chromosomal effects, general DNA damage and neoplastic transformation. Some frequently used asays are listen in Table 5.

TABLE 5—CONVENTIONALLY USED SHORT-TERM ASSAYS

Type of assay	Endpoint	Reference
Mutation:		
(a) Microbial systems:		
Salmonella Typhimurium.	Histidine reversion	42
Escherichia coli	Tryptophane reversion	12
(b) Mammalian cell systems:		
Mouse lymphoma (L5178Y).	Thymidine kinase (Bromodeoxyuridine or Triflurothymidine [TFT] resistence).	39
Chinese hamster ovary (CHO).	Hypoxanthine guanine phosphoribosyl tranferase (6-thioguanine [6TG] resistance).	14
Chinese hamster lung (V79).	Hypoxanthine guanine phosphoribosyl transferase (6TG resistance)..	18
	Na/K ATPase (Ouabain resistance).	....
Human lymphoblasts.	Thymidine kinase (TFT resistance) hypoxanthine guanine phosphoribosyl transferase (6TG resistance).	44
Chromosome effects:		
Many cell types in vitro and in vivo.	Chromosomal aberrations.	17, 19
	Sister chromatid exchanges.	....
	Micronuclei	....
	Chromosome gain or loss.	....
	Translocation	....
DNA damage:		
Unscheduled DNA synthesis (UDA).	Radioactive nucleoside incorporation.	40
Repair deficient bacteria.	Differential growth inhibition.	20
Mammalian cell transformation:		
Cellular:		
Syrian hamster embryo (SHE), Balb/c 3T3, CeH/10T½.	Altered cellular morphology and growth pattern, growth in soft agar, tumorigenicity in appropriate host.	35
Viral enhanced/mediated Syrian hamster embryo (SHE)/Simian adenovirus type 7 (SA7), Fischer rat embryo cells/Rausher leukemia virus.	....	35

A. Gene Mutation

Although it is possible to assay for chemically induced mutation with a variety of methods in a number of organisms and cells, the greatest amount of information on tests for potential carcinogens is derived from a relatively few systems. The system in which the largest number of chemicals has been evaluated is the Salmonella/microsome test (Ames test) (*42, 43*) where several strains of the bacterium *Salmonella typhimurium* have been genetically altered in order to provide increased sensitivity to potential mutagens. In the Ames assay, reverse mutations in the histidine locus (*i.e.* from a histidine requirement to independence) are detected. By virtue of the sensitivity, ease and relatively low cost of performance and duration of the test (48–72 hours), this assay has been used worldwide to test for chemical mutagens. Other microbial systems can also be used (Table 5) but have not been used nearly as extensively as the Salmonella assay. Cultured mammalian somatic cells (which are genetically more similar to cells as they exist in humans than to bacteria) such as the mouse lymphoma (L5178Y) (*39*), Chinese hamster ovary (CHO) *(14)*, and Chinese hamster lung cells (V79) (*18*), have also been used to detect gene mutations induced by a variety of chemical agents and radiations. The various systems that have been developed are capable of detecting forward and/or reverse mutations. However, none of the specific gene loci conventionally used in the bacterial or mammalian cell mutagenesis systems *(his, tk, oau, hgprt*, etc., Table 5) appear to be related in any way to the cellular changes that occur in the induction of neoplasia. These loci are used to measure the capability of a chemical to interact with the DNA of an organism in such a way as to give rise to mutagenic effects. Although it has been widely assumed and theorized that neoplasia arises from mutation, it is known that heritable phenotypic changes also occur in mammalian cells in the process of differentiation and growth and such changes arise without the induction of gene mutation. Consequently, it is necessary to use additional biological endpoints that reflect the capacity of chemicals to cause changes through other types of interactions with the genetic material.

B. Chromosome Effects

Changes in the structure or number of chromosomes occur frequently in tumor cells (*45*) and cells from individuals exposed to radiation show evidence of chromsome damage (*46*). Cytogenetic short-term tests have demonstrated that a number of carcinogens can cause structural alterations of chromosomes (aberrations) in the bone marrow of exposed animals or in cultured cells derived from various tissues (*19*). There is increasing evidence that associates specific chromosomal rearrangements (translocations) with specific neoplastic phenotypes (*47*). Although no definitive causal associations have been defined, the use of molecular and recombinant DNA techniques promises to aid in

resolving the relationship between translocation of large segments of chromosomes and the specific DNA sequences involved. Another assay often used in conjunction with the search for chromosomal aberrations is the induction of sister chromatid exchanges (SCEs) (*48, 49*) that are believed to result from the induction of recombination by DNA damage. However, the association between an increase in the frequency of SCEs in cells *in vitro* or *in vivo* and heritable changes has not not been clearly defined.

It is also possible to identify the production of chromosome fragments (micronuclei) in reticulocytes or erythrocytes (*50*). Induction of micronuclei has been associated with chemical exposure and is also believed to be indicative of the ability of chemicals to induce chromosomal aberrations, but it is also not yet clear that the induction of micronuclei is related to heritable changes in cellular phenotypes.

A long-range potential value of tests of chromosomal effects is that they may be used to assess the comparative (trans-species) toxicity of chemicals. Since the sampling for cells is non-destructive to the exposed animal or individual, and because the assays can be conducted on cultured cells, comparisons are possible between various species and humans. However, such comparisons must be conducted with the understanding that chemical disposition (pharmacokinetics) may vary with species thus giving rise to species-dependent target cell populations. Additionally, the monitoring of human blood cells for chromosomal effects may be a valuable method to assess human exposure to hazardous chemicals (*51*).

C. DNA Damage and Repair

These tests do not measure mutation per se, but DNA damage, or the cellular repair of such damage, induced by the chemcial treatment of microbial or mammalian cells. The most extensively used method is to detect non-S phase DNA synthesis (unscheduled DNA synthesis—UDS) in hepatocytes (*40, 52*), although other cell line (*40*) have also been used. Techniques that measure DNA strand breaks (*23*) or binding of chemcials to cellular DNA *in vivo* or *in vitro* (*53*) are also used as assays for DNA damage. Other assays use strains of *E. coli* or *B. subtilis* and are based on differential growth inhibition of DNA repair proficient and deficient strains (*20, 54*).

D. Cellular Transformation

Exposure of cultured mammalian cells from a variety of tissues and species to carcinogens (biological, chemical and radiations) has been shown to result in certain heritable phenotypic changes. A variety of these change has been described (*35, 55*), but some specific morphological changes have been shown to be highly associated with the capacity of the cells to induce tumors when inoculated into appropriate recipient animals. Because of this ability of transformed cells to produce tumors in recipient hosts, these assays have been considered by some to be the short-term systems most relevant to detection of chemical carcinogens (*6, 35, 51*). A number of cellular systems derived from rodent embryos have been used to identify chemcial carcinogens (Table 5), some of which use concomitant infection with transforming or nontransforming viruses (*35, 55*). There is no clear understanding of the mechanism of *in vitro* transformation, and it is likely that the various heritable alterations can occur through one of several possible mechanisms. Recent innovative advances at the cellular and molecular level and having a major impact on our understanding of this process (*56, 57*). As the implications of studies on oncogenes and transfected transforming genes because better understood and are incorporated into methods for detecting carcinogens (See Chapter 1), there is the promise of significant improvement in the accuracy with which these systems can be used to dectect carcinogenic chemicals.

E. In Vitro Bromothion System

The existence of chemicals which appear to cause (or promote) cancer by mechanisms which may or may not directly involve the genome (See Chapter 1) has necessitated the development of short-term systems that can detect these types of chemicals. However, it should be emphasized that chemicals may have both initiating and promoting activity, and the two properties are not mutually exclusive. The V79-metabolic cooperativity assay (*58*) has been the most thoroughly studied *in vitro* system developed to detect chemicals with promoting activity. A basis for this assay has been attributed to be the interruption of cell-to-cell communication, although the exact mechanism is unknown. Further studies with this and other promotion systems are needed before they could be reliably used as test systems to detect chemicals that act as promoters of tumorigenesis.

F. In Vivo Short-Term Bioassays

Several short-term *in vitro* bioassays to detect carcinogens and/or promoters have been developed. Of these assays, the mouse skin papilloma system and the strain A mouse lung adenoma have been used for testing while the liver foci bioassay is still in developmental stage. These systems are generally viewed as intermediate in biological relevance between *in vitro* systems and the long-term animal bioassay because *in vitro* methabilism and/or chemical disposition are provided for by *in vitro* exposure. However, as the *in vitro* tests, the relevance of the endpoints measured to the development of malignant tumors remains an open question.

The mouse skin papilloma system, first reported by Berenblum (*59*), is based on a multistage (initiation and promotion) hypothesis of cancer induction and has been developed further in other laboratories (*60–64*). The system relies on the induction of benign papillomas, although carcinomas do develop in the later course of the assay. Variations in experimental test design reportedly allow chemicals to be classified as: Initiating carcinogens, promoters, complete carcinogens (chemicals with both initiating and promoting activity), co-carcinogens, and co-promoters (*64*). Several strains of mice have been used in the assay, but the SENCAR stock appears particularly sensitive (*64*). The mouse skin papilloma system is the *in vivo* system in which the greatest number of chemicals have been tested (*65*); however, many of the chemicals investigated have been polycyclic aromatic hydrocarbons, and the usefulness of the system to test other classes of chemicals, or chemicals with organ specificity other than skin, is not presently clearly known.

The strain A mouse lung adenoma system was developed by Shimkin and colleagues (*66–69*). Although lung adenomas do occur spontaneously in mice, the strain A mouse, unlike other strains, has nearly a 100% life-time incidence (*69*).Therefore, the assay appears to rely on decreasing the time-to-tumor onset by the test chemical, although Stoner and Shimkin consider the test chemcial to be inducing rather than accelerating the process (*68, 69*). A wide spectrum of chemical classes has been tested in the lung system (for review, see (*69*) and good correlations of results and the tumorigenicity of the chemicals have been reported although one study using diverse coded chemicals did not find a high correlation (*70*). The system has been criticized because the adenoma is a benign growth and an

analogous tumor does not appear in humans.

The induction of altered foci in rat liver systems have also been studied for use as short-term *in vivo* assays for carcinogens and promoters. However, these systems are relatively new and are still in a developmental stage compared to the mouse skin papilloma and lung adenoma assays. The rodent liver systems have been used by Peraino *et al.* (*71*), Pitot *et al.* (*72*), and Solt and Farber (*73*), to study the initiation/promotion phenomena. The endpoints that have been measured include the appearance of foci in the liver which have increased gammaglutamyl transpeptidase, basophilia, or diaphorase; or foci with decreased glucose-6-phosphatase, adenosine triphosphatase or iron storage (See *74* for review). Chemicals can be tested either for initiating or promoting activity in the liver assays. Since defined protocols have not yet emerged, further development is required before the possible value of the approach in a routine testing program can be assessed. In addition the relationship of the altered liver foci to liver neoplastic nodules remains to be determined.

III. FACTORS IN TEST EVALUATION

A. Metabolic Activities

Most target cells used in short-term tests have limited endogenous metabolic capability for xenobiotics and, therefore, must be supplemented with an exogenous metabolic activation system. The metabolic activation systems employed in short-term tests may be one of the major limitations in these assay systems, because a chemical may show a different metabolic profile *in vitro* from what would occur *in vivo*. From a testing point of view, a major concern is that specific activating enzymes or pathways may be decreased or lost during preparation of the metabolic activation system, thereby giving a false negative result with the test chemical. Alternatively, enhanced activation of certain chemicals would also be possible (false positive). Essentially, three types of metabolic activation systems have been used with target organisms—host-mediated (*24, 75*), intact cells (*76, 77*), and organ homogenates (usually 9000 × g supernatants called S9 [*10,43*]). Most short-term assays have used rat liver S9 preparations because of the ease of preparation and storage, capability to activate many chemical classes, and the relative compatibility (nontoxicity) with most target organisms used in genetic toxicity testing. However, it is also known that liver S9 preparations may cause different responses *in vitro* from what would occur *in vivo* due to factors such as: organotropic differences, chemical induction of S9 preparations, and destruction of certain activation enzymes during S9 preparation, decrease in conjugation capability, etc. Therefore, the inability of S9 preparations to mimic *in vivo* activation has provided the impetus to modify S9 utilization and/or investigate other metabolic activation systems.

Intact cells are another exogenous metabolic activation system used in short-term tests (*77*). These cells can be primary cultures prepared from various organs (liver is most common), embryonic cells that have been cultured only a short time, or in certain cases, established cell lines. For such an activation system to function, the test chemical must be taken up by the activating cell, be metabolized, and the activated product be transferred to the indicator cell. Thus, a negative response with a cellular system may be a function of the uptake of the test substance and the transfer and stability of active metabolites. Since most cells lose significant metabolic capability with time in culture, certain enzyme systems may be functional at reduced levels. However, this loss of activation capability can be at least partially overcome by the use of freshly prepared primary cells.

In the host-mediated assay, the indicator organism is introduced into the peritoneal cavity or injected into the circulatory system of an intact mammal (*24, 75*). The animal is then treated with the substance under test, sacrificed, and the test organism removed and examined for mutation induction. A wide range of indicator organisms has been used (*24*). Because an organism foreign to the host is commonly used as the indicator organism, problems arise such as: (1) Interaction between the host and the indicator organism, including immunological interactions; and (2) the limited time the indicator organism can be kept in the animal. Therefore, this system is unable to detect many carcinogens and chemicals which show *in vitro* mutagenesis.

Attempts to improve metabolic activation systems are certainly worthwhile and have increased our ability to detect carcinogens using *in vitro* methods. One must accept, however, that it is not possible to mimic *in vivo* metabolism in a petri plate and that negative *in vitro* results may represent "false negatives" and positive *in vitro results* may represent "false positives." These possibly erroneous test results due to metabolic activation can sometimes be resolved through the use of short-term *in vivo* rodent assays. A testing scheme has been proposed by Ashby (*78*) whereby short-term rodent assays are employed to study further chemicals falsely identified in the *in vitro* systems.

B. Statistical Evaluation of Short-Term Test Data

The overwhelming majority of short-term test data in the literature have not been statistically evaluated. In general, decisions of "positive" and "negative" are made on a subjective basis or on the basis of simple statistical procedures. Recently, a number of statistical procedures have been proposed for the Salmonella assays (*79–81*), the mammalian cell mutation (*82*) assays, and discussions of statistical approaches appear in the respective Gene-Tox reports, but there is no consensus as to which particular procedures should be used for data evaluation.

C. Factors in the Correlation of Assays With Carcinogenesis

Because short-term tests for carcinogens employ neither the organism (humans) or the biological endpoint (cancer) of concern, there is a need for a great deal of extrapolation of test results, and hence the demand for proving the validity of the tests is great. However, after more than a decade of effort to demonstrate the utility of short-term tests in predicting carcinogenicity, it is still difficult to find sufficient test results to assess: (1) The performance of an assay within or among chemical classes; (2) the activity of a chemical in a variety of tests; or (3) the performance of a group of the tests on even a small group of chemicals. This deficiency in the existing data results largely from two factors. First, there has not been enough of an effort to evaluate systematically a large number of chemicals in a group of selected assays, and second, there is a marked deficiency of genetic toxicity results on noncarcinogens. This latter point presents an immediate obstacle to the evaluation of short-term tests for carcinogens, since in the absence of an adequate data base on noncarcinogens, it is not possible to determine the performance of a single test or group of tests in discriminating between carcinogens and non-carcinogens.

1. Definition of some terms. The reliability of a short-term test system may be defined as its ability to predict accurately a chemical's *in vivo* carcinogenicity. The standard against which short-term test results are most

often compared is the rodent cancer bioassay. A short-term test which correctly identifies, when used without bias, a high percentage of known carcinogens and noncarcinogens is assumed to be capable of predicting the carcinogenicity of chemicals of unknown activity with the same degree of accuracy. The short-term assay's performance can be expressed by three criteria: Sensitivity, specificity, and accuracy. Sensitivity is the proportion of carcinogens giving positive results; specificity is the proportion of non-carcinogens giving negative results; and accuracy is the overall proportion of correct results. Unfortunately many, if not all, previous test system evaluations, have been characterized by an inadequate number of non-carcinogens, and, hence, a determination of specificity and accuracy is severely limited. Also, the accuracy of short-term tests could be altered by the ratio of carcinogens and non-carcinogens in the test sample (*83*). Additionally, the expected accuracy of a test battery (see below) is influenced by the number of tests in the battery and the number of individual tests required to be positive before the chemical is considered to be positive in the battery (*84*).

2. Non-carcinogens and test analysis. The inadequate identification of, as well as the use of, non-carcinogens, as has been noted, is one of the key problems. Many chemicals have been identified as non-carcinogenic merely on the basis of their chemical structure or perfunctory carcinogenesis tests. To date, relatively few chemicals used as non-carcinogens in short-term tests have been tested in lifetime studies using adequate chemical exposures. A major source of information on non-carcinogens is that of the NCI/NTIP rodent bioassay where two sex/species are exposed at sufficient concentration of the chemical and for sufficient duration to insure that the chemical has been tested adequately. However, few of these non-carcinogens have been tested in most genetic toxicity assays (*85*). In addition, factors that determine a "positive" or "negative" short-term test response for a test chemical can vary from laboratory to laboratory or even experiment to experiment within a laboratory. Often parameters such as control background levels, acceptable range of positive control responses, statistical significance of test chemical response, etc., all of which are important in determining whether a chemical is "positive or "negative", can be subjective. Such uncertainties could lead to an inaccurate estimation of the sensitivity and specificity of short-term tests.

3. Test endpoints and carcinogenicity. The development of the Ames test and subsequent short-term tests were predicated on the assumption that most carcinogens were mutagens and, conversely, that mutagenic chemicals will be carcinogenic. Implicit in this reasoning is the conclusion that nonmutagens will not be carcinogenic. However, this conclusion put forth by Ames *et al.* (*5*) and supported by others (*86–90*), that carcinogens and mutagens and the majority of non-carcinogens are not mutagens, may be based on biased data. The majority of the data used to justify this statement comes from studies where the identitity of the chemicals and their carcinogenicity were known to the investigators before they were tested. Because the investigators knew the identity of the chemicals and their "expected" mutagenicity, the test protocols could be modified until the desired results were obtained. While these practices may lead to a true picture of the mutagenicity of a chemical, and are necessary for the assay system development, they probably tend to overestimate the carcinogenicity/mutagenicity correlations.

The correlation of other endpoints (*i.e.* UDS, cell transformation, SCE induction, etc.) with carcinogenesis also is subject to the same limitations. However, the correlations between results from these other systems and carcinogenesis are further complicated by the lack of an adequate data base on chemicals of different classes, and, in some cases, a lack of demonstrated interlaboratory reproducibility of test results.

A method which may establish more objectively the correlations between short-term test results and rodent (or human) carcinogenicity is one where chemicals, coded so their identity is unknown to the persons conducting the assay, are tested, and all the test results are judged by the same criteria. Thus, the one conducting the test does not know whether the short-term test results and conclusions are "right" or "wrong" until after a determination is made. This approach may minimize the proportion of carcinogens detected, but it also eliminates much bias from the correlations. In actual practice, however, additional testing using modified protocols can be done on chemicals whose structure would lead one to believe that the original test conditions were not appropriate. For example, some benzidine-based dyes require reducing conditions, followed by an oxidative system, rather than a strictly oxidative system for their activation. Therefore, choice of test systems should vary with chemical class or nature of the test chemical, but determinations of response should be independent of knowledge of the chemical's identity.

4. Attempts to evaluate short-term test performance. Though not definitive, there have been several systematic efforts to evaluate the short-term assays, singly or in combination, for their ability to detect or predict chemical carcinogens. The largest effort to date was conducted by the Gene-Tox program of the U. S. Environmental Protection Agency and is a multi-phased effort to review and evaluate selected bioassays for mutagenicity and related endpoints using the published literature. Initially, the literature published from 1969–1979 was reviewed to assess test system performances, to determine the chemicals tested, and the ability of the various system to discriminate between carcinogens and non-carcinogens. Reports on the individual systems have been published in Mutation Research (*12–40*). During the second phase, a computerized data base of over 2600 chemical is being analyzed to determine which tests or combination of tests may: (1) Be used for routine screening or need further development; (2) determine if specific types of genetic damage are related to chemical class; (3) discriminate between carcinogens and non-carcinogens; and (4) predict heritable mutagenic risk. To aid in evaluating system performance, the Gen-Tox Program convened a panel to review and evaluate the literature on chemicals designated as carcinogenic and to develop a list of carcinogens and non-carcinogens which could be used in determining system performance.

The International Program for the Evaluation of Short-Term Tests for Carcinogenicity (*41*) was a coordinated effort designed to determine and compare the ability of a series of short-term tests to identify carcinogens and noncarcinogens. Sixty laboratories participated, using their own laboratory protocols, to test coded samples of chemicals supplied from a common source. Test results from this collaborative study led to the following general conclusons. First, while short-term tests are useful in the assessment of potential carcinogenicity, no single test or group of tests was capable fo detecting correctly all carcinogens and noncarcinogens. Secondly, there are *in vitro* eukaryotic assays that may complement the bacterial tests by identifying carcinogens not detected in bacteria.

Following the completion of the collaborative study discussed above, a subsequent study was initiated to focus specifically on the evaluation of *in vitro* eukaryotic assays with potential for identifying carcinogens not detected, or poorly detected, in bacterial mutation tests (*91*).

Other systematic attempts to evaluate the short-term assays include an analysis of their ability to detect pesticides (*92*) and a series of reports prepared for the International Commission for Protection Against Environmental Mutagens and Carcinogens (ICPEMC) (*78, 93*). The NIH/NTP is currently conducting a systematic genetic toxicity assay of chemicals, both carcinogens and non-carcinogens, tested or under test in the rodent, two-sex/species carcinogenesis assay. The major categories of genetic toxicity are represented, and the chemicals are tested under code.

IV. PRACTICAL ASSAY UTILIZATION

A. Assay Selection

The proposed use of short-term tests to detect or predict chemical carcinogens is based on two factors: first, that they require less time to conduct than a rodent carcinogenicity study; and, second, that they are less costly. Currently used tests require from a few days to several weeks to conduct, and the costs may range from 1000 to 20,000 dollars per chemical.

Since there has been little success in establishing a cause-effect relationship between specific genetic changes and cancer, all categories of induced genetic change may be potential mechanisms by which cancers arise, and this is reflected in the variety of endpoints detected by short-term tests. Current short-term testing practices generally involve groups of tests selected on the basis of theoretical prudence rather than on an empirical demonstration of their ability to predict carcinogenicity. By theoretical prudence, it is meant that a range of organisms (bacteria, yeasts, cultured mammalian cells, intact rodents) and endpoints (gene mutations, chromosomal damage, DNA damage, and *in vitro* neoplastic transformation) are employed in an attempt not to miss any effect that might be related to the carcinogenicity of the test agent. However, until the time that basic research on mechanisms of cancer induction provide us with a better understanding of the process, it will be necessary to base testing strategies on a combination of theoretical prudence and correlative evidence obtained by comparing short-term test results with carcinogenicity test results.

In the selection of short-term tests, factors other than ability to discriminate between carcinogens and noncarcinogens must be considered. Reproducibility of test results is a paramount consideration that applies not only to reproducibility within a laboratory but also to agreement of test results among laboratories. When testing chemicals, the ability to reproduce test results qualitatively is more important than quantitative reproducibility. Quantitative differences in test responses represent the degree of response and may be attributed to protocol variables. However, qualitative differences are more serious and affect the judgment of whether a test chemical is "positive" or "negative." Therefore, short-term test selection should be based on reproducibility as well as reliability, sensitivity, specificity, and accuracy. In addition, the degree of development of a particular assay system should also be taken into consideration when selecting tests for use in a screening program. This includes a consideration of the number of laboratories performing the assay, degree of protocol standardization, and a consideration of the number of chemicals and chemical classes which have been tested in the assay.

B. Assay Application

The application of short-term tests to chemical safety evaluation raises questions at several levels of the process. As discussed above, assay performance must be known to determine which tests are useful. Next, it must be decided how many tests to use and in what scheme or order they will be utilized. Tier and Battery testing approaches have been proposed. In the Tier approach, the choice of assay system varies depending on the activity of the chemical in the previous system, beginning usually with the *S. typhimurium* assay. Therefore, decisions are made after each test result is obtained. With the Battery approach, the test chemical is assayed in a group (battery) of systems and a decision on the activity of the chemical made when all results have been obtained.

Once test results from the short-term test scheme have been obtained, it must then be decided how to utilize the results. Within the framework of testing or evaluating chemicals for potential carcinogenicity, where possible regulatory action on the chemical may be involved, short-term test results can be used to influence the process at numerous points including:

(a) The nomination and/or selection of the chemical for prechronic and chronic studies;

(b) The selection of prechronic studies to be conducted;

(c) The decision, used in combination with prechronic results, to proceed with chronic studies;

(d) The evaluation of chronic toxicity results, as a source of additional information;

(e) The design of postchronic studies, again as an additional source of information, to further elucidate mechanisms of activity.

In the industrial setting, short-term tests can be (and are) used extensively in product development, process improvements, product registration, evaluation of occupational environments, etc.

It is generally agreed that a scheme employing multiple tests is a better approach to carcinogen screening than the use of a single test. The tests used should not be limited to prokaryotic or lower eukaryotic test systems and should include assays for more than one genetic endpoint A problem that is immediately encountered when multiple test systems are used, however, is a mixture of positive and negative results. The resolution of this problem is not easy, and several authors *(94–96)* have attempted to deal with the issue. For the present, there are at least three approaches to dealing with such conflicting test results. One may: (1) Use a weight-of-evidence approach to call a chemical positive by some predetermined criterion; (2) use one's best-scientific-judgment to reach a conclusion on a chemical-by-chemical basis; or (3) simply postpone judgment until the time that a sufficiently large data base is available to permit an unequivocal conclusion from any pattern of positive and negative results. This last choice is rarely acceptable, and current practice generally utilizes some combination of the first two. The systematic accumulation of additional knowledge on cross-system testing of various classes of chemicals should provide insight into the proper choices of short-term systems in assessing potential carcinogenic activity.

C. Future Directions

Short-term tests for genetic toxicity and other effects continue to hold promise of improving our ability to identify potential chemcial carcinogens. However, there is at present insufficient data available to justify the use of current tests to supplant animal bioassays. To improve the interpretation of short-term test results, there is a need for a systematic effort to obtain across-test results using *optimal* test protocols and coded chemicals, with particular

emphasis on non-carcinogens. It is clear from available results that no one assay system is adequate, but it is not yet possible to determine which combination of test systems are most appropriate for most carcinogens. Studies are needed to determine if human cells or tissues would be better predictors of human carcinogenicity than the currently used rodent systems. Some carcinogens, by virtue of their intrinsic mechanism of action, will not be identified by available tests, and we must continue to search for assay systems that accommodate the various molecular mechanisms. Concommitantly, we must continue to improve our understanding of the results of rodent assays for carcinogens. Where there is a dichotomy of results between genetic toxicity or other short-term test systems and the results of rodent carcinogenicity, appropriate follow-up studies in the appropriate systems should be conducted to determine the basis for such differences.

V. SUMMARY

While many assay systems have been developed and proposed to detect or assess the genetic toxicity of chemicals, they can be generally grouped into four categories: Gene mutation, chromosome effects, DNA damage, and transformation. The internal momemtum of the field has resulted in the emergence of a few systems or tests in each category that have been used extensively to assess potential carcinogenicity, and the various assays within each grouping have characteristic strengths and weaknesses. The test systems must be demonstrated to have reliability, sensitivity, specificity and accuracy and to be reproducible both within and between laboratories. The various systems also measure different biological endpoints, and there is no reason to expect that all chemicals will cause an effect in all systems. Since no causal relationship has been established between any specific mechanism of induction and any specific type of neoplasia, we are presently limited in our ability to predict potential carcinogenicity of a chemical, particularly when there is apparent disagreement between different *in vitro* test results.

The consensus of available information suggests that short-term tests, when properly used and validated, can provide strong indication of potential carcinogenicity. However, based upon our limited knowledge of the mechanisms of cancer induction, it appears that there will be classes of carcinogens that are not detected by currently available short-term tests. Chemicals which act by a genetic mechanism but which have a high degree or organ, species, or sex specificity may be predictably difficult to detect in any *in vitro* genetic toxicity system since the short-term system may not adequately simulate the basis for these specificities. Additionally, chemicals which appear to act by non-genetic mechanisms would not be detected in currently available short-term tests.

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Chapter 3—Long-Term Carcinogen Bioassay

I. LONG-TERM CARCINOGEN BIOASSAY: GUIDELINES FOR PROTOCOLS

A. Background

The current carcinogen bioassay in rodents has its origins in research efforts, begun in the 1920s, that sought to determine the carcinogenic potential of chemicals as well as to delineate the mechanism by which they produce cancer (*1, 2*). Animal strains were developed and tested for their specific sensitivity or resistance to certain types of cancer. These studies had as their central focus the understanding of the mechanism of carcinogenesis (*1, 2*). While these experiments led to the induction of cancer in laboratory animals and revealed many new carcinogens, most of the investigators did not intend their highly specialized test systems to be used as routine methods for testing large numbers of chemicals. Only in the mid 1960s, with the advent of bioassay program at the National Cancer Institute (NCI), did large-scale systematic testing using a relatively standard protocol really begin (*3*). As a consequence, only recently has there been a reasonably adequate data base on a large number of diverse chemicals tested in a relatively uniform way (*4*). The lack of such a data base, during the earlier developmental period, contributed to some degree of confusion and speculation as to how carcinogens should be tested and how the results from such tests should be interpreted. One finding was clear; known human carcinogens, with the single exception of arsenic, are carcinogenic in appropriately conducted studies in some animal system.

This does not mean that all chemicals found carcinogenic in animals will turn out to be carcinogenic in humans. Many substances are known that seem to be carcinogenic in one animal species but not in another. But a finding of carcinogenicity in rodents is proof that the chemical is carcinogenic in a mammalian species. This must be taken as strong evidence that the chemical can be a carcinogen in man, unless there is compelling epidemiological evidence to the contrary. The knowledge and experience accumulated over the last decade, while by no means answering all questions, have focused the issues and provided a basis for developing some common procedures for conducting and interpreting long-term bioassays.

B. Critical Design Criteria

Several national and international groups have published guidelines on the design and conduct of long-term bioassays (*5–15*), and the issue continues to be pursued. For example new guidelines are currently being developed by the NTP Ad Hoc Panel on Chemical Carcinogenesis Testing and Evaluation. Essentially, the long-term test involves testing compounds in animals to obtain information relevent to humans. While assessing the results of toxicological testing of any chemical, the limitations of the tests must be borne in mind. Of necessity, animal systems are used and the results then extrapolated to humans. Such extrapolations may not always be accurate either qualitatively or guantitatively. In some areas of cancer testing, uncertainty still exists concerning the most appropriate experimental design. For example, there are varying opinions on the optimum duration of a study, the number of doses tested and the number and types of species of test animals that are considered appropriate. It is probably unwise to adopt rigid "standard" test methods for all chemicals; judgment must be exercised to select an appropriate model for a particular chemical (*12*). Obviously, due to limitations of sample size (the number of animals on test), low incidence effects can go unrecognized. Consequently, although the results of carcinogenicity testing will, in many cases, give good indications of possible hazard, they do not eliminate the need for continuing careful observations of humans (*5*). In fact, most known human carcinogens, such as 2-naphtylamine and chlornaphazine, were first discovered by observing humans, and only later were they found carcinogenic in animals. The reverse was true for other compounds such as DES(diethylstilbestrol) vinyl chloride or bis(chloro-methyl)ether.

The following constitutes a series of major issues which should be carefully considered in the design of a carcinogen bioassay.

1. Species and strain of animal. Ideally, the appropriate animal species and strain is one, which, for the chemical tested, is able to predict human response accurately. However, the fact is that only three species have been routinely used in carcinogen bioassay (*16*). All three are rodents: the mouse, the rat and the hamster. The hamster is probably the least desirable because of the lack of comparable familiarity in its use as an animal model. Only these species generally meet the

requirements that the animals be readily available, that the bioassays are likely to be relevant to humans, and that the bioassays be cost effective and relatively quickly performed. Included in these requirements are: (1) The life span of the species must be of reasonable length, long enough to allow time for tumors to develop and short enough to be economically feasible (2–3 years); (2) the animal should be small and should live and breed well in captivity; (3) there should be similarity to humans in regard to metabolism and pathology responses; (4) strains should be those most likely to be susceptible to cancer from the particular chemical yet not hypersensitive. Only three species and a few strains currently meet these specifications, although there are potentially many more genetically derived strains which can provide a wide range of diversity in their metabolism of and response to carcinogens or carcinogenic precursors. There is still no scientific consensus over whether inbred or outbred strains of rodents are better for long-term studies (*17*). While the use of inbred strains may produce more precise and more stable biological responses (*18, 19*), more prosaic strains may more accurately reflect human response, are less expensive, often hardier and less prone to high incidences of spontaneous tumors. When the test substance is chemically related to other human carcinogens, the strain chosen can be that used for the carcinogenic chemical analogue. Extensive experience in using a particular strain is often a strong reason for continuing its use despite its acknowledged limitations (*14*). One major advantage is the availability of abundant data on spontaneous tumor rates at specific organ sites.

Because of genetic differences in tumor susceptibility the use of only a single strain of animal rather than an array of strains represents an experimental compromise, but an essential one given the large cost of a single long-term study. The possibility of a false negative result is reduced by the current recommendation to use two species instead of one in a carcinogen bioassay. More than 90% of the time these species are the rat and mouse. The Fischer inbred (F–344) rat and the B6C3F1 hybrid mouse are commonly chosen for carcinogen bioassay, in large part because of the experience gained by the NCI from their use (*4, 19, 20*). Despite its long history, the continued use of the B6C3F1, hybrid mouse by the NTP is currently under review because of the difficulty in interpreting the significance of proliferative liver lesions (*4–6, 20–22*). The merits of using only a rat strain instead of both a rat and mouse strain have also been recently discussed (*23*). This issue and others relating to species selection will be considered in the NTP Report on Chemical Carcinogenesis Testing and Evaluation.

2. Animal care and diet. Stringent control of environmental conditions and proper animal care techniques are mandatory for meaningful results (*12*). Access to animal facilities should be limited to essential personnel to prevent excessive traffic and the transmission of disease.

Factors such as housing conditions, intercurrent disease, drug therapy, impurities in diet, air, water, and bedding, and animal care facilities can significantly influence the outcome of animal experiments.

The control of intercurrent infectious diseases or parasites is facilitated if rodents are bred and maintained in conditons free from specific pathogenic organisms. Animals from outside sources should not be placed on test without an adequate period of quarantine. Bedding to be used in long-term studies should be absorbent, sterilizable and free of pesticides, other toxic contaminants, or enzyme inducing substances. Animals should be housed in quiet, well-ventilated rooms, having controlled lighting, temperature and humidity. It is advisable to balance dose groups over rows, columns and racks to allow for potential location effects across dosage levels. Alternatively it may be advisable to rotate cages or racks periodically to balance potential sources of variability. Eye lesions (retinal and lenticular degeneration) have been observed with animals kept in cages in closest proximity to light. Experiments should not be initiated until animals have been allowed a one to two week period of acclimatization to environmental conditions.

The absence of biases in selection and allocation of animals between control and treatment groups, as regards diet, husbandry, necropsy and pathology is crucial *(15)*. Since mice are reservoirs of asymptomatic viral disease, it is best to house mice and rats separately where possible. However, it is acceptable practice to house rats and mice in the same room if: (1) They are free of disease; (2) they come from the same supplier; and (3) they are being treated with the same chemical. It is preferable not to mix animals from different suppliers or from two locations of the same supplier. Housing disease-free animals in the same room reduces the chance for differences due to environmental variables. If a population of animals is diseased, then consideration must be given to aborting the study and starting over. Drug therapy should be avoided in bioassays since it is a confounding factor.

Control animals and test animals should be housed in the same room unless there is a possibility of cross-exposure. The availability of hoods, laminar air flow rooms, caging devices and personnel practices all affect the extent to which experiments should be separated or combined. To minimize the chance for inadvertent cross-exposure of animals to the test substance, it is advisable to test only one chemical in each room. If more than one chemical is tested in a room, the actual cross-contamination of chemicals within the room must be measured. Good laboratory practice need not rule out all flexibility nor restrict the use of judgment or impose unnecessary expenses (*26*).

Cages, racks and other equipment must be regularly and thoroughly cleaned. Periodic analysis of the basal diet should be conducted for both nutrients and unintentional contaminants, including carcinogens. The results from such analyses should be retained and included in the final report on each test substance. The essential dietary requirements of rodents are relatively high in protein and low in calories derived from fat (about 2–4% of calories). In the administration of substances by gavage using vegetable oils, the amount of fat ingested in rats can reach 30% (*25*). These facts, coupled with emerging data on the influence of dietary protein and fat on cancer incidence, and the presence of trace levels of carcinogens in animal diets may be important to future re-evaluation of bioassay results. Furthermore, the quality of the agricultural crops that go into animal feed can vary seasonally. Varying amounts of naturally occurring enzyme inducers from plant and fish material in animal feeds may affect the course of a long-term test for carciogenicity. It is therefore important to document and retain pertinent information on diet source and quality.

When the test substance is administered in long-term studies, stability tests are essential. Properly conducted stability and homogeneity tests, prior to the chronic study, should be used to establish the frequency of diet preparation and monitoring required. Similar tests are required for exposure atmospheres (in inhalation tests) to determine the necessity of rotating animals within the exposure

chamber and the frequency of monitoring required. The stability of the neat chemical, if it is to be used for a long time, as in long-term skin-painting studies, should also be determined.

If diets are sterilized, the effects of such procedures on the test substance and dietary constituents should be known. Appropriate adjustments to nutrient levels should be made.

During carcinogenicity tests, investigators should be aware of potential chemical contaminants in the water used. Although water approved for human consumption is generally satisfactory, the investigator should determine the levels of potential contaminants that are likely to influence the outcome of the study. The analyses of the water supply should be included in the reports of these studies.

3. Test and control groups. The number of animals to use has been the subject of great concern because of the fact that the test animals may be serving as surrogates for more than 200 million people in the U.S., and potentialy for far more worldwide. While it is desirable to enhance the sensitivity of the test, it is not feasible to use very many animals. Furthermore, a moderate increase in the group size provides relatively little increase in the statistical power of the test. Therefore, careful attention must be paid to experimental design to assure the maximum reliability of the study and that the results are amenable to statistical evaluation. The use of adequate randomization procedures for the proper allocation of animals to test and control groups is of particular importance. A sufficient number of animals should be used so that at the end of the study enough animals from each group are available for thorough pathological evaluation. For such reasons, it has been recommended that each dose group and concurrent control group should contain at least 50 animals of each sex (*4, 11, 19*). If interim sacrifice(s) is included in the study plan, the initial number should be increased by the number of animals scheduled for the interim sacrifice(s).

At least one concurrent untreated control group, identical in every respect to the exposed groups, except for exposure to the test substance, should be used. An additional concurrent vehicle control group should also be utilized in special circumstances, such as when an emulsifier of uncharacterized biological activity is used as an aid in dispersing the test chemical into the diet, or when it is necessary to use a solvent or vehicle to dissolve the test compound for administration to the test animals. In certain studies, such as with inhalation exposures, that require unique housing conditions, it may also be appropriate to include an additional concurrent control groups housed under conventional conditions.

Occasionally a positive concurrent control group is useful, particularly when there is reason to believe that the strain of animals may be resistant to the particular class of carcinogens or that a previously sensitive strain has become insensitive because of genetic drift. A positive control can also provide some information on the relative carcinogenic potency of the tested chemical. In routine carcinogenicity testing, no positive controls need be used (*4, 11*).

4. Dose levels, frequency and route of exposure. If a single bioassay is to be used both for detection and for risk assessment, at least three dose levels in addition to the concurrent control group are recommended. The question of what maximum dose should be administered may be the most controversial issue concerning bioassays (*10, 26*). The highest dose level currently recommended is that just high enough to elicit signs of minimal toxicity without significantly altering the normal life span due to effects other than carcinogenicity (*7*). The dose of test substance should not comprise so large a fraction of the animal's diet that the required nutrient composition of the diet is altered, leading to nutritional imbalances. This maximum tolerated dose (MTD) is ordinarily determined in a 90-day study; and was initially based on a weight gain decrement, *i.e.,* the highest dose that will produce a slight depression in body weight (approximately 10–12%) if administered over a lifetime. NTP has more recently suggested refinement of MTD selection on the basis of a broader range of biological information. Other signs of toxicity that may be used to establish the MTD include primarily gross and microscopic pathology, but alterations in serum enzyme levels, hematological effects or other physiological, biochemical or pharmacological indices of abnormality may be useful. The important contribution of pharmacokinetic studies to the selection of the MTD is the information such studies provide on the presence of dose-dependent and time-dependent processes affecting the disposition of substances in the body (*27–33*). Such information can identify disproportionate changes in internal concentrations of the chemical or its metabolites with administered dose and also can indicate possible accumulation of the chemical or its metabolites with repeated exposures. When this information is available and effectively applied to dose selection (including the MTD) and utilized in determining the shape of the dose-response curve, carcinogenic responses observed at high doses can be more accurately extrapolated to dose levels in the range of human exposure. To be of maximum use in the dose selection process this information should be obtained early, if possible before or during the prechronic phase of the bioassay.

Lower doses are ordinarily simple fractions of the MTD (½, ¼ etc. . .). The selection of these doses should be based on the results of adequate subchronic studies, pharmacokinetic data, and on anticipated human exposure. The precise objective of the study is also important. Because of cost, a single long-term study may be utilized to obtain data on chronic toxicity, on carcinogenic potential and for carcinogenic risk assessment. The present discussion envisions the combination of both the qualitative objective of a carcinogen bioassay, *the detection of carcinogenic potential,* and the quantitative objective, *obtaining dose-response data for risk assessment,* into a single bioassay. If only one of these objectives is sought, it may be advisable to select the doses differently. The purpose of using the MTD is to provide maximum opportunity for the detection of a neoplastic response. The purpose of several lower doses is to provide adequate information on the shape of the dose response curve for risk assessment and to ensure a meaningful comparison with the control even if the MTD was unfortunately selected at too high a level. The pathological response observed in the subchronic study and the slope of the subchronic dose-responses curve should aid in the selection of the range and number of doses for the long-term study. For example, a steep dose-response curve would suggest closer spacing of intermediate doses than a shallow dose-response curve. The existance of metabolic saturation (as indicated by pharmacokinetic data) at doses well below those identified by pathological criteria suggest a spacing of doses designed to determine both whether the highest dose has any carcinogenic effect and to adequately define the dose-response curve for the saturable process. This may require additional doses. Generally four doses (occasionally three) will be necessary to adequately define the dose-response curve, assuming an appropriately chosen MTD. Since the MTD is an estimate at best, the value of additional doses is generally difficult to judge. If the estimated MTD eventually produces

moderately high or excessively high tumor incidence, additional lower doses may aid in defining the shape of the dose-response curve and may even salvage a possibly invalid quantitative study. If the estimated MTD produces no tumors, or a low incidence of tumors, the additional doses will have been unnecessary. As indicated above, the best guide to the number of doses is the acquisition of knowledge on the pathological and pharmacokinetic criteria upon which a sound, scientific judgment can be based. (The National Toxicology Program [NTP] will be making available more detailed recommendations for prechronic testing and dose selection from its Ad Hoc Panel on Chemical Carcinogenesis Testing and Evaluation). Interruptions in the administered dose may permit some cellular repair and reduce the sensitivity of the test. Therefore, if the chemical is administered in the drinking water or mixed in the diet, it should be continuously available. The frequency of exposure may vary according to the route chosen or may be adjusted according to the toxicokinetic profile or stability of the test substance (See Section 2 above). Protocols for inhalation or for dermal exposure are usually subject to special constraints (*11*).

The route of administration used for the bioassay is critical for both its qualitative and quantitative objectives but in different ways. If the route of administration results in the absorption, distribution and metabolic activation (if required) of the substance, it is reasonable to regard the test as relevant for a *qualitative* demonstration of human carcinogenic hazard. This comparison is obviously most directly made when the route used in the test and the route of human exposure are the same, but as long as the criteria above are satisfied, the results of animal studies are generally considered qualitatively meaningful.

If, because of its palatability, solubility, stability or volatility, a substance can not be given in feed or water, oral gavage may have to be considered. This is clearly less desirable, however, since single large boluses can result in absorption and pharmacokinetic patterns different from those anticipated from human exposures (See also Section 2). Sometimes, the use of gavage can be avoided by gradually increasing dietary concentration of the test substance to gain acceptance, by altering other dietary components to increase the palatability or by microencapsulation.

Certain site specific tumors, *e.g.*, bladder carcinoma following bladder implantation and subcutaneous sarcomas following injection, may not be relevant for human oral exposure. The presence of tumors remote from the site of administration and a knowledge of site-specific metalbolism play a significant role in the final evaluation. Some routes of administration may fail to provide adequate metabolic activation or exposure of target tissues and therefore lead to false negative results (*11*). Such data require careful evaluation.

When an assessment of human risk is desired, it is desirable to obtain sufficient data to permit a reasonably accurate estimate of the dose of the carcinogen reaching the target tissue. In some instances, *e.g.*, for substances used on the skin, this may require a bioassay to detect tumorigenic potential and a separate study of the substance's permeability across the skin to measure the amount absorbed. In inhalation studies, this may require a separate study for determining the amount of inhaled material deposited on, or absorbed by the respiratory tract.

5. Duration of study. Test animals should be exposed for the majority of their normal life spans. While there are certain animal test systems which offer the possibility of inducing cancer within only a few weeks or months, such systems at the present time are useful mainly as research tools to explore specific, well-defined questions. They cannot be relied upon to test unknown substances for carcinogenicity and are insufficient as a primary basis for regulation.

Treatment is preferably begun as soon after weaning as possible (no later than 6 weeks) and continued for the major portion of the animals' lifespans. This is a least 18 months for mice and hamsters and 24 months for rats. In some instances where the cumulative mortality at the planned time of sacrifice is still very low, *e.g.*, less than 10%, a longer duration may be appropriate. Some protocols call for treatment soon after birth (neonatal) or during fetal development (*in utero*). The rationale is based on the greater susceptibility of certain organ systems to carcinogens during their early development. However, there are logistical problems which preclude using neonatal or *in utero* systems for routine bioassays.

Termination of the study is ordinarily acceptable when the number of survivors in the low dose or control group is reduced to 20–25%. Generally, because of difficulties produced by naturally-occurring tumors, long-term studies are conventionally terminated by week 100 for hamsters, 120 for mice and by week 130 for rats, regardless of mortality. When there is an apparent sex difference in response, each sex should be considered a separate study (*9*). If the high does group dies prematurely from obvious toxicity, this should not trigger termination of the lower dose groups or controls. A negative test is ordinarily accepted by regulatory agencies if: (1) No more than 10% of any group is lost due to autolysis, cannibalism or management problems; and (2) survival of all groups (per sex per dose) is no less than 50% at 80 weeks for hamsters, 96 for mice and at 104 weeks for rats (*12*).

Rigid rules for termination are not helpful, the best guide is professional judgment, experience and careful observation of the condition of the animals. The longer the test continues, the greater the opportunity for the development of tumors (*34*). On the other hand, the sensitivity of bioassays decreases with time, because of the natural appearance of age-related tumors in the controls. In theory, it is necessary to know both the incidence and growth rate of naturally-occurring tumors and experimentally-induced tumors at various organ sites in the sex and strain of animal used in order to determine the optimal time for termination. This information is ordinarily not available until the end of the test and while serial sacrifices are useful, they are considered too expensive for routine testing of chemicals.

6. Data collection and reporting. Animals should be checked carefully at least twice each day. Observations should be sufficient to detect onset and progression of all toxic effects, as well as to minimize loss due to diseases, autolysis, or cannibalism. 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. Loss of body weight should trigger careful clinical examination of the animal and its sacrifice, if necessary. Food intake should be determined weekly during the first 13 weeks of the study and then at approximately monthly intervals, unless health status or body weight changes dictate otherwise (*12*).

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

The pathological examination, macroscopic, as well as microscopic, is the cornerstone of the carcinogenicity study. A thorough gross necropsy with subsequent selection of appropriate lesions for histopathology is mandatory. It is vital that the sampling among the test and control tissues be uniform so that no bias is introduced into the comparisons. Microscopic examination of both test and control tissues is required. While an all-inclusive microscopic examination of all tissues is theoretically desirable, the resource limitations may dictate a more selective approach such as the one recently proposed by the National Toxicology Program or earlier by the National Cancer Institute (*35,36*). The quality, extent and completeness of the documentary record of the bioassay are of critical importance in establishing its validity for scientific and regulatory purposes. It is crucial that the physical evidence, *e.g.*, the original tissue-sections and slides, is accurately prepared and maintained. Sponsors of bioassays that are to be submitted to regulatory agencies should be familiar with additional requirements of GLP's (Good Laboratory Practice)(*24*).

II. INTERPRETATION AND EVALUATION OF LONG-TERM CARCINOGEN BIOASSAYS

A. Background

Several attempts have been made over the last 10 years to construct a suitable operational definition for a chemical carcinogen (*6, 7, 10, 11, 37, 38*). Though none of these have succeeded fully, there is common agreement that a chemical carcinogen is a substance which induces cancer by some chemical/biological mechanism. A chemical carcinogen may be a substance which either significantly increases the incidence of cancer in animals or humans or significantly decreases the time it takes a naturally-occuring (spontaneous) tumor to develop relative to an appropriate background or control group. Either phenomenon is said to represent the effects of a carcinogen (*10*).

Just as there were no standardized or uniform protocols for testing chemical carcinogens during the early developmental period of the field, there are now no universally agreed upon ways of interpreting carcinogenicity data. In past years, a substance was not labeled a carcinogen unless the evidence was quite substantial. Usually, the identified carcinogen was used as a research tool to study the process of carcinogenesis, so that many experiments were performed with the compound. This intense study usually dispelled any doubt as to whether the substance was actually a carcinogen. This degree of confirmation gradually decreased as more of the experimentation turned from research on the process of carcinogenesis to detecting as many chemicals as possible that might pose a cancer risk to humans. For this latter purpose, which began in earnest in the mid 1960s with the "screening" bioassays conducted by NCI, it was necessary to devise "decision rules" by which to declare a chemical a carcinogen (*3*). Experience has taught that these should be regarded as general guidelines in evaluating carcinogenicity data and that final decisions are best made after careful communication between pathologists, toxicologists and statisticians. Most useful to the process is the experience and the data derived from large numbers of recent tests using a uniform protocol.

With some oversimplification, the task of the pathologist and toxicologist is to decide whether a lesion is cancerous or pre-cancerous and whether it is related to exposure to the test chemical. The statistician, on the other hand, has the task of calculating the mathematical probability of whether the tumors occurred by chance or as a result of treatment. Neither of these tasks is simple or completely independent of the other and, most importantly, each requires experience and judgment if sensible decisions are to be made.

B. Toxicological Issues in Bioassay Evaluation

1. Use of the maximum tolerated dose (MTD). Largest doses (*i.e.* doses far exceeding human exposure levels) have been strongly recommended by several national and international bodies in order to overcome the inherent low sensitivity of bioassays (*11, 12, 38*). These recommendations have been controversial because high doses may themselves produce altered physiologic conditions which can qualitatively affect the induction of malignant tumors. Normal physiology, homeostasis and detoxification or repair mechanisms may be overwhelmed and cancer, which otherwise might not have occurred, is induced or promoted. If qualitatively different distribution, detoxification or elimination of the test chemical is produced at the highest dose, a toxic response at this dose may not be indicative of effects at low exposure levels. The cancers which arise from high doses of corticosteroids, estrogens, certain sulfonamide compounds and from some instances of bladder implantation may result from such "secondary" effects (*39*, and see Chapter 1). This concern is not easily resolved, for if the dose is not high enough, carcinogens may remain undetected by traditional bioassays.

The concept and operational definition of the MTD have undergone modification during the last several years in response to some of these concerns. The 1971 FDA Advisory Committee on Protocols recommended that: "*Testing should be done at doses and under experimental conditions likely to yield maximum tumor incidence*"(*6*). However, as the use of these tests for human risk assessment increased, it became vital not only to detect carcinogens qualitatively (*i.e.*, avoid false negatives), but also to estimate effects at low dose levels. both the National Cancer Advisory Board Subcommittee (NCAB) (1977) and the Interagency Regulatory Liason Group (IRLG) (1979) (*11*) cautioned against the use of a dose so high that it produced "*unwanted toxic side effects*" (IRLG) or "*unphysiologic conditions [which] may in themselves enhance tumor formation*" (NCAB). Recent publications have emphasized the need to select the doses not only on the basis of the results of subchronic toxicity studies, but also on the basis of what is known about the pharmacokinetics and metabolism of the test substance (*10, 26*). Better techniques to observe and detect significant differences in metabolism and pharmacokinetics thoughout the dose range used are being developed; these should help in a better determination of the MTD (*27, 33*). There should be a reasonable scientific certainty that the dose used meets the objectives of maximally enhancing the sensitivity of the test without introducing qualitative distortions in the results.

2. Promoters vs. Initiators, current experimental status. In Chapter 1 of this report, the evidence for the belief that cancer occurs through at least a two stage process consisting of initiation and promotion was discussed. The basic question of whether tumor promotion in a general feature of carcinogenesis or is only characteristic of some organ systems is still debatable, although more and more evidence for the initiation-promotion process in a variety of organs is becoming available (*40* and Chapter 1.) The question naturally arises as to whether such promoting agents can reliably be distinguished from initiators by traditional bioassays.

While a number of examples of promoting agents are well documented; most of the work on their mechanism has been done on skin, *e.g.*, the phorbol esters in epidermal carcinogenesis.

Other examples are butylated hydroxyanisole in pulmonary carcinogenesis, phenobarbital in hepatocarcinogenesis and estrogenic hormones in breast cancer. Some know promoting agents also have weak tumorigenic activity and some are also initiators, *i.e.* complete carcinogens. Carcinogens may act as promoters at some tissue sites and initiators at others. It is very difficult both in principle and in practice to confirm the claim that a given chemical acts by promotion alone. If such a mechanism is postulated for a specific chemical, data on the purported mechanism at the affected tissue sites and dose response data would be needed.

3. Need for bilogical significance, other data. There is no simple or general solution to the problems which arise when attempting to interpret carcinogenicity data. Over recent years, review groups charged with the responsibility to evaluate carcinogenicty data and come to some decision regarding the carcinogenicity of the chemical have recognized the need to consider all of the available information which may bear on such a decision (*5, 6, 8–10*). Such information includes historical data on control animals, information from *in vitro* mutagenicity or neoplastic transformation systems, toxicological information which might indicate that the organ or tissue which is at risk for carcinogenicity appears to be a target for the toxicity of the compound and any other relevant information. Mutagenicity, DNA repair and neoplastic transformation studies can sometimes be used in aiding judgments on carcinogens (*41*). For example, if the data indicating carcinogenicity are of only marginal significance, negative findings in properly designed and well-conducted *in vitro* tests, depending upon their relevance to the specific situation, may lead to a conclusion that the chemeical is probably not carcinogenic. On the other hand, were the results from *in vitro* testing clearly positive, and if evidence existed to show that the organ in which the cancer question arose was also the toxicological target of the chemical, the conclusion is likely to be that the chemical is carcinogenic. To make these judgements, it is important that there be a good interaction and deliberation among scientists from different disciplines.

C. Issues of Pathology in the Interpretation of Tumor Data

1. Tumor number, type and site in the assessment of incidence. As mentioned earlier, a carcinogen may be defined as a substance which increases the incidence of cancer above the spontaneous backgroung rate Section II, Part A). However, from a partical, point of view, the most important question is: How does one estimate or deduce cancer incidence from tumor data? For instance, should all cancers in the animal be combined and the total number of tumors in treatment groups compared to total cancers in the untreated controls? Alternatively, should carcinomas be separated from sarcomas since they are derived from different tissue? Should the comparisons between treated and controls be restricted to specific organ sites and, if so, should all tumors be conbined, or only certain types? Other questions are: Should benign and malignant tumors be separated or combined; if tumors occur at miltiple organ sites, can they be combined and how should it be done; should cancers which shorten the life of the animal be separated from those which do not? The questions and many more must be answered before cancer incidence(s) can be estimated. Unfortunately, there is no universal agreement among experts in the field relevant to the rules for calculating cancer incidence that is applicable to "all" carcinogens "all" of the time. Generally, most experts agree that the incidence of total tumors at all organ sites is not a very useful expression of cancer incidence, nor in the calculation of the incidence of total benign or total malignant tumors. Most useful appears to be the number of histologically unique tumors at specific organ sites (*42*).

The grouping of lesions for evaluation should be based on commonality of histogenetic origin. Therefore, sarcomas should not be combined with carcinomas for the purpose of establishing a value for the incidence of cancer at a specific organ site. It is often necessary, at least in the judgment of many pathologists, to combine certain benign tumors with malignant ones occurring in the same tissue and at the same organ site. This practice can make the total neoplastic response (benign and malignant) clearly significant despite the lack of statistical significance in the tumors diagnosed as malignant. These pathologists believe that truly benign tumors in rodents are rare and that most tumors diagnosed as benign really represent a stage in the progression to malignancy. For some tissue sites, this view is widely accepted. Examples of this are adenomas versus adenocarcinomas in the pituitary, thyroid, lung, kidney tubules, and according to some experts, in mouse liver. In each of these cases, it is argued that the judgment of the pathologist as to whether the lesion is an adenoma or an adenocarcinoma is so subjective that it is essential they be combined for statistical purposes. It is also argued, in these specific cases, that the adenoma is a precursor of the adenocarcinoma. Indeed, the Subcommittee on Environmental Carcinogenesis of the National Cancer Advisory Board recommended in 1976 that these lesions be combined for statistical purposes (*38*). Most U.S. regulatory agencies have followed this recommendation. Ordinarily the tumor or cancer incidence is calculated as the number of tumor-bearing animals having tumors at a specific organ site divided by the total number of animals that survived long enough on experiment to have been at risk for that specific type of tumor. For reasons discussed in the statistical part of this section, life shortening tumors should not be combined with non-life shortening tumors.

2. Time to tumor. A positive result in a carcinogenesis bioassay can be based on evidence of a substantially decreased latency period, as well as on the induction of an increased incidence of malignant tumors. A good discussion of the precautions that should be observed in order to establish decreased latency is given in the 1979 IRLG report (*11*). Such data, in conjunction with incidence data, can be useful to distinguish between the relative potencies of carcinogens.

3. Problems in tumor diagnosis. A major problem facing those who must make either scientific or public health policy decisions based on carcinogenicity data is that the diagnosis of pathological lesions is subjective and pathologists may disagree on diagnosis. Frequently, the disagreement is not as serious or far ranging as it may appear to nonpathologists. The disagreement may be focused on an issue where neither pathologist is absolutely certain and each is forced to offer his "best opinion." In practice, for most compounds, NCI and NTP have used panels composed of 2 to 4 pathologists to review and analyze the lesions that have been observed (*35, 42*). This procedure helps to provide consistency in diagnosis, but it may not solve all the problems. For instance, most pathologists do not review the microslides "blind." They know which slides come from control animals and which from treated animals. The extent to which this information leads to bias in diagnosis is not known (*35*).

A common problem with the interpretation of pathological data

concerns the selection of the tissues at necropsy, how the tissues were prepared, the slides mounted and so on. For instance, a few years ago most investigators did not open the skulls and carefully examine the brain. In other cases, only brains appearing abnormal were examined in detail. Currently, the brain as well as other tissues such as nasal turbinates are routinely examined histologically (*34, 42*), though the techniques vary somewhat. As a consequence, spontaneous tumors of these tissues are not being reported. Depending upon how extensively these tissues are being sectioned for histological analysis the observed incidence may vary from low to moderately high. Such modifications in the pathology protocol, if not recognized and understood, could lead to serious errors in judgment concerning the carcinogenicity of a chemical.

Critical to decisions about carcinogens is the biological significance and human relevance of certain types of tumors, particularly the liver tumors in the mouse. This matter has been the subject of heated debate for the past 15 years. Some scientists are certain that the mouse liver is overly sensitive and will respond to almost any toxic insult by developing cancer. Other scientists are just as certain that the mouse liver is a reliable indicator of carcinogenicity and as good a predictor of potential human risk as any indicator available. In one review, 61 of 85 chemicals (73%) that increased the incidence of liver tumors in mice also induced tumors in other tissues of mice and/or in rats (*20*). Some toxicologists believe that part of the problem stems from work on C3H strains of mice where the background liver tumor incidence is high and false positive errors are possible. (See below Sec. D.) Generally, there is consensus that the mouse liver model in principle does have significance in terms of human risk, but unambiguous diagnosis between "benign" or "hyperplastic" liver nodules and malignant neoplasia remains elusive and there is no apparent scientific consensus.

In addition to liver tumors, other tumors, including lymphomas in the mouse, which appear to be of viral origin, have been questioned as to their relevance to human cancer. As a general matter, toxicologists must question the relevance to humans of any experimental tumor which has a known viral background where the virus may be unique to the animal. This includes a variety of lymphomas and sarcomas in rats and mice, as well as mammary tumors in the mouse. Likewise, the relevance to human exposures of the induction of bladder cancer in rodents which are shown to have bladder stones and crystaluria only at high doses of the test substance is questionable.

D. Evaluation of Statistical Significance

1. Statistical tests and procedures. For the analysis of tumor incidence data, a widely-used method for comparing experimental and control groups is Fisher's exact test (*43*). This analysis is generally carried out in conjunction with a Cochran-Armitage trend test (*44, 45*) which examines the relationship between tumor incidence and dose. The trend test has the advantage of utilizing cancer incidence data for all doses and as a result is somewhat more sensitive than pairwise comparisons to the detection of carcinogenic effects.

The statistical procedures mentioned above for the analysis of tumor incidence data do not take survival differences into account (*15*). This can be a problem with chemicals that produce a life-shortening effect. Although "survival-adjusted" tumor incidence analyses (*15, 46, 47*) are more complex, they have the advantage of adjusting for intercurrent mortality and of being sensitive to shortened latency period as well as to increased tumor incidence. These procedures require the determination of whether tumors in individual animals are "lethal" or "incidental" with respect to the underlying cause of death. This determination may be difficult if not impossible to make for many animals, but experimentalists should try to distinguish in their records between lethal and incidental tumors.

While it is customary practice in the conduct of bioassays to regard an increased tumor incidence that is statistically significant (*e.g.*, $p < 0.05$) as reasonable assurance that the effect is real and not due to chance alone, the final judgment regarding a compound's carcinogenicity is rarely based on a single finding of statistical significance (*47*). One must be assured that the effects observed are biologically significant as well. Other factors that must be taken into account include: (1) The reproducibility of the effect in other doses and/or in other sexes or species; (2) the historical incidence of the tumor in question; (3) the survival histories of dosed and control animals; and (4) evidence of hyperplasia, metaplasia or other signs of an ongoing carcinogenic process.

The 0.05 p-value is a reasonable statistical benchmark with a history of use in the bioassay field. It would be imprudent to disregard it as an index of significance without good reason. It would be equally unwise to regard it as an absolute requirement or a *sine qua non* without considering the weight of biological evidence. For example, doubling or tripling the incidence of a tumor that occurs at low incidence in the controls may not be statistically significant, but may indicate a biological effect that should not be dismissed. Similarly, the finding of nominal statistical significance ($p = 0.05$) at a single tumor site may be less important to the final evaluation than knowledge of the target site and the lack of consistency with related biological findings. As stated by IARC (*15*):

> The p-value from a good experiment is an objective fact, subject to public agreement. The weight to be given it and to the available pieces of information is not.

2. Problem of False Positives and False Negatives. Because of the time and expense involved, most chemicals, if tested at all, are tested in only one bioassay. It is vital that this test have good sensitivity since otherwise many true carcinogens would remain undetected (a false negative). The test must also possess a high degree of *specificity* since there are economic losses as well as disincentives for testing if safe, useful substances are mistakenly declared carcinogens (a false positive).

a. False Negatives

The typical bioassay, due to inherent limitations (low number of animals and biological variation) cannot reliably detect an increased tumor incidence of much less than 10–15%. High test doses are one way around this limitation, but as discussed above (Sec. II, Part B, 1), this can introduce confounding factors if the dose is to high. In addition, the possibility always exists that for a specific substance, the most sensitive species may be inappropriate for qualitative extrapolation to humans. Until more is known concerning the mechanism of cancer and the similarity in response between man and animal, this uncertainty will persist.

Confidence in an apparently negative bioassay increases with: (1) The number of animals used; (2) the extent to which its duration approaches the animals' expected lifetime; and (3) the adequacy of the gross and histological examination of both control and treated groups. Corroborative evidence such as experimentally consistent biological results in different species or sexes, or at different doses, strengthens the likelihood that the bioassay is correctly interpreted. Rigid rules or definitions are not very useful. The ultimate decisions

must be based on the knowledge of experienced pathologists and toxicologists, the weight of corroborative evidence and a careful statistical evaluation.

The spontaneous tumor incidence can be of considerable importance in the interpretation of results from carcinogenicity studies. If, for example, the effect of a chemical is to double or triple the background tumor incidence, tissue sites with low spontaneous tumor rates are more likely to yield false-negative results than are sites with high spontaneous tumor rates. For example, if a tumor is a rare tumor even a slight increase in incidence may be biologically significant and may be considered adequate evidence of carcinogenicity (*43*). This factor must be taken into account in the overall evaluation of the data. For such tumors (*e.g.* with spontaneous tumor rates of 1% or less), the utilization of historical control data may be particularly useful in increasing the sensitivity of the study for detecting carcinogenic effects. All recent work emphasizes the fact that the evaluation of the bioassay is not a simple nor routine statistical exercise, but a multidisciplinary process requiring the interaction of toxicologist, pathologist and statistician.

b. False Positives.

Appropriate precautions in the design and evaluation of bioassays can minimize the chance of false positive results. The test substance must be free of carcinogenic contaminants and appropriate doses and routes of exposure must be used. However careful attention to these and to other factors cannot eliminate entirely the possibility of either false positive or false negative results. These problems are fundamental to the proces of drawing inferences from observed sample data.

Many investigators familiar with data from bioassays believe that in practice the overall false positive rate does not greatly exceed 0.05 (*43,44*). Others are not as certain, and all admit that this is an important problem area that must be considered in the interpretation of carcinogenesis bioassay data. The very effort to be as thorough and as complete as possible in the selection of tissue at necropsy, and in the detection of responses from various doses, increases the chance for a false positive error. Typical bioassay protocols require sectioning and analysis of tissues from 20–30 sites in two species, in two sexes, and, at the least, two dosage levels. It has been suggested that the multiple tests for significance applied to these many sites produce an unacceptability high overall false positive rate. (The argument is that if 'n' independent tests are concluded at the 'a' significance level, the probability of finding a significance somewhere [the false positive rate] is $[1-(1-a)^n]$. For $n=20$ and $p=0.05$ for a single dose-sex comparison, this probability is about 0.64.)

However, recent investigators have shown that this argument overestimates the likely false positive rate of the bioassay. Gart, *et al.* (*43*), using historical incidence rates for B6C3F1 mice, found the probability of at least one significant result at the 0.05 level at some site in at least one sex was only about 8%. In a commonly used strain of rat (F–344) the same probability was approximately 21%. These findings are will below the 64% value cited above. One reason for this difference is that tumor incidences are discrete court data, and in this situation, the actual significance level of test procedures comparing tumor rates is always less than the nominal level, often considerably less.

This is particularly true for tumors having a low spontaneous incidence. Even the estimated false positive rates reported by Gart, *et al.* (*43*) may be too high, since (as noted earlier) the final judgment that a compound is carcinogenic is rarely based on a single isolated "significant" increase in tumor incidence. Generally more stringent evidence is required, such as the reproducibility of the effect in other doses and/or other sexes or species. For example, if a chemical is judged carcinogenic only if it is positive ($p<0.05$) at some tissue site for *both* the low and high doses, the overall false positive rate for a two sex, two species experiment has been estimated to be 5.1% (*49*).

In a recent examination of 25 NTP feeding studies, a simple statistical rule was derived by Hasemen (*48*) which appears to mimic closely the scientific judgment process used in these experiments. This "rule" was as follows: Regard as carcinogenic any chemical that produces a high-dose increase in a common tumor that is statistically significant at the 0.01 level or a high-dose increase in an uncommon tumor that is statistically significant at the 0.05 level. The overall false positive rate associated with this particular decision rule (which appears to approximate closely the overall evaluation process) was estimated by Haseman and found to be no more than 7–8% for the current NIP two-sex, two-species protocol (*50*).

In summary, in the interpretation of tumor incidence data an investigator should be aware of the false positive issue. In the overall evaluation process, one should be guided by (but not rely exclusively upon) the statistical significance of an observed tumor increase. This awareness will help minimize the likelihood of a false positive result.

3. Use of historical controls. It is extremely important for the interpretation of a bioassay that the variability of the incidence of spontaneous tumors in the strain of animals used be established with the highest precision possible. These variations present problems in interpreting the results of bioassays. NTP has recognized this problem and has continued to accumulate data from untreated and corn-oil gavage control groups for the strains of animals used (*19*). With such data, the toxicologist may conclude that a study yielding statistical significance is not biologically significant or conversely that an effect is real, even though there is no "significant" difference between test and concurrent controls. Of 27 bioassay study reports issued by the NCI from 1978–1980, historical controls were used in 14 studies to show that an apparent increase of a specific tumor was not related to treatment (*50*). In 13 studies, the use of historical controls showed that an increase in tumors was related to treatment. Historical control data can be valuable when used appropriately, especially when the differences in incidence rates between treated and concurrent negative controls are small and can be shown to be within the anticipated historical incidence. However, the use of historical control data is not without its own problems. Over the course of time, the thoroughness of pathologic evaluations has improved and pathologic diagnosis has evolved and nomenclature has changed. There is also the possibility that the genetic susceptibility of the strain has shifted. The sources of variability in historical control data should be identified and, if possible, controlled. Obviously one has more confidence in the most recent historical control data from the same laboratory conducting the current study than in a compilation of pooled older data from other laboratories. Several procedures have recently been proposed for utilizing historical control data in a formal testing framework (*51–53*). The relative merits of these methodologies are currently being investigated by the NTP.

III. SUMMARY

Although the identification of carcinogenic substances has long been

of interest to the scientific community, until the 1960's the focus of most studies was research, not testing for substances which may pose a public hazard. The National Cancer Institute, and later the National Toxicology Program, have conducted a number of test-oriented studies, the chronic bioassays, and have also attempted to standardized many important parameters in conducting these studies. Type and number of animals, dose level and route, and study duration are fairly uniform in an effort to make the data base as comparable as possible from test to test.

Certain issues remain as problems. One of these is the use of the maximum tolerated dose (MTD) in long-term studies and the appropriateness of dose route, schedule, and length of exposure. Use of the MTD, if not an ideal solution to the problem of bioassay sensitivity, is appropriate if it is properly determined. Another problem is the interpretation of tumor data. This includes tumor number, type, time to tumor, tumor diagnosis, etc. and the significance of certain tumors. Certain guidelines have been recommended, with the proviso that they are to be used in conjunction with sound judgment.

Furthermore, although several methods of statistical analyses of long-term test results have been developed, the questions of false negatives and false positives and the use of historical controls, suggest that the evaluation of a long-term test is not a routine statistical exercise, but is instead a multidisciplinary process requiring the interaction of toxicologist, pathologist, and statistician. The utilization of long-term animal studies for quantitative human risk assessment has accentuated and focused attention on the real scientific uncertainties involved in high-to-low dose and animal-to-human cancer risk extrapolations. Nonetheless such animal studies, with all their limitations, remain the best way of predicting effects in humans when epidemiological data is unavailable.

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Chapter 4—Current Views on Epidemiological Methods

I. INTRODUCTION

Epidemiology is generally defined as the study of the distribution and determinants of disease in human populations, and thus it involves two major approaches (*1*). Descriptive studies examine the "distribution" of disease and are usually employed to generate etiological hypotheses, while analyticl studies are used mainly to test hypotheses and identify the "determinants" of disease. A primary objective of epidemiology is to identify and quantify relationships between exposure to chemicals and deleterious health effects. The associations may lead to causal inferences (*2*), which in turn provide the basis for instituting preventive measures for various diseases.

II. STRENGTHS AND LIMITATIONS OF EPIDEMIOLOGY

A. Strengths

In contracts to studies in other biological systems, epidemiology directly evaluates the experience of human populations and their response (risk of disease) to various environmental exposures and host factors. Thus, it if often possible to evaluate the consequences of an environmental exposure in the precise manner in which it occurs and will continue to occur in human populations. This include such important considerations as dose, route of exposure and concomitent exposures to other exogenous and endogenous factors. Through epidemiological studies human cancer has been linked to a number of lifestyle and other environmental hazards, including tobacco products and alcohol, ultraviolet and ionizing radiation, certain occupational and medicinal chemicals, dietary factors, and some infectious agents (*3–5*). Epidemiology has played a central role in detecting carcinogenic exposures, and it has complemented studies in laboratory animals in clarifying the carcinogenic potential of specific agents (*61*). Another strength of the epidemiologic approach is its ability to provide insights into the mechanisms of huma carcinogenesis. Thus, epidemiological observations have complemented experimental evidence that carcinogenesis is a multi-stage process, and that many cancers may result from the cumulative effect of environmental factors and host susceptibility states that accelerate the transition rates at various stages of carcinogenesis (*7*).

B. Limitations

Although epidemiology is the only means of assessing directly the carcinogenic risks of environmental agents in humans, the method has several limitations that are difficult to overcome (*1, 8*). One problem is that evidence of an environmental hazard is usually obtained from persons with high or intermediate levels of exposure. Just as for studies in laboratory animals, detecting causal relationship at low exposure levels is difficult in epidemiology, since the observed associations with disease are usually less pronounced and may have alternative explanations, including those related to chance, errors, biases or confounding variables. To provide a valid basis for risk estimates, large numbers of human subjects are often needed, especially if the exposure is low or rare, or if the excess risk is small compared to that ot the baseline incidence rate. Another obstacle to epidemiology is the long latency period between exposure and the development of cancer. This complicates the detection of relationship and, of course, makes it impossible to identify the carcinogenic risks to humans of agents newly introduced into the environment. Another common problem in epidemology it that of exposure assessment. Often the specific exposure of interest cannot be measured directly so that surrogate measures must be used (*e.g.* occupation, place of residence). Since exposure data are usually derived from historical records generated for other purposes or from the recollections of subjects, opportunities for either random or biased misclassification of exposure are frequently encountered. In addition, appropriate study groups are often simply unavailable or inaccessible. Furthermore, it may be difficult to implicate specific carcinogens when environmental hazards involve complex exposures to a variety of agents, the effects of which are to difficult to disentangle. Still another difficulty is the inability of epidemiological studies to adjust for unknown risk factors, since control can be introduced only when the risk factors are already recognized. Thus, when a particular factor is related to exposure and disease outcome, it may be confounding and give the appearance of an association when in fact none exists, or it may inflate or decrease the magnitude of an association. In view of these difficulties, it is not surprising that epidemiological data exist for only a small proportion of the many chemicals that have been shown to be carcinogenic in laboratory animals.

III. DETERMINING CAUSALITY

In interpreting epidemiological findings, one is guided by the magnitude of the risk estimates, their statistical significance (likelihood of being due to chance), and the rigor of the study design to avoid various kinds of bias, including those related to selection, confounding, classification, and measurement (*2, 9*). A determination of causality in epidemiology is bolstered by dose-response relationships, the consistency and reproducibility of results, the strength and specificity of the association, its biological plausibility, and other considerations. Thus inferences from epidemiology are not made in isolation, but should take into account all relevant biological information. Although epidemiological and other observations can accumulate to the point that a causal hypothesis is

likely, it is not possible to ever prove causality (in the strict sense, a hypothesis can only be disproven). Nevertheless, a causal can be sufficently probable, as in the case of cigarette smoking and lung cancer, to provide a reasonable and even compelling basis for preventive and public health action.

IV. EPIDEMIOLOGICAL STUDIES

A. Descriptive and Correlational Studies

Descriptive (or demographic) studies are concerned with identifying the distribution or patterns of disease in populations (*1*). It is a basic tenet of epidemiology that diseases, including cancer, do not occur randomly, but fluctuate according to factors such as age, sex, race, time and geographical location. The use of rates as measures of diseasa frequency is fundamental in describing patterns of cancer among these population groups. Prevalence, incidence and mortality rates of cancer define the levels of risk prevailing in different populations and permit comparisons between groups. Descriptive surveys of cancer occurrence have been valuable in formulating etiological hypotheses and providing direction for analytical studies, which are then necessary to establish whether risks are associated with particular exposures (*10*). Thus, improtant leads to etiology have come from population-based cancer surveys, have revealed substantial international variations in cancer incidence, shifts in risk among migrant populations, changes in risk over time, and geographical peculiarities from mapping cancer morality at the county level (*4*).

Descriptive studies may utilize the correlational (or ecological) approach, in which the rate of disease in a popluation is compared with the spatial or temporal distribution of suspected risk factors (*10*). This type of study may be particularly helpful in developing or refining hypotheses about carcinogenic risks, but falls, short of establishing causal relationships. Correlational studies have the advantage of being much less expensive and time-consuming than analytical studies, because they often utilize mass statistics previously collected for another purpose (*9*). The primary weakness of such studies, as with descriptive studies generally, is that data are collected on populations, rather than individuals. In other words, the rate of disease and the prevalence of exposures to variables of interest are known for various population groups, but information on the exposure status of persons, who have the disease and those within each population is not known. Thus, one cannot infer from the correlations of the population levels that the exposure of concern is associated with the risk of developing disease within each population (*9*). For example, in early surveys of lung cancer, the international variation in mortality rates and temporal increases among males appeared consistent with the reported patterns of cigarette smoking, but these correlations by themselves may have been circumstantial rather than causal, since a variety of other exposures (e.g., occupational hazards, air pollution) also varied concomitantly with the patterns of lung cancer. It took the analytical studies that pursued these tests to establish cause-and-effect relationships between smoking and lung cancer. Correlational studies also may provide supporting evidence in evaluating relationships detected by analytical studies. This is illustrated by the more recent temporal increases in lung cancer among females, who have lagged about 20 years behind males in their adoption of smoking habits. Another example is the temporal variation in endometrial cancer incidence, showing a rise and fall in rates asociated with the usage of estrogenic drugs for menopausal symptoms (*11*). Because correlational studies deal with aggregate exposures and disease occurrence at the population level, they are often seriously limited by the imprecise measurements of exposure and the many potentially confounding variables. However, occasionally the correlational approach may provide evidence that strongly suggests a practical causal relationship, as with aflatoxin, which has been linked to primary liver cancer based on concomitant geographical variation with the intake of contaminated foodstuffs.

B. Analytical Studies

In order to test etiological hypotheses and to identify and quantify carcinogenic risks to man, it is necessary to conduct analytical epidemiological studies (*9–10*). These studies are the principal means for determining the human health hazards of specific environmental exposures and agents. In contrast to descriptive surveys, data are obtained on disease occurrence and putative risk factors for specific individuals, using mainly the case-control or cohort method. Thus, by grouping exposed individuals and comparing them to those unexposed, after controlling for all other relevant variables, the risk of disease associated with exposure can be estimated. While it is important not to impose unnecessary constraints on epidemiological investigation, there are some methodological guidelines to consider in designing a study. In particular, the study groups should be sufficiently large, and the time intervals between initial exposure and tumor onset sufficiently long, to identify the lowest excess risk considered important to detect. Reliable and valid estimates of exposure should be sought, with quantitative measurements to permit dose-response evaluations. Studies should be designed in a manner that minimizes potential sources of bias, and permits detection and control of confounding variables. Because the putative exposure may act either at an early stage or at a late stage of carcinogenesis (or both), it is possible to provide insight into the mechanisms of action by examining risk in relation to several temporal aspects of exposure. The studies should also strive to identify interactions of particular exposure with other risk factors that may contribute to the carcinogenic process.

Analytical epidemiological investigations are essential to achieve a better understanding of the causes and means of preventing cancer. These studies cover a broad range of exposures with special relevence to public policy. Occupational studies have been a time-tested means of identifying carcinogens, but they require further emphasis to assess industrial hazards suspected on the basis of experimental, clinical, and field observations. Radiation studies also need increased emphasis to clarify the effects of low-level exposure and the shape of the dose-response curve. Drug studies have been useful both for direct evidence of risk associated with taking certain medications and also for insights into mechanisms of human carcinogenesis. They require an expanded effort to evaluate the late effects of estrogenic compounds, immunosuppressive and cytotoxic agents, and other medications suspected of having carcinogenic activity. Because of increasing clues about nutritional risk factors, emphasis is needed to clarify the role of dietary components including fat, fiber, micronutrients, trace elements, and food additives as well as cooking practices. The influence of general environmental pollutants remains an epidemiological challenge and requires more intensive evaluation through analytical studies, some of which may integrate appropriate environmental and body-burden measurements. Finally, multidisciplinary projects combining epidemiologic and experimental approaches to chronic disease have recently gained impetus, and should be encouraged to evaluate the influence of

oncogenic viruses, dietary and metabolic factors, host susceptibility, general environmental pollution, and other causative factors that may continue to elude detection by traditional observational methods.

1. Case-control studies. These studies start by identifying persons with a particular disease (cases) and a group of similar persons without the disease (controls). Information of past exposure to known or suspected risk factors is then collected from interviews, questionnaires, medical records, occupational logs, or other sources. The frequency of a particular exposure among the cases is compared with that in the control group, after making appropriate adjustments for other relevant differences between the two groups. If the proportion of cases with a certain exposure is significantly greater than that of the controls, an association between exposure and disease may be indicated. The case-control approach is especially well-suited in studying relatively rare conditions, such as most cancers, where the putative exposure is common in the general population (*e.g.* menopausal estrogens and endometrial cancer), or when the exposure is rare but accounts for a large portion of a particular cancer (*e.g.* vinyl chloride and liver angiosarcoma) (*12*).

2. Cohort Studies. These start by identifying a group of individuals with a particular exposure and a similar group of unexposed persons and follow both groups over time to determine subsequent health outcomes. The rates of disease in the exposed and unexposed groups are then compared. Information on disease frequency and other factors may be identified from medical records, occupational records, physical examinations, interviews, questionnaires or death certificates. An association between exposure and disease may be indicated if the rates of disease are greater in the exposed group than in the unexposed group. These investigations may be based on current exposure and future health outcomes (prospective cohort study), but more commonly they utilize past exposure information and disease occurrence (retrospective cohort study). Instead of an unexposed comparison group, general population mortality or incidence rates (specific for age, sex, race, and calendar time) are often used to determine the expected number of cases of disease. This method assumes that in the absence of specific exposure the study group would have had the same probability of developing the disease as the general population, but differences in ethnic, socio-economic and other variables must be considered in evaluating the validity of this assumption. The cohort approach is used mainly when it is possible to evaluate heavy exposures in clearly defined subgroups of the population. Thus it has been especially helpful in assessing the carcinogenic risk from occupational hazards or medical exposures such as radiation and certain drugs.

The statistical measures of association most often used in analytical studies are the odds ratio and the relative risk. The odds ratio is usually associated with the case-control study and represents the odds or probability of disease occurrence in the exposed compared with unexposed individuals. An odds ratio of 1 indicates no association between exposure and disease, assuming that proper adjustments for confounding factors have been made. The relative risk is a measure of association in cohort studies and is defined as the ratio of the disease rate in the exposed group to that in the unexposed group. For this reason it is also known as the rate ratio, and it permits a direct estimate of the risk of disease associated with a particular exposure. When the disease is rare, when incident cases are collected, and when cases and controls are representative of the same populations, the odds ratio is a good estimate of the relative risk (*12*). The magnitude of the odds ratio or relative risk is a measure of the strength of an association, although they are both subject to unknown or unmeasured biases which may distort their true value. Another term often used in epidemiology is the population attributable risk or etiologic fraction which is the absolute amount of disease contributed or caused by a specific exposure.

Both the case-control and cohort methods are characterized as having certain strengths and weaknesses, although they complement each other in the testing of specific etiological hypotheses. Case-control studies provide a more efficient means of studying rare diseases, with fewer individuals needed for study as compared with the cohort approach; a shorter time period for study completion and generally lower costs as compared with the cohort method; an opportunity to evaluate simultaneously several causal hypotheses as well as interactions (the extent and manner in which two or more risk factors modify the strength of one another); and a capacity to evaluate the effects of common exposures as well as those rare exposures which may account for a large proportion of the cases. On the other hand, the case-control approach has some problems in directly estimating the risk associated with a particular exposure, except in special circumstances noted above; in reducing certain biases (*e.g.* selection, historical recall) that affect the comparability of cases and controls; and in providing detailed and precise information on exposures occurring in the past (*1*). Such investigations also, by definition, can only evaluate one disease or outcome at a time.

The advantages of cohort studies are their capacity to estimate directly the risks attributed to a particular exposure, since incidence or mortality from disease is actually being measured; to reduce subjective biases by obtaining information before the disease develops; to determine associations between a particular exposure and multiple health outcomes; and to evaluate temporal relationships such as latency period and duration of effect. However, cohort studies are usually expensive and complex untertakings. They require large numbers of exposed individuals, particularly when relatively rare events such as most cancers are being investigated; long periods of follow-up to accomodate the latency period for chronic diseases such as cancer; and special handling of problems associated with persons lost to follow-up and with biased estimates of risk as from the "healthy worker effect" of occupational studies (*1*).

3. Intervention Studies. Also referred to as experimental studies (*7*), these represent a third strategy of analytical epidemiology, which is especially useful in confirming causal relationships suggested by case-control or cohort studies. This approach may be applied in programs designed, for example, to reduce cigarette smoking and alcohol intake, modify diet, control occupational pollutants, or evaluate candidate preventatives (*e.g.* vitamin A supplements, hepatitis-B vaccine). Ethical considerations are obviously critical when developing this approach, and after intervention the statistical procedures resemble those employed for cohort studies.

V. BIOCHEMICAL EPIDEMIOLOGY

It seems likely that some limitations of cancer epidemiology may be overcome by incorporating laboratory methods in analytical investigations. This has been a valuable routine practice in infectious disease epidemiology for the past century. This approach, sometimes called biochemical or molecular epidemiology (*13*), has only

recently developed in cancer epidemiology. There is current enthusiasm for these kinds of investigations, since they merge the strengths of observational human studies with newly developed experimental probes to derive information that could not be developed by epidemiology or laboratory study alone. The laboratory aspect may make it possible to define past exposures and subclinical or preclinical response to initiators, promoters, and inhibitors of carcinogenesis, or to evaluate host-environmental interactions. There is special interest at present in using this technique to clarify carcinogenic risks associated with nutritional influences or specific environmental agents that can be detected in tissues or body fluids. Opportunities are also available to assess specific host factors that influence susceptibility to carcinogenesis, including endocrine parameters, immunocompetence, and genetic markers. Techniques are being refined to detect and quantify particular carcinogens or their metabolites in tissues or body fluids through chemical analyses, mutagenesis assays or immunologic detection techniques. It is already possible to measure the interaction of specific agents with cellular target molecules, for example, through adduct formation with proteins and nucleic acids, excretion levels of excised adducts, or markers of altered gene expression (*13*). The task of identifying the effects of lifestyle and other environmental and host factors is obviously formidable. Biochemical epidemiology represents an innovative approach that may help to elucidate further the causes of cancer and the actual mechanisms of carcinogenesis.

IV. IMPLICATIONS OF NEGATIVE STUDIES

Epidemiological observations often provide regulatory agencies with health information which is helpful in establishing sound, defensible rules governing workplace exposures as well as exposure to general environmental contaminants. The clinical and laboratory observations, and subsequent epidemiological confirmation, linking hepatic angiosarcoma and exposure to vinyl chloride led directly to safeguarding of workers by regulatory limitation of permissible exposure levels. Although epidemiological studies can never "prove" the absence of an association, sufficiently large and well-controlled studies that fail to detect a hazard can also be useful. Specifically, for the types of populations studied, for the doses and duration of exposure to the putative agent, and for the assessed time following exposure, likely upper bounds on the estimates of risk can be made, as well as the statistical likelihood of the study to identify an effect. As an example, the results of a large collaborative study of bladder cancer and artificial sweeteners were interpreted as indicating that saccharin use was not a significant public health problem (*14*).

The likelihood of a study to identify an effect, if one is really present, is referred to as the statistical "power" of the study. Its estimation involves consideration of the size of the study group, the number of subjects exposed, and the level of excess risk which is considered important not to miss. With this information, the power of case-control and cohort studies can be calculated and thus some index of confidence can be attached to the observation of no association. The statistical power of "negative" investigations should be routinely considered when such studies are reviewed.

Regulatory agencies are often in the position of making decisions not simply on the effect of an exposure on the "average" exposed population, but on the effects among particularly susceptible subgroups of this population (*e.g.*, the concern over the acute effects of air pollution on infants, the very old, and the infirm). The risk of malignancy following exposure to a carcinogen is rarely uniform across all subpopulations but is routinely modified by genetic constitutions, demographic characteristics (*e.g.*, age, race, sex), and exposures to other substances. Such risk modifiers can act either to eenhance risk, such as the capacity of cigarette smoking to multiply the risk of lung cancer associated with asbestos exposure, or to reduce risk, such as the recently emerging evidence that certain dietary factors may inhibit the carcinogenic process. Epidemiological data are particularly, and often uniquely, relevant to these issues. Because of this, analyses of risk among subgroups of an exposed population are particularly germane to regulatory agencies. At the same time, these types of analyses are challenging to interpret, since variation between groups can easily occur due to chance or the differential operation of some type of bias.

Because of the central importance of epidemiology in cancer risk assessment, it is important to develop and strengthen programs of epidemiologic research, and to ensure the conduct of studies to measure health effects whenever relevant human exposure has occurred. Collaborative studies among Federal agencies and other groups are critically needed to evaluate urgent issues involving scientific, regulatory or public policy concerns, as well as to stimulate the epidemiological application of technical and data resources that are used by the government and other institutions for different purposes. Because of the international peculiarities in cancer occurrence, collaborative studies with investigators in other countries should also receive greater emphasis to pursue a wide variety of etiological leads. In addition, further statistical investigation is needed to develop and test multi-cause and multi-stage models of carcinogenesis, and to clarify the many issues involved in extrapolating results from experimental testing to the human experience.

VII. SUMMARY

Epidemiological investigations comprise one of the major strategies in creating the scientific base necessary for regulatory decision-making. Descriptive epidemiological studies, including ecological or correlational approaches are useful in generating and refining hypotheses about potential cancer risk factors. Well-designed, well-conducted, and well-evaluated analytical epidemiological studies of either the case-control or cohort variety can test such hypotheses and provide the basis for practical causal inferences that are especially useful for public health decisions. The strengths of these studies lie in their capacity to assess directly the carcinogenic risks of environmental agents in humans (often with the dose levels and manners of exposure that are most relevant) and in their ability to provide insights into mechanisms of human carcinogenesis that can assist in extrapolation of risk estimates to dose levels or similar agents not yet studied. The lack of evidence of a hazard from an epidemiological investigation can also be useful in that, within the scope of the study, likely upper bounds on the estimates of risk can be made, as well as an estimation of the statistical power of the study to have detected an effect, if one were really present. However, the epidemiological method is often hampered by the long latent period that exists between exposure to a carcinogenic agent and the development of cancer, by the inability to control for the confounding influences of unknown risk factors, by problems in assessing specific agents when the human exposures are to mixtures, by the frequent absence of appropriate groups

for study, and by a variety of difficulties associated with accurate and unbiased historical exposure assessment or disease ascertainment. In addition, the epidemiological method shares in the difficulties encountered by experimental studies in the direct detection of relatively low-level risks. In general, the strengths and weaknesses of the epidemiological method form a useful complement to the strengths and weaknesses of laboratory approaches to carcinogenesis. This, coupled with the direct assessments of risks and the results of interventions allowed by epidemiology, makes a strong argument for the conduct of epidemiological studies and inclusion of their results in regulatory decision-making, whenever relevant exposure has occurred in human populations.

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Chapter 5—Chemical Exposure Assessment

I. INTRODUCTION

For the purpose of this document, a chemical exposure assessment is that process which seeks to define the quantity of a chemical which comes into contact, or may come into contact, with human populations. This information then can be coupled with toxicity data to allow estimates of the potential risk from a carcinogen. Exposure assessments may also be used to identify and predict the effects of prospective control options or to measure the effectiveness of intervention activities designed to limit exposure. An assessment may address sources of human exposure, the intensity, routes and conditions of exposure, the frequency and duration of exposure, and the segments of population exposed. In certain simple situations an assessment may only need to consider well defined individual exposures that can be measured directly. In more complicated circumstances, broad systematic surveys and extensive mathematical computations may prove necessary (*1–3*). Exposure assessment is often the most resource demanding portion of the evaluations of chemical risks performed by the various Federal agencies.

> Many authorities agree that the weakest link in our understanding of the environmental health studies is our knowledge of human exposure. (*4*)

The great diversity of different sources and routes of chemical exposure complicates a review of this area. In the Federal Government, the source of a specific chemical exposure has served as a guiding principle in defining the responsibilities of different regulatory agencies. Separate responsibilities exist for exposure to chemicals through the ambient environment (air, water, and waste sites), food, drugs, consumer products, and the workplace environment. In each area an array of specialized estimating techniques, data sources, and expertise have been developed. The hundreds of thousands of possible combinations of factors which might be included in a specific exposure assessment make it extremely unlikely that any two exposure assessments of the same chemical will be identical. Each Federal agency tends to focus on those aspects of exposure which are relevant to the laws which it administers (*5–25*). This chapter addresses some of the common considerations in methodology, as well as the broad issues and difficulties that are relevant to exposure assessments. Three appendices provide more detail on aspects of exposure assessments dealing with food, consumer products, and potential carcinogens in the environment.

Despite the overall grouping according to source of exposure, it is important to recognize that the path that a chemical follows from its source to the exposed target can be quite complex and may involve a number of different media. For example:

(a) Chemicals released into one medium (*e.g.*, the air) may later transfer to other media (*e.g.* soil or water);

(b) Low ambient levels of a compound may be magnified through bioaccumulation of the compound through the food chain;

(c) Chemicals may be degraded and new compounds formed during environmental transport and desposition;

(d) A chemical used in an article may be released into the environment years later when the article is disposed of as waste; and,

(3) Workers may carry a chemical home on their work clothing leading to a continuing exposure of their families.

II. METHODOLOGY OF EXPOSURE ASSESSMENT

There is a diversity of procedures for estimating exposure (*26*). Although exposure estimating techniques are becoming more sophisticated at this time there is no universally accepted minimum set of specifications for the reliable estimation of exposure (*27*). Exposure assessment is rapidly developing as a technical specialty, and even recent major reports on risk assessment only mention exposure briefly (*28–32*). There are, however, a number of general approaches discussed below which may be applicable to the conduct of most exposure assessments.

As a useful simplification, exposure assessment studies can be divided among those that rely more heavily on direct measurement of chemical exposure and those where exposure is predicted, or "modeled".

A. Monitoring Studies

The direct monitoring approach is exemplified by the development of practical personal monitors which measure directly concentrations of chemicals in the air which individuals breathe (*33, 34*). An exposure assessment can be based on the range of personal exposure data generated by these monitors when used with an appropriate study group. However, it should be noted that the detection limits attained by personal monitors may pose

limits in some studies and that most personal monitoring techniques currently provide information on average exposure but not on the time course or peak levels of exposure.

Monitoring of pollutants in the air and water has probably generated the greatest quantity of data among all exposure study approaches. Some examples of these type of studies include:

(a) Carbon monoxide exposure has been examined extensively using personal and population monitoring (*35–42*);

(b) Air pollution in the city of St. Louis was studied for several years (*43–44*), producing over one million data points per day during much of the program;

(c) The plum from a power plant (*45*), was studied intensively to define the transport and chemical transformation of the emissions from a single point source;

(d) Other techniques have been used to monitor emissions from entire cities to refine our understanding of the atmospheric transport of chemicals over large geographic distances (*46*);

(e) Significant monitoring studies have also been conducted for a wide range of organic and inorganic compounds in marine, freshwater, and surface environments (*47–60*);

(f) Extensive studies have been conducted to determine the levels of bioaccumulated pesticides and other chlorinated compounds in edible fish and shellfish.

In the workplace monitoring is the principle methodology for determining the levels of exposure to specific chemicals. The monitoring is usually site-specific, and generally is not used to estimate the total exposure of the entire workforce. The Occupational Safety and Health Administration (OSHA) and the National Institute of Occupational Safety and Healthy (NIOSH) have established data management systems for assimilating and aggregating data from workplace measurements. At times, these two agencies collaborate in the preparation and publication of occupational health surveys that describe exposures to certain chemicals across broad segments of an industry or occupation.

There is no monitoring system in place for determining the levels of exposure to substances in consumer products. Exposure estimates are generally based on *ad hoc* short-term surveys, laboratory studies and mathematical modeling. For example, monitoring systems have provided for many years extensive information, on the levels of pollutants in the ambient air from the combustion of fossil fuel. No such parallel exists for these same pollutants generated from the combustion of fuel indoors. However, several monitoring studies of indoor air pollutants have now generated data on exposures to combustion products from gas or kerosene appliances in the home (*61–63*).

Chemical contaminants in the U.S. food supply are monitored through the FDA's Total Diet Studies, an annual program which involves purchase and analysis of approximately 200 foods in grocery stores across the United States. Other monitoring systems used to measure exposure to chemicals in food are described in Appendix A entitled "FDA Information Sources for Exposure Estimates" (Sec. IV A).

Chemicals are also monitored in humans as a means of exposure evaluation (*64–74*). In a national program, human tissue has been analyzed on a regular basis to monitor selected, persistent chemicals such as DDT and PCBs (*75, 76*). Tissue, secreta or excreta, or any combination of these, can be assessed and compared to an appropriate reference (*77*). Such data can be used not only as a measure of overall individual exposure, but also as a measure of the effectiveness of regulatory actions when the monitoring is conducted for sufficiently long times to reveal trends. Chemicals or classes of substances for which there is some evidence that biological monitoring may be useful for detecting evidence of a substantial internal dose on an individual or group basis include: inorganic and organometallic substances, aliphatic, alicyclic and aromatic hydrocarbons, aromatic amines, ketones, aldehydes, phenols and pesticides (*77–80*). In summary, monitoring can provide estimates of individual exposure and data to define the connection between the sources of a chemical and exposure to humans. Monitoring activities can encompass studies of the sources of chemicals, the ambient environment, the human environment of the home and workplace, the food and water consumed, as well as the doses absorbed and retained by the human body.

B. Modeling

Modeling is a second broad approach utilized in exposure as sessment. Here the term modeling is used broadly to describe the construction of a methodology through which diverse data on different factors can be combined to predict levels of human exposure in the absence of complete monitoring data. Models are predictive tools used when direct measurement of exposure would be prohibitively time-consuming and expensive, and when the relationship between the sources of a chemical and the eventual exposure is complex.

Models often begin as an effort to describe and generalize theoretically a phenomenon observed in the laboratory or field. Monitoring data bases are used to construct mathematical relationships among variables of concern (*e.g.*, meteorological parameters and the temporal/spacial distribution of chemicals emitted from a source). For example, the air monitoring data from the St. Louis and the power plant plume studies cited above have been used to build and refine modeling tools for predicting population exposure to contaminants in the air.

Given the wide variety of possible situations requiring exposure assessment, it is not surprising that many different models have been developed. A recent catalogue of the models available for use in regulatory decision-making identified 156 models, most of which can be applied to exposure assessments (*81–83*). A review of these models is beyond the scope of this chapter, but descriptions of various types of models are informative and can be found in the references (*17, 27, 60, 84–111*).

In general, models are most helpful when average, or idealized estimates of exposure are satisfactory. Even though the level of sophistication of a particular model may be high, the predictions may still be crude, because any model is an simplified description of the natural phenomenon it depicts. There is a need for more research to validate most models, since the accuracy of their predictions is often unknown or only roughly known. In addition, the accuracy of the exposure estimates made by models can be no better than the quality of the input data used. The use of models does not compensate for poor data, and in certain instances, may obscure the uncertainty inherent in the exposure predictions. The use of scientific judgement in interpreting results is essential.

C. Microcosms

The use of microcosms to study pollutant behavior provides an approach that combines features of both monitoring and modeling studies.

The transport and transformation processes which occur between the source of a chemical and the eventual human exposure can be difficult and expensive to study in nature, and in the absence of data on these processes, models cannot be constructed. Microcosms, which attempt to recreate

portions of the environment in the laboratory, can provide an alternate means for defining these processes. They have the added advantage that many variables can be controlled. Ideally, a microcosm, when properly validated, faithfully mimics the processes occuring in nature. By adding a chemical to such a system, its subsequent partitioning amongst the components of the environment, together with the associated chemical and biological conversions, can be studied in a single integrated experiment.

Marine, freshwater, terrestrial, subsurface or aquifer, and atmospheric microcosms have been studied (*84, 112–130*). For example, the laboratory microcosm concept has received increasing attention recently in studies which seek to predict residential exposure to products of combustion or air pollutants (*131, 132*). Limited validation of predicted, versus measured, levels of products of combustion in a residence has shown such predictions to be useful in assessing exposure (*129, 130*).

D. Human Factors

In exposure studies using monitoring, modeling, or a combination of approaches, the actual behavior of the human population under study must be taken into consideration. Some examples illustrate this: (1) The amount of time that an individual spends indoors compared with outdoors will affect the risks associated with either outdoor or indoor air pollution; (2) failure of workers to wear protective equipment can increase their occupational exposure to hazardous compounds; and, (3) consumer exposure to vapors during the use of various products will decrease proportionally with increases in the amount of ventilation provided in the home.

Human behavior is often evaluated using information gathering techniques somewhat similar to opinion polls or marketing studies. In such a study, a randomly selected population is asked a series of questions that define aspects of their behavior that can effect chemical exposure. On an occupational setting "time-motion" studies of the periods of employee contact with varying work environments can contribute to an exposure assessment.

The exposure to direct and indirect food additives is monitored by the FDA using a number of complex studies of human eating habits. Surveys of food additive use have been undertaken since 1973 with the help of the National Academy of Sciences, as have studies of the migration of chemicals from packaging materials into foods (*15, 19, 23, 25*). Other related work has determined the frequency of consumption of individual foods and individual food serving sizes, with the data compiled in such a way as to be compatible with the population data found in the U.S. Census (*22*). These surveys and studies have produced a complex array of information which then is converted by the FDA into estimates of population exposure to direct and indirect additives in human food (*16, 24, 135*). Some of the same data are used by EPA in setting tolerances for pesticide residues in food crops (*14*).

Structure-Activity Relationships

Data now exist on the chemical, physical, and toxicological characteristics of a large number of chemicals, as does information on their transport, transformation, and environmental fate. Thus, some correlations now can be made between the structure of certain chemicals and the properties they exhibit, a process called structure activity analysis. Under favorable conditions, these correlations allow rough predictions to be made of the characteristics of chemicals which have not been studied, reducing the amount of testing necessary to make at least an initial judgement of the risks which they may pose (*99, 136–142*). Structure-activity relationships can be used to delineate the general properties of interest, but they are not generally a substitute for actual measurements.

The use of structure-activity relationships may allow preliminary exposure estimates to be made even when there is a scarcity of data on a specific chemical. Structure-activity analysis is a relatively new fields, and the available tools are still crude. The user must exercise scientific judgement in interpreting the results, because substantial work remains to be done in refining and validating these techniques.

F. Integrated Exposure Assessments

Most assessments performed in support of rule-making consider only a single source or route of exposure. Such assessments are appropriate in the case when only one source or route of exposure is significant. However, certain recent exposure assessments on chemicals with multiple routes of exposure have considered all routes simultaneously (*144*). In a pilot effort, an exposure assessment has been prepared which considers six related halogenated solvents and all routes of exposure simultaneously (*145, 146*). This assessment, in combination with hazard data, attempts to provide a risk assessment data base for all six solvents and all routes of exposure. Using this approach, all regulatory options and combinations of options may be considered in seeking the most effective means of reducing risk.

The preparation of such integrated exposure assessments for widely used chemicals is very difficult and resource intensive and requires the contributions of specialists in many disciplines. The difficulty and expense of integrated assessments may limit the broad application of these valuable tools.

Monitoring of the levels of chemical contamination in human tissue, blood and urine, as discussed above, can provide a second method of assessing the total human exposure to certain chemicals depending on metabolism and persistence in the body.

G. Data Bases and Data Management

Research by the Federal regulatory agencies on human exposure has produced a large body of data which can be used in performing assessments. One recent index identified 319 data bases, many of which could be of value in exposure assessment (*82*). For example, monitoring data are reported regularly for National Pollution Dicharge Elimination System (NPDES) permits, issued under the Clean Water Act, thereby affording information on 133,000 sources of chemicals found in surface water (*147*). The EPA's *Handbook for Performing Exposure Assessments* lists 12 regularly used automated data bases on chemicals in the environment (*148*). Monitoring data on air emissions from many sources and ambient air quality data from over 4,000 active air monitoring sites across the country are included in the Aerometric and Emissions Reporting System (AEROS) (*149*). In addition, many major research efforts (*e.g.*, the St. Louis air pollution study) generate large data bases useful in exposure assessment (*43, 44*).

Many data bases which do not deal directly with chemicals are also useful. The data of the U.S. Census provide information helpful to determine populations at risk. The National Climatic Center maintains detailed weather records, while the U.S. Geological Survey has a massive data base on water on a regional and state basis (*150, 151*). The EPA has been constructing a comprehensive data base on organic chemical manufacturing (*152*).

As the number of data bases and models available for exposure assessment grow, it will become increasingly difficult to access and manipulate the large quantities of information and broad assortment of

techniques without computer systems. Preliminary exposure assessments, reviews of the relative effectiveness of regulatory control options, and many other assessment-related tasks, which in the past required substantial effort, can be accomplished rapidly with limited accuracy at a computer terminal, calling upon data bases and models as needed (*153–157*).

III. BROAD ISSUES AFFECTING EXPOSURE ASSESSMENTS

A. Extrapolation and Non-Representative Systems

Exposure assessments, in a manner often analogous to risk assessments, may require extrapolation from a limited number of sites, to a large population. Such extrapolations carry with them uncertainties that need to be reflected in the final assessment.

In some cases, the exposure measured may not correlate well with the actual experience of the larger populations. In such cases, the non-representative nature of the exposure measurement may give rise to an assessment that is inaccurate. Such errors can arise especially when microcosm measurements and models are used instead of extensive monitoring networks.

B. Quality Assurance

Measurements of chemicals at the trace levels of concern, as needed for many exposure assessments, is a difficult exercise in analytical chemistry and can lead to errors and uncertainties which should be considered in preparing an assessment. Until recently, extensive quality assurance information was not a routine part of most research, testing, and monitoring programs. To improve this situation some agencies have instituted quality assurance procedures which require that records be maintained on the accuracy and reliability of every chemical measurement associated with research, monitoring, and compliance enforcement (*158, 159*). Two examples of such state-of-the-science projects are the EPA-Chemical Manufacturers Association Joint Study on water treatment systems and the Love Canal Monitoring Study (*160, 161*).

The quality assurance portion of the Love canal Monitoring Study provided information on the accuracy and reliability of the analyses of chemicals at trace levels in air, water, and soil. The results demonstrated the limitations inherent in the measurement of complex mixtures in a variety of matrices. The overall results in the Love Canal studies suggest that the most advanced analytical techniques correctly identify trace levels of an organic chemical in complex environmental mixtures with approximately 70 percent certainty.

To date, the major efforts on quality assurance have been devoted to chemical analyses of environmental materials. As discussed, other data contribute to the exposure assessment, *e.g.*, information generated through microcosm studies, human behavior, or modeling. There is a need for further validation of such tools to assure their accuracy and reliability in estimating exposure (*162–165*).

C. High risk populations

Subgroups of the population may be particularly at risk from exposure to a chemical. These groups may be exposed to unusually high levels of a chemical or may be especially sensitive to the toxic properties of a compound. The existence of subgroups of either type could lead to a compound producing a substantial risk even though the average exposed individual may experience little, if any, risk. A thorough exposure assessment needs to consider the possibility that high risk populations or situations exist. Many, or most, current exposure assessments do not have enough data to allow an evaluation of the effects of a chemical on high risk populations.

D. Refinement of Assessments

At the initial consideration of a chemical, only crude data relating to exposure will generally exist. Such crude data may allow a rough estimate of likely exposure; this preliminary estimate may be useful to decision makers in assigning priorities and resources. It is also possible that crude data may allow an estimate of the maximum or minimum exposure that may be expected to occur. Such an analysis may effectively rule out, or indicate the presence of, a significant risk. Specifically, a "worst case" estimate, where all assumptions are chosen so as not to underestimate the possible exposure, may indicate that little risk exists if significant exposures are not predicted.

As more resources are devoted to an exposure assessment and more studies conducted, a refined assessment is generated. Often there will be several stages of refinement of an assessment, and the degree of refinement and accuracy finally required will be related to the certainty needed to enable risk management decisions.

E. Relationships to Biological Dose

The data generated in an exposure estimate must ultimately be coupled with biological data on the hazards presented by a chemical in order to determine the actual risk presented by the exposure. In order to achieve this coupling, the estimated human dose must be related to the doses received in the human or experimental studies that generated the information on the biological hazard. The type of exposure data needed can vary for different risk estimation procedures. For example, for risk estimation using a dose-response relationship that is not linear in the range of human exposure, information on the frequency distribution of different exposure levels in the population is needed, instead of simply an estimate of the average exposure.

This comparison can be complicated by differences in the frequency, duration, intensity, and route by which the two doses were received. This is particularly relevant since human exposures to a single chemical may follow many different patterns ranging from a high single exposure to a low level lifetime exposure. Humans will also often be exposed through several routes: Ingestion, inhalation and dermal. Because of the diversity of human exposures frequently there will not be toxicological data available which was obtained under the same exposure conditions. In those cases, careful review and expert judgment enter into the application of the exposure data.

Recently, there has been considerable interest in the possibility of relating human chemical exposures to hazard studies by utilizing biological indices of the effects of the different exposures. Specifically, it has been suggested that the quantity of a carcinogen reaching and interacting with DNA be directly compared in exposure and hazard studies. This topic receives fuller discussion in Chapters 1 and 4 of this document. It should be noted that at the present time, data to allow such comparisons are generally not available, and considerable research still is needed in order to assess the practical applicability of this approach.

F. Overall Limitations of Exposure Assessments

Many exposure assessments are unable to distinguish all of the parameters that are important in determining possible health effects. These include variations of exposure with time, including peak versus average exposures, and annual versus daily exposures. In addition, exposure assessments rarely, if ever, are able to estimate the dose that actually reaches a biological site where an effect may occur. As a consequence, some information is often available about the

magnitude, frequency, or duration of exposure, but rarely is information available that is adequate to accurately determine all three. In addition, the population whose exposure is being assessed will often be exposed by more than one route from a number of different sources. As a result, estimates addressing single routes or sources of exposure are often inadequate to represent what is actually occurring. Environmental monitoring data, models, and microcosms may sometimes be inadequate, and of limited value, and may lead to a false sense of security if broadly applied without consideration and enumeration of their limitations.

Finally, there are a number of factors that are a part of both a hazard assessment and an exposure assessment which usually are not completely determined. These factors include the age distribution of the exposed population, special sub-populations that may be particularly sensitive to low doses, cumulative exposures, latency, host factors, or populations which may be exposed to particularly high concentrations. As a result, there are generally unknown data and uncertainties associated with exposure assessments at many points. It is important that the assessor describe the data that are missing, the associated uncertainties at each step of the assessment, and the assumptions made when filling in the gaps.

At present, therefore, there is a great deal that can be improved in carrying out exposure assessments. Informed decision making often requires consideration of a complex array of information, rather than a single numerical estimate of exposure. To the extent possible the ultimate use of the data should be defined, and the exposure assessment tailored accordingly. The limits of the assessment, both conceptual and methodological, need to be described, and all the uncertainties enumerated. Quality assurance programs and efforts should be included as well as a description of efforts made to validate laboratory studies and mathematical modeling; in general, careful scientific review of all elements of an exposure asssessment should be stressed.

IV. APPENDICES

A. Appendix A—FDA Information Sources for Exposure Estimates

Survey, epidemiological, toxicological, and analytical data used by the Bureau of Foods in determining the human health consequences of exposure to many of the categorical agents which the agency regulates are derived from a variety of sources. For example, NIOSH and OSHA provide data on occupational exposure and CDC provides data for a variety of exposure sources. The scientific literature and various reports provide a wide variety of animal and human data. In addition, data are obtained on types and amounts of foods eaten, concentration of food contaminants and human health effects.

1. Food consumption. Some principle sources of food consumption data include: (1) The U.S. Department of Agriculture (USDA) Nationwide Food Consumption Survey (NFCS); (2) National Health and Nutrition Examination Survey (NHANES); and (3) Market Research Corporation of America (MTCA) survey data. Sources of food composition data include: (1) USDA: Handbook 456, Handbook 8, National Nutrient Data Bank file; (2) NHANES: Model Gram and Nutrient Composition files; and (3) MRCA: Lead content of foods and methylxanthine content of foods (based on data from Total Diet Study and industry). Additional information of some of these sources is provided in the following discussions:

a. Nationwide Food Consumption Survey. The results of the Nationwide Food Consumption Survey (NFCS) 1977–78 provide data on the kinds and quantities of foods and nutritive values of diets ingested by men, women and children of different ages classified by various household characteristics. Dietary information obtained for individuals included the kind and amount of each food eaten; the time the food was eaten, the type of service, and the cost. Individuals were also asked if the day's intake was typical and if they were on a special diet, were vegetarians, or took vitamins, minerals or other supplements. Reported are average intakes per day and per eating occasion for foods most commonly eaten by individuals who participated for 3 days in the NFCS conducted by the U.S. Department of Agriculture.

b. National Health and Nutrition Examination Survey.

Data were collected for NHANES II through response to questionnaires on medical history, food consumption, and health-related behavior. Data also were collected through direct medical examination. NHANES II was conducted on a nationwide probability sample of approximately 20,000 persons ages 6 months to 74 years from the civilian non-institutionalized population of the United States. Food consumption data is of the form of quantities of food consumed and frequency of eating in a 24-hour recall period.

c. Market Research Corporation of America Surveys.

One of the largest proprietary surveys of food intake is one conducted by the Market Research Corporation of America (MRCA). This corporation conducts the Menu Census survey which is designed to collect detailed information for one year on the ultimate disposition of all food products in terms of their consumption by individuals or their use in preparation of other dishes consumed by the individuals. Only information on frequency of eating is reported; that is, quantities are not included. Each Menu Census consists of 4,000 households. Each household reports all food preparation and consumption at home and away from home for 14 consecutive days in daily diaries. Brands and food packaging materials are also described. The sample is balanced within each quarter as closely as possible to the U.S. Census by various demographic characteristics, including region, household size, household income and other socioeconomic variables.

Pertinent comparisons among these three data sources are given in the following table:

Characteristic	USDA NFCS 1977–78	NHANES 11 1976–80	MCRA Menu Census VI 1977–78
Sample size	30,770	20,322	11,150.
Length of dietary survey	3 days	1 day	14 days.
Dietary methodology	24-hr. recall (one-day) records (two days).	24-hr/recall	Daily diary.
Collection methodology	Recall: interviewers record: self-administered.	Interviewer	Self-administered via mail.
Reporter	Individual subject	Individual subject	Main food preparer.
Place of recording	Home	Mobile exam center	Home.
Food quantities	Yes	Yes	No.
Drinking water	Yes	No	No.
Processing/packaging information.	No	No	Yes.
Other surveys in this series	1965–66	1971–74	1957–75.

2. Total diet studies. The Food and Drug Administration uses several surveillance and regulatory programs to monitor the amount of chemical contaminants in the U.S. food supply. Most of these programs measure

contaminants in raw or unprocessed foods.

The Total Diet Studies monitor levels of chemical contaminants and essential minerals in table ready food and consider dietary intake of these substances by various age-sex groups. The Total Diet Study is an annual program which involves the purchase of approximately 200 foods in grocery stores across the United States and the analysis of these foods for essential minerals, toxic elements, radionuclides, industrial chemicals and pesticides. The food list and diets, beginning in April 1982, were revised to reflect the present food supply and the current food consumption habits. The number of age-sex groups was increased to eight (infants, young children, male and female teenagers, male and female adults, and male and female older persons) in order to expand the coverage of the U.S. population. Foods were analyzed individually, rather than in commodity groups, to obtain information on the contribution of specific foods to the daily intake of minerals and contaminants.

The diets are based on data from the 1977–78 USDA Nationwide Food Consumption Survey (NFCS) and the Second National Health and Nutrition Examination Survey (NHAMES II) carried out by the National Center for Health Statistics in 1976–80. Two hundred and thirty-four foods are collected from four geographic regions (northeast, south, west, and north central) and analyzed by the Kansas City Field Office Laboratory for 11 essential minerals and more than 120 chemical contaminants. The essential minerals analyzed for are copper, iron, magnesium, manganese, potassium, phosphorus, calcium, sodium, iodine, selenium, and zinc. The daily intake by weight of these 234 foods has been weighted to represent 100% of the usual diet for the eight age-sex groups. These estimated food intakes are used to assess daily contaminant and mineral intake.

3. Food Additives.

a. National Academy of Science Survey Data.

The NAS surveys provide the agency with a data base for the concentration of additives used in food. To ensure confidentiality the NAS maintains the raw data from the surveys. These data have been obtained from mailed questionnaires that have been sent to over 2000 firms with approximately 1100 responding. The values obtained from the NAS are used as the substance concentration value in estimating intakes.

The surveys approach the question of usage of food additives from two directions: (1) How much poundage is used annually; and (2) what is the level of use in each of the firms products.

b. Bureau of Foods Data Base.

The Bureau of Foods' database for food additives comprises food additive safety information for substances added directly to food in the United States. Nearly 1600 items (over 1300 synthetic flavoring substances, nearly 300 direct additives, and some 70 color additives and color diluents) supply data on human exposure levels, chemical structure, and toxicological parameters. The computer database is searchable by software programs that permit sorting of the data on many parameters, including lowest effect levels, test animal species, toxicological effects and organ sites, as well as exposure and chemical structure categories.

This database comprises one module of the Bureau's SIREN (Scientific Information Retrieval and Exchange Network) system, the other two being the food additive information system (FAIS) and the new animal drug information system (NADIS) containing over ½ million indexed records across two FDS bureaus.

4. Census Data. Census data on the count and characteristics of the population are obtained from the Bureau of the Census. The agency has census files for 1960 and 1970 for the total population counts for each U.S. county or county equivalent, such as independent city and within each county equivalent by sex, race, and 5-year age interval. These data are used in calculating rates in the population. Population estimates for the years 1971–1978 are available.

5. Fish Intake Data. Fish intake data are important to FDA in regulating food safety and has been used in evaluating the hazard of mercury, dioxins, and other contaminants of fish. The sources of national fish production data are the National Marine Fisheries Service (NMFS) and the USDA. NMFS calculates an annual estimate of the per capita consumption of seafood from data on the total catch of commercial seafood. The estimate does not include commercial fresh water fish, recreationally caught fish or marine fish sold at roadside stands. The NMFS also supplies a database on the mercury content found in fish. The USDA calculates annual per capita fish intake by adding an estimate of recreationally caught fish and the other type of data to the commercial seafood production to calculate the total fish consumption.

6. Natural Toxicants. A natural toxicants literature file is maintained which contains reprints of articles relative to the occurrence and health effects of mycotoxins including plant and marine toxins. In addition, information is maintained on the level of mycotoxins in various commodities in the U.S.

7. Elemental analyses. Data from the U.S. Geological Survey on elemental analysis of soils, waters, and vegetation provides background information on geographic differences in elements likely to be in the human diet.

8. Special studies. Consumer interview surveys are conducted periodically on topics of special interest, which may grow out of immediate or potential health risks to the public or the need for information not currently available in areas of regulatory concern to the agency. The studies are designed internally, executed under extramural contract and the data are delivered on computer tape for internal tabulation and analysis.

9. Literature. A bibliography file for articles written by FDA employees is maintained. This file together with computerized cross index contain articles on such subject as analytical methods and food contaminant levels.

B. Appendix B—Exposure Assessment at EPA

1. Introduction. An exposure assessment is an estimate of the amount of a given chemical substance that is absorbed by individuals over time. This integral portion of the risk assessment is a growing technical speciality whose complexity has long been noted but has only recently been addressed using the sophisticated scientific tools recently been addressed using the sophisticated scientific tools available (*28,29*).

The process involves consideraton of the magnitude, frequency and duration of encounters between individuals and the chemical in question. In some instances (*e.g.*, prescribed dosing with drugs), the information is rather precisely known. In the case of exposure to chemicals of environmental concern, however, the assessment process in often associated with a dearth of data and assumptions/approximations must be made to fill in the gaps. For example, while some data may be available on the "direct exposure" from the emissions of a manufacturing plant, estimates of the "indirect exposure" resulting from potential bioaccumulation in the food chain may be less precisely known.

In recent years, data bases have been compiled, computer models developed, laboratory/field measurement taken and "standard operating procedures"

adopted (*30–32*) that have narrowed the uncertainty associated with some of these estimates.

2. Defining the exposure assessment. Before beginning an exposure assessment, it is necesary to define clearly the product needed in terms of purpose, breadth, depth and approach. This conceptual scheme is being developed in detail in a seven volume set of information by the EPA (*166*).

Depending on the answers provided to these questions, different resources are available to construct the exposure asessment. Some of the more important resources are discussed below.

3. Monitoring. In many cases, site-specific monitoring is performed in order to generate qualitative or quantitative data for an exposure assessment. For example, more than 5000 water, soil, and sediments samples have been analyzed for the presence of 2,3,7,8-tetrachlorodibenzo-p-dioxin in the state of Missiour; groundwater and vertical soil cores have been analyzed for ethylene dibromide in Hawaii, California, and Georgia; and stack emissions have been sampled for heavy metals.

In addition to site-specific information, qualitative and quantitative exposure information is available from the various monitoring networks which have operated over the years to collect data on chemicals in different envonmental media. For example, the Mussel Watch Program (*53,59*) has analyzed shellfish in the U.S. estuatine waters for a variety of pollutants. Shellfish are filter feeds and are generally efficient bioconcentrator of water contaminants; the residues in these organisms serve as sensitive markers for upstream pollution of water which feed the estuaries.

In addition, great quantities of analytical chemical information on U.S. waters can be accessed through the computer data based, STORET. These data represent information gathered over the past years from every major watershed in the U.S.

Increasingly, the problem of groundwater contamination is of concern. Little systematically collected information on this environmental compartment is available at this time, although more effort is being expended to identify and quantitate levels of certain chemicals such as aldicarb and ethylene dibromide at selected sites.

EPA has gathered information on atmospheric pollutants for many years. As part of the national efforts to the quality of the ambient air, innumerable measurements have been made at various times and locations on certain pollutants, namely carbon monoxide, sulfur oxides, nitrogen oxides, particulates, hydrocarbons, and photochemical oxidants. Much of this information is available in computer files maintained by EPA, Ofice of Air Programs in Research Triangle Park, NC.

In a broader prespective of "monitoring"—where the chemical is in the environment—EPA's Office of Toxic Substances maintains and has access to file which identify chemcial substances which are manufactured in or imported into the U.S. Some types of geototal production figures are generally available, along with graphic information on the location of production sites. This information is useful in generating a "material balance" of the a chemical in the environment).

EPA often relies on data generated by other Federal agencies (*e.g.* FDA, NIOSH and the Department of Labor) to estimate the exposure likely to be encountered from eating food or being in the workplace.

Increasingly, interest is expressed in determining more direct measurement of human exposure to environmental pollutants. For years, EPA has maintained the National Human Adipose Tissue Network which collects adipose tissue from selected cadavers in such a way as to obtain a rigorous statistical sample of the U.S. population. These tissue samples are analyzed for a variety of organic chemical residues so that trends and potential problem chemicals can be identified. Further, the Agency publishes a summary of studies on residues in humans which have appeared in the scientific literature.

An ambitious program underway at the EPA, called the Total Exposure Assessment Methodology (TEAM) study (*66–71*), combines personal monitor results (*34*) with analyses of food, beverages, water, human blood plasma and serum, urine, breath and mothers' milk to characterize the individual exposure of large test populations to a variety of organic chemicals over extended periods of time. Such data also will be useful in determining what portion of a particular chemical is actually absorbed by the body and how absorption varies from one individual to another at different exposure levles. The actual dose of a chemical which determines the health outcome from the exposure is often different from the apparent exposure level (*167*). The goal of the TEAM program is to develop an individual exposure monitoring methodology for use in estimating population exposures, including analytical chemical techniques of proven accuracy and reliability, tools for conveniently collecting detailed information on the activities of individuals on a 24-hour a day basis, and tools for managing, analyzing, and interpreting the data produced by such studies.

In sum, the Agency has broad environmental monitoring data on a number of chemical substances in various media. The information too often is not sufficient in depth, detail, and validation to provide precise data in a given exposure situation. Further uncertainties are introduced when attempts are made to extrapolate these data to absorbed dose. Therefore, while the Agency is indeed building an exposure data base, the range of uncertainty surrounding any exposure situation can be significant.

4. Modelling. The questions of "indirect exposure" ofter center on the transformation, transport, and ultimate fate of a chemical once it is released into the environment. While direct monitoring can provide some data in this regard, important detailed qestions of chemical kinetics, distribution between media, potential uptake by biological organisms, and the like cannot easily be derived from such information. Two approaches to filling this gap are through the use of microcosms and through the use of mathematical modelling.

a. Microcosms. A microcosm is a recreation of a portion of the environment in the laboratory—a physical model of nature. Ideally, the microcosm faithfully mimics the processes occurring in nature. By adding a chemical to such a system, its subsequent partitioning amongst the components of the environment, together with the associated chemical and biological conversion, can be studied in a single integrated experiment.

Marine, freshwater, terrestrial, atmospheric and subsurface or aquifer microcosms are available and have demonstrated utility. Much of our knowledge of the chemistry which occurs in the atmosphere has been generated using smog chambers (*80, 112*), *i.e.* atmosphere microcosms. Marine microcosms are now being used to understand the complex processes which affect the fate of a chemical in the ocean where it can be transformed by the benthate community, and eventually concentrated up the food chain leading to human food (*113–116*). Terrestrail micorcosms are being used to define more precisely the fate of a chemical in soil-plant-water systems (*127–129*). Subsurface or aquifer microcosms have been created using special drilling techniques which allow a column of material to be removed from an aquifer

in an undisturbed and uncontaminated state (*130*). Such colums are then used to study the chemical and biological conversions underground.

One of the continuing questions raised about microcosms is how well do their results actually mimic what would occur in the "real" environment itself. In recent years, increased effort has been aimed at validating these systems and gaining an appreciation of the limitations of their data. At this time, however, the risk assessor must recognize this inherent potential source of error.

b. Mathematical modeling. One of the most frequently used tools in exposure assessment is mathematical modeling. Most such models begin as an effort to describe theoretically the phenomena observed in the laboratory or field. Large monitoring data bases are used to construct mathematical relationships between variables of concern (*e.g.*, meteorological parameters and the temporal/spacial distribution of chemicals emitted from a source). These relationships constitute the model which, because of the inherent complexity, often requires a computer for its use. Models are predictive tools used when direct measurement of exposure would be prohibitively time consuming and/or expensive and when the relationship between the sources of a chemical and the eventual exposure is complex.

Given the wide variety of possible situations requiring exposure assessments, it is not surprising that many different models have been developed. A recent catalogue of the models available for use in regulatory decision-making (*81*) identified 156 models, most of which can be applied to exposure assessments (82, 83). A description of the general features of some advanced models is informative (*85*).

Ambient air modeling has reached the highest level of complexity with a capability for considering numerous point, line, and area emission sources simultaneously (*86–88*). Several models (*84*) predict the complex chemical conversions occurring in air during transport of pollutants. One model allows prediction of the half-life and reaction products for an organic chemical in air (*89*). Models currently under development address the problems of predicting exposure on a regional basis (*90*) and in areas with rough terrain (*91*). The *Handbook for Performing Exposure Assessments* (*166*) lists a number of routinely used air models available through NTIS.

Models are also available for the indoor air environment. The breadth and complexity of such modeling varies in proportion to the complexity of the environment being modeled. Even a single room or residence model may become quite complex when considerations of air filter efficiencies, particulate agglomeration/planting-out, nonspecific heterogeneous decay of reactive pollutants, multiple sources, sinks and varying sources strengths are encountered. Additional difficulties of assessing infiltration rates, and the partitioning of ventilation rates among discrete volumes in a building have led to the use of compartmented models (*92*). The contribution of varying levels of ambient pollutants to the indoor environment has been evaluated by a time-segmented model which has undergone limited field validation (*134*).

As a result of the Great Lakes research program (*93–97*) and research into the environmental fate of pesticide (*98–99*), models are available which describe the movement of chemicals in fresh water aquatic environments. Studies on the ocean disposal of municipal wastes have led to models for chemicals in coastal waters (*100–102*). Recent work on terrestrial microcosms has provided input data for a terrestrial model (*103*) which includes consideration of plant uptake. A number of models are available which describe leaching and runoff of chemicals to streams (*81*). The newest areas of modeling are ground water (*60,104–108*), human micro-environments and activity patterns (*109–111*), food consumption (*17*) and multimedia environmental partitioning (*168, 169*).

As models increase in complexity, the ability to predict exposure generally becomes more accurate. This additional accuracy, however, is purchased at the price of additional input data (*135*). The choice of models available for a particular assessment may be reduced by a limited data base.

In general, models are most helpful when rough estimates of exposure are required. Even though the level of sophistication of a particular model may be high, the predictions are still crude because any model is an oversimplified description of the natural phenomenon it depicts. There is a need for more research to validate most models, since the accuracy of the exposure estimates made by models can be no better than the quality of the input data. The use of models does not compensate for poor input data, but in certain instances may obscure the true uncertainty inherent in the exposure predictions. In sum, the modelling as well as in monitoring, the Agency can obtain valuable information. At the same time it needs to be recognized that this information might be accompanied by potential significant uncertainties. Nevertheless, the use of scientific judgment in interpreting results is essential.

C. Appendix C—Chemical Exposure Assessment for Consumer Products

Assessing human exposure to toxic chemicals from consumer products is a vital part of CPSC's chemical hazard work and is the element which most distinguishes CPSC's efforts from the activities of other Federal agencies. The range of uses of chemical compounds in consumer products is great and the ways that consumers utilize a single product may vary considerably. For example, the techniques required for assessing exposure to a gas that may be emitted from building materials into home air and for a dye or other agent contained in cloth that may contact the skin have little in common. Most experience in exposure assessment from products has been gained over the past few years. Because of this, CPSC approaches exposure assessments on a case-by-case basis with each study in general requiring original experimental or theoretical work. Nonetheless, the general activity of exposure assessment can be described as a four step process:

(1) Determine the fact of the release of a compound from products and estimate the quantity of release. The degree of difficulty of this step can vary greatly. For example, it is straightforward if the compound of interest is contained in an aerosol product. It may require extensive experimental study if the compound is contained in a plastic matrix that is subjected to a variety of uses. More often than not in CPSC's experience, experimental studies are required. The presence of a compound in a consumer product does not imply that release actually occurs.

(2) Relate the quanity of release with the level of human exposure (to the outside of the body) from use of the products. A series of analyses are required for this step. First, use patterns for the products must be determined; both the most typical use patterns and those that could lead to lower or higher degrees of exposure must be considered. Experience of staff, information from industry, and consumer surveys can provide this information. Then, for each specific use, the data on chemcial release must be used to estimate the actual amount of contact the user has with the chemical. For a chemical emitted into the home air, data on release rate, use patterns, home volume, and air exchange rate can be combined in a mathematical model to estimate the average and peak levels in the air. On

the other hand an experimental study could be conducted to measure actual air levels from a certain product use. As another example the quantity of a liquid that comes into contact with skin can be measured.

(3) Determine the entry of the material into the body. This step can be straight forward if the toxicity information on a chemical was obtained by a route of exposure comparable with consumer use. On the other hand, particularly in the case of dermal exposures, data in entry of a compound into the body is fequently lacking. In that case experimental studies, such as painting the skin of an experimantal animal with a radio-labeled quantity of the compound of interest, may be conducted.

(4) Combine the data in steps 1 through 3 for a complete estimate of human exposure to a compound. This estimate can then be combined with health hazard information (toxicity) in evaluating human risks; it can serve as the basis required for a mathematical estimation of risk.

These steps are presented as one sequence however, exposure estimation is often an interactive procedure. One uses available, limited information to make preliminary estimates of risk that may (if sufficiently conservative) eliminate concern about an exposure or may be successively refined by gathering data where it is most needed.

In addtion, in certain studies, some of the above steps may be combined rather than considered separately. For example, a chemically treated cloth may be placed in contact with the skin of an experimental animal and the amount of uptake of the chemical by the animal measured. This experiment provides information that could separately be obtained by measuring both quantities released from the cloth and rates of penetration of the chemical through the skin. As another example, personal air monitoring can take into account both release rates from products and patterns of product use.

Exposure assessment for chemicals in consumer products is a developing science which makes innovative use of standard physical and chemical data, demands development of new experimental procedures, and combines relatively "hard" experimental data with "soft" data on widely varying human behavior patterns. While each exposure assessment is now a new undertaking, certain basic techniques (such as air level modeling) are finding repeated application. In the next several years the knowledge now being gained should lead to more readily standardized and more easily performed exposure assessments.

V. SUMMARY

Exposure assessment is a co-equal component, along with hazard assessment, in the evaluation of human risk. The assessment of exposure to chemicals by direct routes (*e.g.*, breathing emissions from an industrial stack) and indirect routes (*e.g.*, *pollutants accumulating through the food chain) depend upon analytical chemical monitoring data, results of modelling estimates, and assumptions needed to fill in data gaps. The field of exposure assessment is rapidly developing and only recently have attempts been made to develop systematic approaches to the problem. Although current efforts are often associated with considerable uncertainty, the increasing sophistication being brought to bear on the issue holds the promise of greater confidence in these estimates in the future.*

Methodologies can be broken up into two broad categories, monitoring studies, which depend more on the measurement of exposure, and mathematical modeling, which use analysis to define the exposure. Microcosms, as samples of the environment, provide data for both approaches. Human factors, however, are important and must be considered.

An important part of the assessment process is the marshalling of the appropriate data bases, and some of the data bases used by three regulatory agencies are listed in appendices.

Broad issues affecting assessments include the question of extrapolation from representative systems to large populations, quality assurance, the question of high-risk populations; and the approach of coupling exposure data to actual biological dose to improve assessments, are emphasized, with a discussion of the limitations of assessment as it is being done today.

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Chapter 6—Utilizing Scientific Data in Assessing Human Cancer Risk Associated With Chemical Exposure

I. INTRODUCTION

Assessment of human cancer risk associated with some specified chemical exposure is a complicated scientific endeavor that requires careful review of all pertinent information by appropriately trained individuals. This process relies heavily on information derived from epidemiological, clinical, and long-term animal studies. Short-term test results and information on structure-activity relationships, comparative metabolism, pharmacokinetics and mechanisms of action also contribute, in varying degrees, to the assessment.

While there is general agreement within the scientific community about the steps involved in the risk assessment process, a number of specific issues related to these various steps are still uresolved *e.g.* the combining of positive and negative data when evaluating carcinogenic potential and the selection of a dose-response model for making low-dose risk projections. The execution of any given risk assessment may also be hampered by the existence of critical gaps in the data underlying the assessment. Because these issues and data gaps can be pivotal in the process, it may be necessary to make judgments about these unresolved issues and assumptions to adjust for deficiencies in the underlying data base that will permit risk assessments to be completed. These judgments and assumptions should be plausible and, to the extent, possible, be based on the scientific information on hand. However, the validity of these judgments and assumptions often cannot be conclusively established, so the risk assessment process should not be viewed as strictly "scientific" in the usual sense of the word. Instead, risk assessment involves a complex blend of current scientific data, reasonable assumptions and scientific judgments that permit decisions to be made in the absence of complete information.

With the passage of time the scientific data base grows, information gaps are filled, and perceptions change. This process of evolution and maturation results in a need for periodic reformulations and restatements of the appropriate guidelines for risk assessment.

The present chapter describes the basic framework for carcinogenic risk assessment and attempts to reflect what general agreement currently exists within the scientific community. It is intended to provide a bridge between the scientific data base on carcinogenicity developed in previous chapters and the various Federal regulatory agencies that are charged with the responsibility of protecting the general public from exposures to carcinogenic agents.

II. STEPS IN THE ASSESSMENT PROCESS

There are four steps or components (1) that are typically involved in carcinogenic risk assessment. The first, which is often referred to as hazard identification, entails a qualitative evaluation of both the data bearing on an agent's ability to produce carcinogenic effects and the relevance of this information to humans. The second, exposure assessment, is concerned with the number of individuals who are likely to be exposed and with the types, magnitudes, and durations of their anticipated exposures. The third component, hazard or dose-response assessment, uses the information on carcinogenicity from the hazard identification phase together wtih mathematical modeling techniques to estimate the magnitude or an upper bound on the magnitude of the carcinogenic effect at any given dose level. Finally, one may combine the information from the first three components or steps to characterize the carcinogenic risk associated with the expected human exposure to the compound of interest.

A. Hazard Identification

Due to the lack of complete information, there may be some uncertainty involved in determining whether or not an agent poses a carcinogenic hazard to humans. While information on human exposures and effects is particularly valuable, too often such data either do not exist or are inadequate, and one must rely primarily on information from long-term animal bioassays in assessing the potential for carcinogenic response in humans. In reviewing animal studies, each relevant study must be evaluated as to the limits of inference implied by the experimental design, the results, and the conclusions. Although strict criteria for evaluating carcinogenicity data have not been developed for all circumstances, there seems to be reasonably good agreement amony scientific groups as to the general elements to be included as a part of qualitative carcinogenic assessments (See also Chapter 1) (*2-8*).

Epidemiological studies can, under certain circumstances, provide direct measures of carcinogenic effects in humans (See also Chapter 4). For example, some epidemiological investigations are able to associate cancer incidence or mortality with exposure to specific chemicals, industrial processes, or components of an individual's life style. Analytical studies, i.e., case control and cohort studies, are especially important indicators of possible human carcinogenic risk, but case reports and descriptive studies may also be supportive. Consistent results in independent studies, freedom from bias and confounding factors, reliable exposure data, reasonable follow-up time, dose-related responses and high levels of statistical significance are among the factors leading to increased

confidence in a conclusion of carcinogenicity. Even if an epidemiological investigation fails to demonstrate an increased incidence of carcinogenicity among the exposed study members, an upper confidence limit on the risk measure used in the study can indicate the maximal level of risks likely to be incurred by a similarly composed segment (*i.e.* in terms of age, race, sex, etc.) of the general population.

Evidence from long-term animal bioassays (see also Chapter 3) constitutes the second major class of information bearing on the carcinogenicity of chemicals. The primary factors that are considered in the evaluation of carcinogenicity are higher tumor incidence and shorter latency in treated groups relative to controls. Findings are strengthened by the existence of positive effects in more than one treated group or sex. Additional support comes from positive results observed with different routes of exposure, in replicated experiments, in different animal strains and species, and in multiple organs or tissues. Attention is also given to the magnitude of tumor increase in treated animals, the existence of dose-related trends, the degree and extent of malignancy at a given site as a function of dose and time, the evaluation of concurrent non-neoplastic pathology in treated attempting to use epidemiological data in cancer risk assessment is the lack of appropriate historical exposure information.

In an exposure analysis, a group or groups of individuals are identified and described with respect to their population size and composition, and the route (*e.g.* inhalation, dermal, ingestion), magnitude, frequency, and duration of their exposures. In addition, consideration must be given to both direct exposures (*e.g.* drugs or occupation) and indirect exposures (*e.g.* food or air) that result from a chemical's transport through various environmental compartments before impacting on humans.

For a given exposure source the concentration or amount of the chemical in that medium is determined by measurement, estimated by modeling, calculated from physical-chemical properties and other information on the agent, or projected from data on surrogate chemicals. Common values might include ambient airborne concentrations given as parts of chemical per million parts of air or the number of milligrams of a drug per dose. Measurements, estimates or assumptions then need to be made concerning the exposure of humans to the chemical through a given medium, *e.g.* the frequency of consumption of a specific food containing a pesticide residue or the frequency, pattern, duration and conditions of use of a consumer product containing a cleaning solvent. Next, assimilation of the chemical into the body is considered; again there is reliance on whatever pharmacokinetic data or estimates are available. Values may be expressed in a number of ways such as the percentage of dose absorbed from the gastrointestinal tract, the movement of so many micrograms of chemical across a given area of skin over a certain time period, or the percent of the inhaled material absorbed in the respiratory tract.

All the above information, *i.e.* population identification and characterization, chemical concentration in a given medium, the nature and pattern of exposure and finally the assimilation of the chemical into the body is assembled and analyzed. Information on multiple sources of exposure may then be combined into a single measure of overall exposure.

Tremendous variability in exposure exists among members of a population. People change jobs or place of residence, convert from well water to municipal water, occasionally take prescription/non-prescription drugs, etc. Each of these differences among persons contributes to the uncertainty associated with any exposure value. To the extent possible, the degree of uncertainty in exposures should be discussed and estimated.

C. Hazard or Dose-Response assessment

Hazard or dose-response assessment is a quantitative exercise that attempts to describe the expected human response to any given level of a carcinogenic exposure. The response is typically characterized in terms of either a specific risk estimate of an upper bound on the underlying risk. This step in the risk assessment process relies heavily on data from exposed humans and on observations of animals employed in long-term cancer studies. Long-term animal screening studies have traditionally focused on the detection of carcinogenic potential. As a result, they are usually based on exposures at or near the test animal's maximum tolerated does level. Since these doses are often order of magnitude higher than those encountered by humans, some form of mathematical low-dose extrapolation procedure is required to estimate the expected response at the exposure levels typically of concern in the environment. In addition a species extrapolation or scaling factor is usually employed when projecting anticipated human cancer risk from experimental data. Low-dose extrapolation may also be an important consideration for epidemiologically-based assessments, since the available human data may often involve relatively high level occupational exposures.

1. Low-dose extrapolation. Low-dose carcinogenic risk estimation is based on mathematical modeling that attempts to characterize explicitly the unknown, underlying relationship between exposure and response or to place an upper bound on the dose-response relationship.

Linear extrapolation procedures are often employed for estimating the low-dose risk. In the absence of background response a commonly used approach is to draw a straight line from an upper (binomial) confidence limit on the observed response rate at the study exposure level to the origin. If background is present, the procedure can be modified by making specific assumptions about the nature of background in order to take its effect into account (*9, 10*). Two of the more appealing features of this procedure are that it is easy to apply and that it will generate an upper bound on the unknown, underlying cancer risk in most instances. The most frequent criticisms directed against it are that it may be unduly conservative and that it fails to utilize all of the study data if several exposure levels are employed. Recently, an interesting modification of the linear extrapolation procedure has been proposed by a number of investigators (*11, 12*). They suggested that some type of mathematical dose-response model should be fit to the experimental data, the estimated response and associated model-based confidence limit for a preselected level of exposure should be determined, and that linear extrapolation should then be used to project an upper bound on risk for the low-dose level of interest.

When low-dose extrapolation is based on an attempt to characterize the underlying dose-response relationship, one of three general classes of models (*i.e.* tolerance distribution models, mechanistic models, or time-to-tumor models) is usually employed.

The tolerance distribution models assume that each member of a population has a threshold or tolerance level below which that individual will not respond to the exposure in question. It is also presumed that the variability among individual threshold levels can be described in terms of a probability distribution. For this class of models the

probability that a particular individual will exhibit a carcinogenic response when exposed at dose level *d* is the same as the probability that the individual's tolerance level is less than *d*. The familiar probit, logit, and Weibull dose-response models can all be generated by adopting different probability distributions to describe the population's tolerance variability (*13*). The best known of the low-dose extrapolation techniques based on tolerance distribution models is the Mantel-Bryan procedure (*14, 15*), which employs a probit model. Although the Mantel-Bryan procedure was originally regarded as a conservative approach to the estimation of low-dose risk since it employs a fixed, presumably conservative estimate of slope and extrapolates from an upper confidence limit on the observed experimental response, subsequent research (*16*) has shown that in the low-dose region the probit model tends to produce relatively high "safe-dose" estimates when compared to other extrapolation procedures. As a result of this characteristic and other criticisms that have been raised against the Mantel-Bryan procedure (*16*), its use in quantitative risk assessment has markedly declined.

The class of mechanistic models, *i.e.* the one-hit, multistage, and multi-hit models, derives its name from the presumed mechanism of carcinogenesis upon which the models in the class are based. Each of these models reflects the assumption that a tumor originates from a single cell that has been damaged by either the chemical or one of its metabolites.

The simplest of the mechanistic models is the one-hit model (*13*) which assumes that damage to the target cell sufficient to cause a tumor results from a single "chemical hit." This model is essentially linear in the low-dose region. Since it has only a single parameter other than background, it will not always provide an adequate fit to the experimental observations.

The multistage model, developed by Armitage and Doll (*17*), is perhaps the most frequently employed of all low-dose extrapolation models currently in use. It reflects the observation that a developing tumor goes through several different stages, which can be affected by the carcinogen in question, before it is clinically detectable. The multistage model will often be approximately linear in the low-dose region, and so its risk estimates are sometimes regarded as being relatively conservative. Furthermore, the procedures most commonly used to generate upper confidence bounds for the multistage model lead to estimates that are linear at low doses (*18, 19*).

The multi-hit model (*13, 20*) assumes that the target cell must absorb at least *k* chemical hits before the cell achieves its carcinogenic potential. Some investigators regard the generalized multi-hit model, where *k* can be any non-negative value, as being more flexible than the multistage model; and its use in risk assessment has been recommended (*21*). However, there are research results (*22*) that indicate a number of important, practical problems associated with its application, such as the fact that model hyperlinearity may produce extremely conservative estimates of safe dose levels, or that the multi-hit model can also indicate a "safe dose" at levels higher than some doses that actually produced deleterious effects.

The last class of models employed in low-dose extrapolation is the time-to-tumor models. These models, which attempt to describe the complex relationship between dose, tumor latency, and cancer risk, include the lognormal distribution (*9*), the Weibull distribution (*23*), and the Armitage-Doll (*17*) and the Hartley-Sielken (*24*) models. While time-to-tumor models can lead in many instances to a more complete characterization of the underlying carcinogenic process, the quality of the available data may not permit their application or, at the very least, is often not sufficient to allow any discrimination among such models using goodness-of-fit criteria. Furthermore, a recent analysis (*25*) of a simulated data base containing information on time-to-tumor occurrence indicated that low-dose risk estimates generated by a variety of extrapolation procedures occasionally differed from the actual risk by three or more orders of magnitude even when this additional information was included in the modeling process. It also appeared that, on the whole, incorporation of time-to-tumor data did not substantially increase the precision of low-dose risk estimation relative to modeling based only on quantal response information.

When data on the time of tumor occurrence or detection are available, some investigators have proposed that alternative measures or risk based on the concept of time-to-tumor, such as the "mean-free" dose or "late-risk" dose (*26*), be used in the cancer assessment process. However, a detailed evaluation of these measures (*27*) indicated that they suffer from a number of shortcomings and are probably most meaningful when used in combination with the more traditional measures, like the lifetime probability of tumor.

As the preceding discussion has indicated, some uncertainty is associated with the use of most if not all of the commonly employed extrapolation models. Furthermore, goodness-of-fit to the experimental observations is not an effective means of discriminating among models (*28*). Thus, no single low-dose extrapolation procedure has yet gained universal acceptance within the scientific community.

Model selection is further complicated when background tumor incidence is present and presumed to be induced by a mechanism or mechanisms distinct from that associated with the chemical under study. In this instance the various mathematical models may fit the observable data equally well and still generate low-dose risk estimates that may differ by many orders of magnitude. On the other hand if background additivity is assumed, *i.e.* if it is presumed that there is a common mechanism of tumor induction, then all models are essentially linear in the low-dose region (*29*). Even if only a small portion of the background incidence is associated with the same mechanistic process as the study chemical, linearity will tend to prevail at sufficiently low doses (*30*).

All of the preceding discussion has implicitly assumed that the carcinogenic dose-response relationship of interest is that which associates the probability of tumor onset with the administered dose or exposure levels. However, in the case of experimentally-derived data, saturation of critical enzyme systems may occur at high dose levels, and so the real dose of interest may be the biologically effective dose that reaches the actual target site. Since the relationship between the effective dose and the administered dose may be non-linear, pharmacokinetic considerations can significantly modify low-dose risk estimates regardless of the model employed (*31–34*).

2. Other estimation procedures. Traditionally, the approach to quantitative hazard evaluation for non-carcinogenic, toxicological endpoints has been based on the use of safety factors. With a safety factor approach an "acceptable" exposure level is determined by dividing the no observed effect level (NOEL) from laboratory-based chronic toxicity tests by an appropriately chosen safety factor. In theory the safety factor that is selected attempts to reflect both the possibility of an increased sensitivity of humans relative to laboratory animals and the

variation in susceptibility within the human population.

In spite of its common use, there are a number of potential problems associated with the safety factor approach. The observation of no treatment-related effects at a given dose level may depend, at least in part, on the number of animals exposed at that particular level. Furthermore, the determination of a NOEL ignores the shape of the dose-response curve, even though it would seem that a curve that has a shallow slope in the experimental NOEL region potentially represents a greater toxicological hazard than one that rises steeply in this region. In addition, there is no biological justification for the general use of any specific safety factor. Another important consideration that would argue against the use of a safety factor approach in cancer risk assessment is the fact that this approach assumes the existence of a true population threshold below which no adverse effects can occur. Even if the concept of individual thresholds could be supported, the well-recognized genetic variability in the human population would effectively prevent the estimation of a general population threshold value. Moreover, given the high level of background cancer present in the human environment, it seems unlikely that one could rule out the possibility that a new chemical exposure, however limited, might augment an already ongoing mechanistic process and thereby produce a collective or additive exposure that exceeds the unknown threshold level *(35)*. While recent attempts to categorize carcinogens as acting directly or indirectly on the genome have renewed interest in the possible use of the safety factor approach, most scientists believe that there is not sufficient biological understanding of these processes to make such classifications for the carcinogenic effect of an agent on the total organism at the present time *(36)* (See also Chapter 1).

Another measure that is occasionally proposed for use in cancer hazard evaluation is the relative potency index. Relative potency calculations are based on standard measures of toxicity, such as the ED50. Such measures are limited from an extrapolation viewpoint in that they attempt to compress an entire dose-response curve into a single number suitable for comparative purposes. Therefore, depending upon which toxicological measure underlies its derivation, a relative potency index may offer little insight into the relative risk in the low-dose region.

3. Extrapolation of low-dose risk estimates to target populations. Low-dose risk estimates derived from laboratory animal data still must be extrapolated to the human population. This process is complicated by a variety of factors that differ among species and potentially affect the response to carcinogenic exposures. Included among these factors are differences between humans and experimental test animals with respect to life span, body size, genetic variability or population homogeneity, existence of concurrent disease, pharmacokinetic effects such as metabolism and excretion patterns, and exposure regimen (See also Chapter 1).

The traditional approach to the species extrapolation issue has been to use some standardized baseline for making interspecies comparisons. Commonly employed standardized dosage scales include mg/kg (body weight)/day, ppm in the diet or water, mg/m^2 (body surface area)/day, and mg/kg (body weight)/lifetime. A number of studies *(37–39)* have used the few epidemiologic and laboratory data sets available in the literature involving comparable carcinogenic exposures to evaluate the relative reliability of these various scales for animal-to-human extrapolation. While no single scale emerged as the clear choice in all cases, it does appear that the best agreement between observed and predicted human cancer risk is often obtained when a mg/kg/day or a mg/m^2/day dosage scale is employed *(38, 39)*. However, it must be emphasized that experience in this area is very limited and that the use of any standardized dosage scale for species scale-up is only a crude approximation to a more detailed adjustment that considers the spectrum of factors that can lead to differential species' responses. Furthermore, recent research *(40)* into this issue has indicated that the previously observed *(39)* strong quantitative correlation of various agents' carcinogenic potencies in mice and rats may be explained by a corresponding correlation of maximum tolerated dose levels for these same agents. The general implications of this finding for quantitative species extrapolation are not fully understood at this time.

In addition to the empirical comparisons discussed above, there are a number of biological issues that bear on the choice of a particular dosage scale in species scale-up. For example, it may be important to know whether the parent compound, one or more of its metabolities, or some combination of the two, is responsible for the observed carcinogenic activity. Similarly, the nature of the biochemical target site and the various factors determining the rate, duration, and extent of the toxicologic reaction may be relevant to the selection process. However, while any meaningful information on the underlying carcinogenic mechanism(s) should, if possible, be taken into account when choosing a dosage scale, such specific information often will not be available.

While no extrapolation across species is involved when epidemiological data are used in risk estimation, the marked variability in human sensitivity must be taken into account. Furthermore, there are a number of other problems that may be encountered with attempting to use epidemiological data to construct estimates of liftime risk for a subset of the general population. In the absence of specific knowledge about the synergistic or antagonistic potential of the exposure of interest, additivity of effects is often assumed. This assumption can lead to either an under or over estimation of the true carcinogenic risk presented by the exposure under consideration.

Epidemiological investigations are often conducted for only a limited period of time, and failure to adjust properly for incomplete follow-up of subjects can significantly affect the estimation of life-time risk. Similarly, estimation of lifetime cancer risks is also complicated if the available epidemiologic data involves exposures that fall far short of a normal lifespan. For example, if a multistage mechanism is assumed, then the effect of early termination of exposure will be dependent on the stages of the carcinogenic process that are influenced by the exposure *(41, 42)*.

D. Risk Characterization

The final step in the risk assessment process, risk characterization, usually involves a total evaluation of the qualitative evidence, the exposure information, and the quantitative results. The final product of this evaluation is, typically, the generation of a quantitative estimate of the human cancer risk associated with the projected exposure profile.

As discussed earlier, the qualitative evidence is evaluated by a weight-of-the-evidence approach. Uncertainties in the data are resolved using best scientific judgement. While no overall measure can be given of the uncertainty, the decision-maker needs to be aware of the strengths of these qualitative data when making this dichotomous determination. The results of a total risk assessment should be expressed in such a way that there is a clear distinction drawn between a chemical for which the

qualitative evidence is overwhelming and a chemcial for which the qualitative evidence is only marginally persuasive.

As developed in Chapter 5, the exposure assessment may contain numerous uncertainties. For example, the assessment may reflect inherent limitations, such as those associated with analytical monitoring or modeling techniques. Further, assumptions are sometimes necessary in the absence of data in order to generate an assessment; *e.g.*, the amount of soil ingested by a child. In many such cases, "reasonable worst case" assumptions are made so that one errs on the side of pubic safety. The exposure assessment should be expressed in such a way that these uncertainties and assumptions are evident to the decision-maker.

Description of uncertainy is also important in the development of the quantitative estimate of human cancer risk. Unfortunately, the description of uncertainty in this step of the assessment process is often limited to the statistical uncertainty involved in low-dose extrapolation as expressed through the calculation of confidence limits for risk estimates.

As has already been noted, the choice of a particular low-dose extrapolation model can have a pronounced influence on the estimated low-dose risk. Therefore, it has been proposed *(43)* that an indication of the variability introduced by model selection be obtained by consideration the range in the magnitude of low-dose risk estimates associated with the more commonly employed models.

Furthermore, if laboratory data are utilized in estimating low-dose risk, some attempt can be made to describe the biological variability associated with the process of species scale-up by contrasting estimates based on different dosage scales and animal test systems.

Finally, it is important in the characterization of human cancer risk to summarize briefly any judgements or assumptions that may have entered into the risk assessment process to insure that they are clearly differentiated from scientific fact *(1)*.

III. EMERGING AREAS OF SCIENCE EXPECTED TO IMPACT ON REGULATORY ACTIONS

Although no one can predict with certainty what and when major breakthroughs will occur in science, many emerging areas of science can be expected to affect significantly cancer risk assessment and related regulatory decisions in the future.

Greater knowledge of the metabolic pathways of chemical compounds and the pharmacokinetics of such agents in various animal species should help regulatory agencies to ascertain whether carcinogenicity tests have been performed in an animal assay system which is relevant to man. Metabolic pathways for activation of a chemical carcinogen known to be important in humans but not present in the animal assay system used lessen the validity of the data for human risk evaluation. Similarly, factors such as diet, age, stress, sex, hormonal status, etc. can influence the metabolic conversion of chemicals to ultimate carcinogens and may be important in hazard evaluations conducted on proposed compounds.

DNA adducts remaining in organs *in vivo*, in the absence of tumors in a test animal species, may be innocuous, or they may indicate physiological peculiarities in the test system that prevent progression to overt tumors. Such inhibitory factors may or may not be present in other assay systems or in man. Alternatively, failure to demonstrate tumor formation may indicate the need for promoter activity or interaction with other modifying agents. Substantial adduct formation without resulting biological manifestations may suggest either that the lesions induced are innocuous or dictate that more testing is needed to access the human exposure experience (*i.e.* multiple exposure factors, promoting agents, nutrition, etc.).

Most prescreen assays for chemical carcinogens presently rely primarily on chemical interaction with DNA (point mutation assays, DNA damage and repair). This approach may be inadequate since certain chemicals may not induce cancer by direct interaction with the DNA. New assays for carcinogenicity are being developed to include animal models where these factors can be taken into consideration in the evaluation of chemical agents.

Since tumor induction is generally considered to be a multi-stage process, each stage may be under independent genetic control. Test systems are being developed and used that are based on animals believed to be defective in genetic factors similarly to those defects that predispose humans to cancer induction. Such test systems may also be used to screen sensitivity to selected carcinogens for subpopulations of humans with similar genetic characteristics.

A relationship seems to exist between altered DNA and carcinogenesis. The presence of carcinogen-altered DNA is being explored as a possible test system to detect persistent damage at the molecular level after exposure to suspect carcinogenic chemicals. Such a system could be used to screen exposed human populations for altered DNA bases using monoclonally-derived specific antibodies to modified bases. Such assays may also help place in perspective effects on the DNA arising from endogenous vs. exogenous DNA damaging agents. This screening would be an example of using a marker for altered cells important to the carcinogenic process. Additional biomarkers should be developed for cells in all stages of carcinogenesis. Such tests might be expected to identify the carcinogenic potential of suspect agents and the population(s) at risk for possible future development of cancers.

Certain DNA base alterations may occur in regions of the genome that seem to be unimportant for cancinogenesis. It is important to know which alterations are necessary for initiation of a tumor cell and which are masked or not expressed. Information is being assimilated on types of alterations in "control" regions and the relationship of the number of altered bases in these regions to tumor formation. These data will provide information needed to differentiate those insults to DNA that are critical from those that are not. If multi-critical sites must be altered in a particular manner, this approach may be used to examine multi-stage phenomena of cancer. The measurement of risk from particular carcinogens at the cellular level would then be based on a more selective effect than the level of total DNA adducts.

Other areas of research that may in time have an effect on regulatory decisions include the development of model systems to assay for compounds influencing promotion and progression of initiated populations, elucidation of factors controling cellular differentiation, and tests for chromosomal rearrangements and oncogene activities. Each of these areas of study will not only expand our knowledge of mechanisms but also permit a more realistic assessment of risk. Besides improving assessment, increased knowledge of mechanism may also result in a greater ability to render carcinogenic agents biologically innocuous, removing hazards.

IV. SUMMARY

The assessment of human cancer risk associated with different chemical exposures is seen to be a complex mixture of currently available scientific data, assumptions and judgments based on prevailing scientific thought, and policy decisions. The process involves the identification of potential human cancer hazards, usually based on epidemiological, clinical, and long-term

animal studies, the determination of potential human exposure profiles, the estimation of the unknown, underlying relationship between exposure and response, and, finally, the combination of the results of these exercises to characterize the expected human cancer risks. Some degree of uncertainty is involved in all phases of this process, and as a result, no simple, standardized format has yet been developed for evaluating human cancer risk, even though a number of basic principles have emerged. It is apparent that human cancer risk assessment is still in an evolutionary state and that a number of emerging areas of science will impact on this process in the future.

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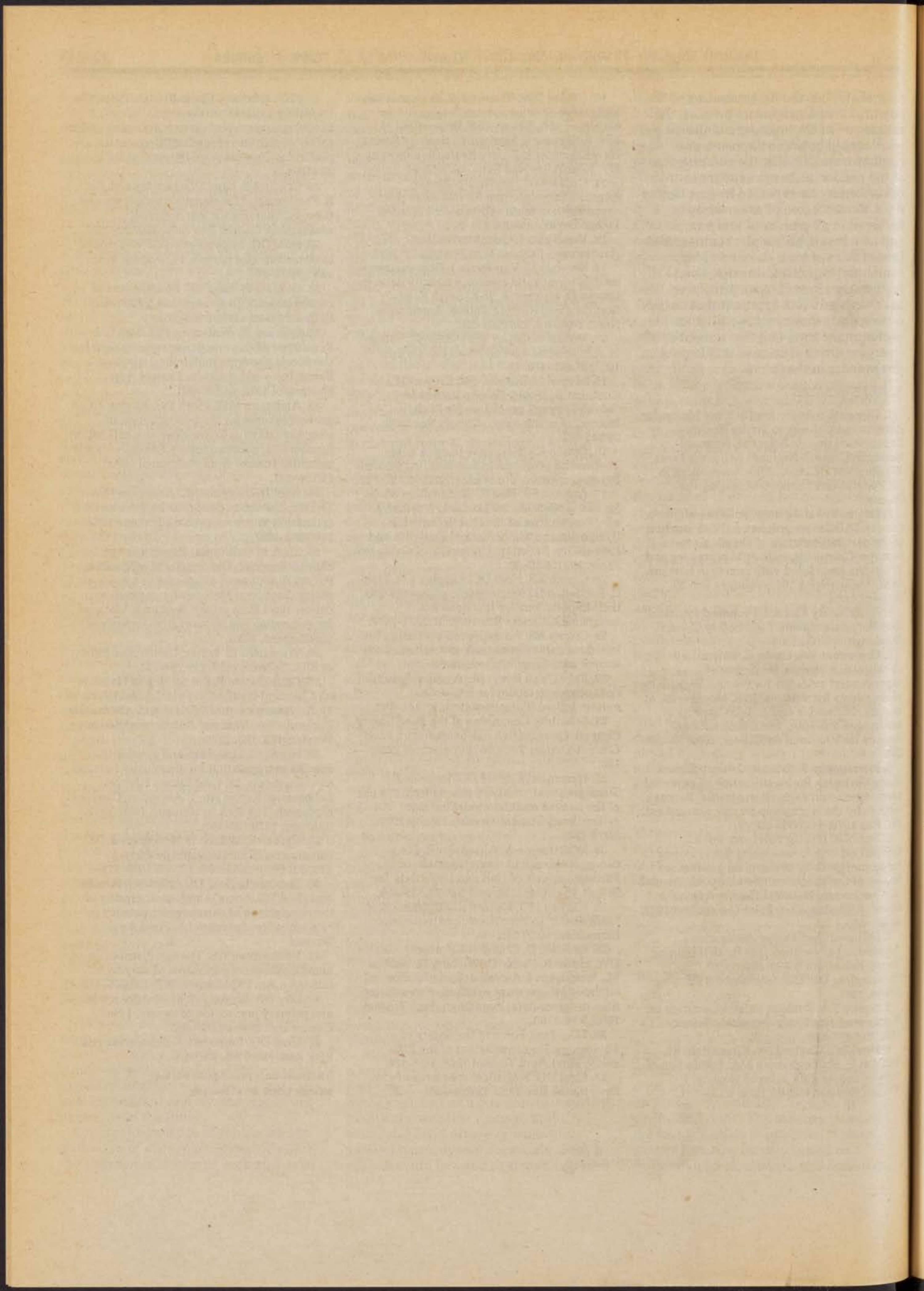

federal register

Tuesday
May 22, 1984

Part III

Department of the Interior

Fish and Wildlife Service

50 CFR Part 17

Endangered and Threatened Wildlife and Plants; Review of Invertebrate Wildlife for Listing as Endangered or Threatened Species

DEPARTMENT OF THE INTERIOR

Fish and Wildlife Service

50 CFR Part 17

Endangered and Threatened Wildlife and Plants; Review of Invertebrate Wildlife for Listing as Endangered or Threatened Species

AGENCY: Fish and Wildlife Service, Interior.

ACTION: Notice of review.

SUMMARY: The U.S. Fish and Wildlife Service identifies invertebrate animal taxa native to the United States being considered for addition to the List of Endangered and Threatened Wildlife. Although "candidate" species receive no substantive or procedural protections under the Endangered Species Act, the Service encourages federal agencies and other planners to take such taxa into account in environmental planning under the National Environmental Policy Act and similar statutes. Also identified in this notice are those invertebrate taxa that were previously under consideration for listing, but that are currently presumed either to be extinct, to not be valid species or subspecies, or to be more abundant and widespread than previously thought and/or not subject to identifiable threats.

DATE: Comments may be submitted until further notice.

ADDRESSES: Interested persons or organizations are requested to submit comments to: Director, U.S. Fish and Wildlife Service, Department of the Interior, Washington, D.C. 20240. Comments and materials relating to this notice are available for public inspection by appointment during normal business hours at the Service's Office of Endangered Species, Suite 500, 1000 North Glebe Road, Arlington, Virginia.

Information relating to particular taxa may be obtained from appropriate Service Regional Offices listed below:

Region 1.—California, Hawaii, Idaho, Nevada, Oregon, Washington, American Samoa, Guam, Territory of the Northern Marianas, and Trust Territories of the Pacific.

Regional Director (ARD/FA), U.S. Fish and Wildlife Service, Suite 1692, Lloyd 500 Building, 500 N.E. Multnomah Street, Portland, Oregon 97232 (503/231-6131 or FTS 8/429-6131).

Region 2.—Arizona, New Mexico, Oklahama, and Texas.

Regional Director (ARD/AFF), U.S. Fish and Wildlife Service, P.O. Box 1306, Albuquerque, New Mexico 87103 (505/766-3972 or FTS 8/474-3972).

Region 3.—Illinois, Indiana, Iowa, Michigan, Minnesota, Missouri, Ohio, and Wisconsin.

Regional Director (ARD/AFF), U.S. Fish and Wildlife Service, Federal Building, Fort Snelling, Twin Cities, Minnesota 55111 (612/725-3596 or FTS 8/725-3596).

Region 4.—Alabama, Arkansas, Florida, Georgia, Kentucky, Louisiana, Mississippi, North Carolina, South Carolina, Tennessee, Puerto Rico, and the Virgin Islands.

Regional Director (ARD/FA), U.S. Fish and Wildlife Service, The Richard B. Russell Federal Building, 75 Spring Street, S.W., Atlanta, Georgia 30303 (404/221-3583 or FTS 8/242-3583).

Region 5.—Connecticut, Delaware, District of Columbia, Maine, Maryland, Massachusetts, New Hampshire, New Jersey, New York, Pennsylvania, Rhode Island, Vermont, Virginia, and West Virginia.

Regional Director (ARD/FA), U.S. Fish and Wildlife Service, Suite 700, One Gateway Center, Newton Corner, Massachusetts 02158 (617/965-5100 ext. 316 or FTS 8/829-9316, 7, 8).

Region 6.—Colorado, Kansas, Montana, Nebraska, North Dakota, South Dakota, Utah, and Wyoming.

Regional Director (ARD/FA), U.S. Fish and Wildlife Service, P.O. Box 25486, Denver Federal Center, Denver, Colorado 80225 (303/234-2496 or FTS 8/234-2496).

Region 7.—Alaska.

Regional Director (ARD/AFF), U.S. Fish and Wildlife Service, 1101 East Tudor Road, Anchorage, Alaska 99503 (907/263-3539 or FTS 8/263-3539).

FOR FURTHER INFORMATION CONTACT: John L. Spinks, Jr., Chief, Office of Endangered Species, U.S. Fish and Wildlife Service, Washington, D.C. 20240 (703/235-2771), or the appropriate Regional Office.

SUPPLEMENTARY INFORMATION:

Background

The Endangered Species Act of 1973 (16 U.S.C. 1531 *et seq.*) requires a determination of whether species of wildlife and plants are endangered or threatened based on the best available scientific and commercial data. For many years, the U.S. Fish and Wildlife Service (hereafter the Service) has been gathering data on taxa of invertebrates (sponges, crustaceans, arachnids, insects and mollusks), native to the United States, that have appeared, at least at times, to warrant consideration for addition to the List of Endangered and Threatened Wildlife. The accompanying table identifies many of these taxa (including, by definition, biological subspecies) and assigns each to one of the following three categories.

Category 1 comprises taxa for which the Service currently has substantial information on hand to support the biological appropriateness of proposing to list the species as endangered or threatened. Currently, data are being gathered concerning essential habitat needs and, for some taxa, data concerning the precise boundaries for critical habitat designations. Development and publication of proposed rules on such species is anticipated.

Category 2 comprises taxa for which information now in possession of the Service indicates that proposing to list the species as endangered or threatened is possibly appropriate, but for which conclusive data on biological vulnerability and threat(s) are not currently available to support proposed rules at this time. Taxa in this category for which there have not been authenticated records since 1963 are indicated by an asterisk (*); some of these are possibly extinct, but further biological research and field study are needed to ascertain their status with any confidence. It is likely that many of the taxa in this category will not warrant listing, while some will be found to be in greater danger of extinction than some taxa in category 1. It is hoped that this notice will encourage the necessary research on vulnerability and/or threats for these taxa.

Category 3 comprises taxa that are no longer being considered for listing as endangered or threatened. Such taxa are included in one of three subcategories depending on the reasons for removal from consideration.

3A. Taxa for which the Service has persuasive evidence of extinction. If rediscovered, however, such species might acquire high priority for listing. At this time, the best available information indicates that the taxa included in this category, or the habitats from which they were known, are in fact extinct or destroyed, respectively.

3B. Names that, on the basis of current taxonomic understanding, usually as represented in published revisions and monographs, do not represent taxa meeting the Act's definition of "species." Such supposed taxa could be reevaluated in the future on the basis of subsequent research.

3C. Taxa that have proven to be more abundant or widespread than was previously believed and/or those that are not subject to any identifiable threat. Should further research or changes in land use indicate significant

decline in any of these taxa, they may be reevaluated for possible inclusion in category 1 or 2.

The Service hereby solicits data concerning the taxa in the accompanying table. Especially sought is information—

(1) Indicating that a taxon would more properly be assigned to a category other than the one in which it appears;

(2) Nominating a taxon not included in the table;

(3) Recommending an area as critical habitat for a candidate taxon, or indicating why it would not be prudent to propose critical habitat for a taxon, or why critical habitat may not be determinable for a taxon;

(4) Documenting threats to any of the listed taxa;

(5) Pointing out taxonomic changes for any of the taxa;

(6) Suggesting new or more appropriate names; or

(7) Noting errors, such as in the indicated distributions.

The Service intends to consider all data received in response to this notice, to make appropriate amendments to the accompanying table, and to indicate intentions with regard to future listing actions. Substantive changes in status may be announced by periodic notice in the **Federal Register.**

The following table is arranged in a general systematic order, beginning with sponges and ending with mollusks. For each taxon, the assigned category appears on the left, followed by the common name, the scientific name, the family name, and the known distribution, usually indicated by abbreviations of State names. For mollusks the authority and date for the scientific name is given, because of unusual instability in systematics of those groups. The species may no longer occur in some of the areas shown. Some taxa have been included that have not yet been formally described in the scientific literature. Such taxa are indicated by the abbreviation "sp." after the generic name, or "ssp." after the generic and specific names.

A provisional vernacular name in English or Hawaiian is given for most species. Some are vernacular names actually in common use, and in some groups whose systematics most need revision these are about as informative as the current scientific name in Latin. Most of the taxa are rare, however, and the obscurity of many almost guarantees that no name has much history of use. Group names such as snail, amphipod, or dragonfly have been appended to many names to clarify distinctions from other invertebrates or plants with similar or confusing names; such extra qualifiers would probably be dropped from any name that came into truly common use. These provisional names may be changed or discarded if their use is later found to be inappropriate.

This notice was prepared by the zoologists in the Service's Office of Endangered Species in Washington that specialize in invertebrate species, and the Endangered Species Program staff of the Service's Regional Offices and Field Stations.

List of Subjects in 50 CFR Part 17

Endangered and threatened wildlife, Fish, Marine mammals, Plants (agriculture).

Dated: April 24, 1984.

G. Ray Arnett,

Assistant Secretary for Fish and Wildlife and Parks.

Category and common name	Scientific name	Family	Distribution
SPONGES			
3B—Muscular sponge	*Anheteromyia biceps*	Spongillidae	MI.
2—Carolina sponge	*Corvomeyenia carolinensis*	Spongillidae	SC.
2—Oklawaha sponge	*Dosilia palmeri*	Spongillidae	FL, Mexico.
2—Kissimmee sponge	*Ephydatia subtilis*	Spongillidae	FL.
2—Pennsylvania sponge	*Heteromeyenia longistylis*	Spongillidae	PA.
2—Oneida sponge	*Spongilla heterosierila*	Spongillidae	NY.
3B—Spongy sponge	*Spongilla sponginosa*	Spongillidae	SC.
FLATWORMS (Turbellaria)			
2—(No common name)	*Kenkia rhynchida*	Kenkiidae	OR.
2—(No common name)	*Macrocotyla glandulosa*	Kenkiidae	MO, IA.
2—(No common name)	*Procotyla typhlops*	Kenkiidae	MD, VA.
3A—Holsinger's groundwater planarian	*Sphalloplana holsingeri*	Planariidae	VA.
2—Refton Cave planarian	*Sphalloplana pricei*	Planariidae	PA.
3A—Bigger's groundwater planarian	*Sphalloplana subtilis*	Planariidae	VA.
BRANCHIOPODS (Crustaceans, Subclass Branchiopoda)			
2—Mono Lake brine shrimp	*Artemia monica*	Artemiidae	CA.
ISOPODS (Crustaceans, Order Isopoda)			
2—Clifton Cave isopod	*Caecidotea barri*	Asellidae	KY.
2—Bat Cave isopod	*Caceidotea macropoda*	Asellidae	OK.
2—Nickajack Cave isopod	*Caceidotea nickajackensis*	Asellidae	TN.
2—Rye Cove Cave isopod	*Lirceus culveri*	Asellidae	VA.
2—Lee County Cave isopod	*Lirceus usdagalun*	Asellidae	VA.
AMPHIPODS (Crustaceans, Order Amphipoda)			
2—Central Missouri cave amphipod	*Allocrangonyx hubrichti*	Crangonyctidae	MO.
2—Oklahoma cave amphipod	*Allocrangonyx pellucidus*	Crangonyctidae	OK.
3C—Kansas well amphipod	*Bactrurus hubrichti*	Crangonyctidae	KS, MO, OK.
3C—Anomalous spring amphipod	*Crangonyx anomalus*	Crangonyctidae	IN, KY, OH.
3C—Appalachian Valley cave amphipod	*Crangonyx antennatus*	Crangonyctidae	AL, IL, TN, VA.
2—Pennsylvania cave amphipod	*Crangonyx dearolfi*	Crangonyctidae	MD, PA.
2—Florida cave amphipod	*Crangonyx grandimanus*	Crangonyctidae	FL.
2—Hobbs' cave amphipod	*Crangonyx hobbsi*	Crangonyctidae	FL.
3C—Minor cave amphipod	*Crangonyx minor*	Crangonyctidae	IA, IL, IN, MI, Canada.
3C—Packard's cave amphipod	*Crangonyx packardi*	Crangonyctidae	IN, KY.
2—Illinois cave amphipod	*Gammarus acherondytes*	Gammaridae	IL.
2—Bousfield's amphipod	*Gammarus bousfieldi*	Gammaridae	KY.
2—Noel's amphipod	*Gammarus desperatus*	Gammaridae	NM.
2—Diminutive amphipod	*Gammarus hyalleloides*	Gammaridae	TX.
2—Pecos amphipod	*Gammarus pecos*	Gammaridae	TX.
2—Kauai cave amphipod	*Spelaeorchestia koloana*	Talitridae	HI.
3C—Allegheny cave amphipod	*Stygobromus (=Stygonectes) allegheniesis*	Crangonyctidae	MD, NY, PA.
2—Tidewater interstitial amphipod	*Stygobromus (=Apocrangonyx) araeus*	Crangonyctidae	VA.
2—Arizona cave amphipod	*Stygobromus arizonensis*	Crangonyctidae	AZ.

Category and common name	Scientific name	Family	Distribution
2—Balcones cave amphipod	*Stygobromus* (=*Stygonectes*) *balconius*	Crangonyctidae	TX.
2—Barr's cave amphipod	*Stygobromus* (=*Stygonectes*) *barri*	Crangonyctidae	MO.
2—Bifurcated cave amphipod	*Stygobromus* (=*Stygonectes*) *bifurcatus*	Crangonyctidae	TX.
2—Bowman's cave amphipod	*Stygobromus* (=*Stygonectes*) *bowmani*	Crangonyctidae	OK.
2—Clanton's cave amphipod	*Stygobromus* (=*Stygonectes*) *clantoni*	Crangonyctidae	KS, MO.
2—Burnsville Cove cave amphipod	*Stygobromus* (=*Stygonectes*) *conradi*	Crangonyctidae	VA.
2—Cooper's cave amphipod	*Stygobromus* (=*Stygonectes*) *cooperi*	Crangonyctidae	WV.
2—Cascade Cave amphipod	*Stygobromus* (=*Stygonectes*) *dejectus*	Crangonyctidae	TX.
2—Elevated Spring amphipod	*Stygobromus* (=*Stygonectes*) *elatus*	Crangonyctidae	AR.
2—Greenbrier Cave amphipod	*Stygobromus* (=*Stygonectes*) *emarginatus*	Crangonyctidae	MD, WV.
2—Ephemeral cave amphipod	*Stygobromus* (=*Apocrangonyx*) *ephemerus*	Crangonyctidae	VA.
3C—Central Kentucky cave amphipod	*Stygobromus exilis*	Crangonyctidae	AL, KY, TN.
2—Ezell's Cave amphipod	*Stygobromus* (=*Stygonectes*) *flagellatus*	Crangonyctidae	TX.
3C—Shenandoah Valley cave amphipod	*Stygobromus* (=*Stygonectes*) *gracilipes*	Crangonyctidae	MD, PA, VA, WV.
2—Grady's cave amphipod	*Stygobromus gradyi*	Crangonyctidae	CA.
2—Devil's Sinkhole amphipod	*Stygobromus* (=*Stygonectes*) *hadenoecus*	Crangonyctidae	TX.
2—Hara's cave amphipod	*Stygobromus harai*	Crangonyctidae	CA.
2—(No common name)	*Stygobromus heteropodus*	Crangonyctidae	MO.
2—Malheur Cave amphipod	*Stygobromus hubbsi*	Crangonyctidae	OR.
2—Tidewater amphipod	*Stygobromus* (=*Stygonectes*) *identatus*	Crangonyctidae	VA.
3C—Iowa amphipod	*Stygobromus iowae*	Crangonyctidae	IA
2—Long-legged cave amphipod	*Stygobromus* (=*Stygonectes*) *longipes*	Crangonyctidae	TX.
3A—Rubious Cave amphipod	*Stygobromus* (=*Apocrangonyx*) *lucifugus*	Crangonyctidae	IL.
2—MacKenzie's cave amphipod	*Stygobromus mackenziei*	Crangonyctidae	CA.
3C—Southwestern Virginia cave amphipod	*Stygobromus mackini*	Crangonyctidae	TN, VA.
2—Mountain cave amphipod	*Stygobromus* (=*Stygonectes*) *montanus*	Crangonyctidae	AR.
2—Morrison's cave amphipod	*Stygobromus* (=*Stygonectes*) *morrisoni*	Crangonyctidae	VA, WV.
2—Bath County cave amphipod	*Stygobromus* (=*Stygonectes*) *mundus*	Crangonyctidae	VA.
2—Norton's cave amphipod	*Stygobromus* (=*Apocrangonyx*) *nortoni*	Crangonyctidae	TN.
2—Onondaga Cave amphipod	*Stygobromus onondagaensis*	Crangonyctidae	MO.
3C—Oregon cave amphipod	*Stygobromus oregonensis*	Crangonyctidae	OR.
2—Ozark cave amphipod	*Stygobromus* (=*Stygonectes*) *ozarkensis*	Crangonyctidae	AR, MO, OK.
2—Minute cave amphipod	*Stygobromus* (=*Apocrangonyx*) *parvus*	Crangonyctidae	WV.
2—Peck's cave amphipod	*Stygobromus* (=*Stygonectes*) *pecki*	Crangonyctidae	TX.
2—Pizzini's amphipod	*Stygobromus* (=*Stygonectes*) *pizzinii*	Crangonyctidae	DC, MD, PA, VA.
2—Wisconsin well amphipod	*Stygobromus putealis*	Crangonyctidae	WI.
2—Reddell's cave amphipod	*Stygobromus* (=*Stygonectes*) *reddelli*	Crangonyctidae	TX.
2—Alabama well amphipod	*Stygobromus smithi*	Crangonyctidae	AL.
2—Spring cave amphipod	*Stygobromus* (=*Stygonectes*) *spinatus*	Crangonyctidae	WV.
2—Stellmack's cave amphipod	*Stygobromus* (=*Stygonectes*) *stellmacki*	Crangonyctidae	PA.
2—Subtle cave amphipod	*Stygobromus* (=*Apocrangonyx*) *subtilis*	Crangonyctidae	IL, MO.
3C—Potomac groundwater amphipod	*Stygobromus* (=*Stygonectes*) *tenuis potomacus*	Crangonyctidae	DC, MD, PA, VA.
2—Wengerors' cave amphipod	*Stygobromus wengerorum*	Crangonyctidae	CA.
CRAYFISHES AND SHRIMPS (Crustaceans, Order Decapoda)			
1—Shasta (=Placid) crayfish	*Pacifastacus fortis*	Astacidae	CA.
2—Alabama cave shrimp	*Palaemonias alabamae*	Atyidae	AL.
1—California freshwater shrimp	*Syncaris pacifica*	Atyidae	CA.
2—Mona cave shrimp	*Typhlatya monae*	Atyidae	PR, West Indies.
2—(No common name)	*Cambarus batchi*	Cambaridae	KY.
2—Big South Fork crayfish	*Cambarus bouchardi*	Cambaridae	KY, TN.
2—Greensboro burrowing crayfish	*Cambarus catagius*	Cambaridae	NC.
2—New River riffle crayfish	*Cambarus chasmodactylus*	Cambaridae	NC, VA, WV.
2—Chickamauga crayfish	*Cambarus extraneus*	Cambaridae	GA, TN.
2—Obey crayfish	*Cambarus obeyensis*	Cambaridae	TN.
3C—Conchas crayfish	*Orconectes deanae*	Cambaridae	NM.
2—Louisville crayfish	*Orconectes jeffersoni*	Cambaridae	KY.
2—Nashville crayfish	*Orconectes shoupi*	Cambaridae	TN.
2—Palm Springs Cave crayfish	*Procambarus acherontis*	Cambaridae	FL.
2—Pee Dee lotic crayfish	*Procambarus lepidodactylus*	Cambaridae	NC, SC.
2—(No common name)	*Procambarus connus*	Cambaridae	MS.
2—Texas cave shrimp	*Palaemonetes antrorum*	Palaemonidae	TX.
1—Squirrel Chimney cave shrimp	*Palaemonetes cummingi*	Palaemonidae	FL.
SPIDERS (Arachnids, Order Aranea)			
2—Dolloff Cave spider	*Meta dolloff*	Araneidae	CA.
2—Torreya trap-door spider	*Cyclocosmia torreya*	Ctenizidae	FL.
2—Key gnaphosid spider	*Cesonia irvingi*	Gnaphosidae	FL.
2—Kauai cave wolf spider (pe'e pe'e maka 'ole)	*Adelocosa anops*	Lycosidae	HI.
2—Rosemary wolf spider	*Lycosa ericeticola*	Lycosidae	FL.
2—Lake Placid funnel wolf spider	*Sosippus placidus*	Lycosidae	FL.
2—Santa Cruz teleman spider	*Telema* sp. (undescribed)	Telemidae	CA.
PSEUDOSCORPIONS (Arachnids, Order Pseudoscorpiones)			
2—Empire Cave pseudoscorpion	*Microcreagris imperialis*	Neobisiidae	CA.
ROCKHOPPERS AND BRISTLETAILS (Insects, Order Archeognatha)			
2—Hawaiian long-palp bristletail	*Machiloides heteropus*	Machilidae	HI.
2—Perkin's club-palp bristletail	*Machiloides perkinsi*	Machilidae	HI.
MAYFLIES (Insects, Order Ephemeroptera)			
2—Berner's two-winged mayfly	*Heterocloeon berneri*	Baetidae	GA.
2—American sand-burrowing mayfly	*Dolania americana*	Behningiidae	FL, GA, SC, NC.
2*—Yellow brachycercus mayfly	*Brachycercus flavus*	Caenidae	LA*.
2*—Frison's ceratellan mayfly	*Ceratella frisoni*	Ephemerellidae	AL*, IL*.
2*—Spiculose ceratellan mayfly	*Ceratella spiculosa*	Ephemerellidae	TN*, NC*.
2—Argo ephemerellan mayfly	*Ephemerella argo*	Ephemerellidae	IL, IN.
2*—Colorado burrowing mayfly	*Ephemera compar*	Ephemeridae	CO*.
2*—West Virginia burrowing mayfly	*Ephemera triplex*	Ephemeridae	WV*.
3A—Robust pentagenian burrowing mayfly	*Pentagenia robusta*	Ephemeridae	OH*.
2—Meridion blackwater mayfly	*Pseudirion meridionalis*	Heptageniidae	FL, GA.

Category and common name	Scientific name	Family	Distribution
2—Cahaba sand-filtering mayfly	*Homoeoneuria cahabensis*	Oligoneuridae	AL, MS.
2—Blackwater sand-filtering mayfly	*Homoeoneuria dolani*	Oligoneuridae	FL, GA, SC.
3A—Pecatonica River mayfly	*Acanthometropus pecatonica*	Siphlonuridae	IL*.
2—False ameletus mayfly	*Ameletus falsus*	Siphlonuridae	AZ*.
2*—Diverse isonychian mayfly	*Isonychia diversa*	Siphlonuridae	TN*.
DRAGONFLIES AND DAMSELFLIES (Insects, Order Odonata)			
2—Barrens bluet damselfly	*Enallagma recurvatum*	Coenagrionidae	MA, NY, NJ.
1—San Francisco forktail damselfly	*Ischnura gemina*	Coenagrionidae	CA.
2—Adytum megalagrion damselfly	*Megalagrion adytum*	Coenagrionidae	HI.
2*—Fallax megalagrion damselfly	*Megalagrion amaurodytum fallax*	Coenagrionidae	HI*.
2*—Pele megalagrion damselfly	*Megalagrion amaurodytum peles*	Coenagrionidae	HI*.
2*—Waianae megalagrion damselfly	*Megalagrion amaurodytum waianaenum*	Coenagrionidae	HI*.
3A—Jugorum megalagrion damselfly	*Megalagrion jugorum*	Coenagrionidae	HI*.
2—Leptodemas megalagrion damselfly	*Megalagrion leptodemus*	Coenagrionidae	HI.
2—Molokai megalagrion damselfly	*Megalagrion molokaiense*	Coenagrionidae	HI.
3A—Nesiotes megalagrion damselfly	*Megalagrion nesiotes*	Coenagrionidae	HI*.
2—Nigrohamatum megalagrion damselfly	*Megalagrion nigrohamatum*	Coenagrionidae	HI.
2—Blackline megalagrion damselfly	*Megalagrion nigrolineatum*	Coenagrionidae	HI.
2—Oahu megalagrion damselfly	*Megalagrion oahuensis*	Coenagrionidae	HI.
2—Oceanic megalagrion damselfly	*Megalagrion oceanicum*	Coenagrionidae	HI.
2—Pacific megalagrion damselfly	*Megalagrion pacificum*	Coenagrionidae	HI.
2—Orange-black megalagrion damselfly	*Megalagrion xanthomeles*	Coenagrionidae	HI.
1—Say's spiketail dragonfly	*Cordulegaster sayi*	Cordulegastridae	FL, GA.
2—Apalichicola twilight skimmer dragonfly	*Neurocordulia clara*	Corduliidae	AI, FL.
2*—Ohio emerald dragonfly	*Somatochlora hineana*	Corduliidae	OH*, IN*.
2—Big Thicket emerald dragonfly	*Somatochlora margarita*	Corduliidae	TX.
2—Banded bog skimmer dragonfly	*Williamsonia lintneri*	Corduliidae	NY, NJ, MA, RI, NH.
2—Sandhills clubtail dragonfly	*Gomphus (=Hylogomphus) carolinus*	Gomphidae	NC, SC.
3B—Riffle clubtail (syn.)	*Gomphus adelphus (=G. brevis)*	Gomphidae	MA, NY.
2—Cherokee clubtail dragonfly	*Gomphus consanguis*	Gomphidae	SC, AL, NC, TN, VA.
2—Tennessee clubtail dragonfly	*Gomphus sandrius*	Gomphidae	TN.
2—Septima's clubtail dragonfly	*Gomphus septima*	Gomphidae	AI, NC.
2—Bronze clubtail dragonfly	*Gomphus townesi*	Gomphidae	FL, AL, SC, NC, TN.
2*—Edmund's snaketail dragonfly	*Ophiogomphus edmundo*	Gomphidae	NC*.
2—Midget snaketail dragonfly	*Ophiogomphus howei*	Gomphidae	MA*, NY, PA, VA, KY, NC, TN.
2—Alleghany snaketail dragonfly	*Ophiogomphus incurvatus alleghaniensis*	Gomphidae	WV, VA, AL, TN?.
2—Variegated clubtail dragonfly	*Progomphus bellei*	Gomphidae	FL, NC.
2—Wabash belted skimmer dragonfly	*Macromia wabashensis*	Macromiidae	OH*, IN* TX.
STONEFLIES (Insects, Order Plecoptera)			
2—Lake Tahoe benthic stonefly	*Capnia lacustra*	Capniidae	CA, NV.
3A—Robert's alloperlan stonefly	*Alloperla roberti*	Chloroperlidae	IL*.
2—Schoolhouse Springs leuctran stonefly	*Leuctra szczytkoi*	Leuctridae	LA.
2—Meltwater lednian stonefly	*Lednia tumana*	Nemouridae	MT.
2—Wahkeena Falls flightless stonefly	*Nemoura wahkeena*	Nemouridae	OR.
2—Fender's soliperlan stonefly	*Soliperla fenderi*	Peltoperlidae	WA.
2—Georgia beloneurian stonefly	*Beloneuria georgiana*	Perlidae	GA, NC.
2—Cheaha beloneurian stonefly	*Beloneuria jamesae*	Perlidae	AL.
2—Henson's appalachian stonefly	*Hansonoperla appalachia*	Perlidae	TN, MA.
2—Leon River taeniopteryx stonefly	*Taeniopteryx starki*	Taeniopterygidae	TX.
GRASSHOPPERS, CRICKETS AND KATYDIDS (Insects, Order Orthoptera)			
2—Idaho point-headed grasshopper	*Acrolophitus pulchellus*	Acrididae	ID.
2—Michigan bog grasshopper	*Appalachia arcena*	Acrididae	MI
2—Siskiyou chloealtis grasshopper	*Chloealtis aspasma*	Acrididae	OR.
2*—Superb spharagemon grasshopper	*Spharagemon superbum*	Acrididae	TX*, OK*
2—Pinaleno monkey grasshopper	*Eumorsea pinaleno*	Eumastacidae	AZ.
2—Desert monkey grasshopper	*Psychomastix deserticola*	Eumastacidae	CA/NV.
2—Howarth's cave cricket	*Caconemobius howarthi*	Gryllidae	HI
2—Schauinsland's bush cricket	*Caconemobius schauinslandi*	Gryllidae	HI.
2—Kaumana Cave cricket	*Caconemobius varius*	Gryllidae	HI
2—Keys scaly cricket	*Cycloptilum irregularis*	Gryllidae	FL
2*—Prairie mole cricket	*Gryllotalpa major*	Gryllidae	KS*, IL*, MO*, MS*, OK*.
2—Oahu deceptor bush cricket	*Leptogryllus deceptor*	Gryllidae	HI.
2—Laricis tree cricket	*Oecanthus laricis*	Gryllidae	MI/OH.
2—Volcanoes cave cricket	*Thaumatogryllus cavicola*	Gryllidae	HI.
2—Kauai thin-footed bush cricket	*Thaumatogryllus variegatus*	Gryllidae	HI.
2—Arizona giant sand treader cricket	*Daihinibaenetes arizonensis*	Rhaphidophoridae	AZ.
2—Kelso giant sand treader cricket	*Macrobaenetes kelsoensis*	Rhaphidophoridae	CA.
2—Coachella giant sand treader cricket	*Macrobanetes valgum*	Rhaphidophoridae	CA.
2—Samwell Cave cricket	*Pristoceuthophilus sp.*	Rhaphidophoridae	CA.
2—Tanner's black camel cricket	*Utabaenetes tanneri*	Rhaphidophoridae	UT.
2—Kelso Jerusalem cricket	*Ammopelmatus kelsoensis*	Stenopelmatidae	CA
2—Port Conception Jerusalem cricket	*Ammopelmatus muwu*	Stenopelmatidae	CA.
2—Coachella Valley Jerusalem cricket	*Stenopelmatus cahuilaensis*	Stenopelmatidae	CA.
2—Navajo Jerusalem cricket	*Stenopelmatus navajo*	Stenopelmatidae	AZ.
2—Sierra pygmy grasshopper	*Tetrix sierrana*	Tetrigidae	CA.
2—Torreya pygmy grasshopper	*Tettigidea empedonepia*	Tetrigidae	FL.
2—Nihoa banza conehead katydid	*Banza nihoae*	Tettigoniidae	HI.
2—Big Pine Key conehead katydid	*Belocephalus micanopy*	Tettigoniidae	FL.
2—Keys short-winged conehead katydid	*Belocephalus sleighti*	Tettigoniidae	FL.
3A—Remote conehead katydid	*Conocephaloides remotus*	Tettigoniidae	HI*.
2—Middlekauf's shieldback katydid	*Idiostatus middlekaufi*	Tettigoniidae	CA.
3A—Antioch Dunes shieldback katydid	*Neduba extincta*	Tettigoniidae	CA*.
2—Santa Monica shieldback katydid	*Neduba longipennis*	Tettigoniidae	CA.
COCKROACHES (Insects, Order Blattodea)			
2—Tuna cave roach	*Aspiduchus cavernicola*	Blattidae	PR.
ZOROAPTERANS (Insects, Order Zoraptera)			
2—Swezey's zoroapteran	*Zorotypus swezeyi*	Zorotypidae	HI.

Category and common name	Scientific name	Family	Distribution
TRUE BUGS (Insects, Order Hemiptera)			
2—Saratoga Springs belostoman bug	*Belostoma saratogae*	Belostomatide	CA.
2—Mauna Loa metrargan seed bug	*Metrarga obscura*	Lygaeidae	HI.
2—Kauai band-legged neseis seed bug	*Neseis alternatus*	Lygaeidae	HI.
2—Mt. Haleakela neseis seed bug	*Neseis haleakalae*	Lygaeidae	HI.
2—Villosan flightless seed bug	*Nesocryptias villosa*	Lygaeidae	HI.
2—French Frigate Shoal nysius seed bug	*Nysius frigatensis*	Lygaeidae	HI.
2—Fullaway's nysius seed bug	*Nysius fullawayi*	Lygaeidae	HI.
2—Necker goosefoot nysius seed bug	*Nysius neckerensis*	Lygaeidae	HI.
2—Nihoa nysius seed bug	*Nysius nihoae*	Lygaeidae	HI.
2—Necker bunchgrass nysius seed bug	*Nysius suffusus*	Lygaeidae	HI.
2—Bryan's oceanides seed bug	*Oceanides bryani*	Lygaeidae	HI.
2—Perkins' oceanides seed bug	*Oceanides perkinsi*	Lygaeidae	HI.
2—Rough-headed oceanides seed bug	*Oceanides rugosiceps*	Lygaeidae	HI.
2—Dry Creek cliff strider bug	*Oravelia pege*	Macroveliidae	CA.
2—Aaa water treader bug	*Cavaticovelia aaa*	Mesoveliidae	HI.
3A—Phyllostegian leaf bug	*Cyrtopeltis (=Engytatus) phyllostegiae*	Miridae	HI.
2—Lanai kalanian leaf bug	*Kalania hawaiiensis*	Miridae	HI.
2—Oahu kalanian leaf bug	*Kalania* sp. (undescribed)	Miridae	HI.
1—Ash Meadows naucorid bug	*Ambrysus amargosus*	Naucoridae	NV.
2—Amargosa naucorid bug	*Pelocoris shoshone*	Naucoridae	CA, NV.
2—Pulchrus thread bug	*Empicoris pulchrus*	Reduviidae	HI.
2—Ana wingless thread bug	*Nesidiolestes ana*	Reduviidae	HI.
2—Mt. Tantalus wingless thread bug	*Nesidiolestes insularis*	Reduviidae	HI.
2—Robert's wingless thread bug	*Nesidiolestes roberti*	Reduviidae	HI.
2—Selium wingless thread bug	*Nesidiolestes selium*	Reduviidae	HI.
2—Smith's sicellan reduviid bug	*Siacella smithi*	Reduviidae	HI.
2—Annectans rhopalid bug	*Ithamar annectans*	Rhopalidae	HI.
2—Hawiian rhopalid bug	*Ithamar hawaiiense*	Rhopalidae	HI.
CICADAS AND ALLES (Insects, Order Homoptera)			
3A—Red-veined prairie leafhopper	*Felexamia rubranura*	Cicadellidae	IL*.
2—Kauai parti-colored oliarus planthopper	*Oliarus consimilis*	Cixiidae	HI.
2—Oliarus wild cotton planthopper	*Oliarus discrepans*	Cixiidae	HI.
2—Lanai oliarus planthopper	*Oliarus lanaiensis*	Cixiidae	HI.
2—Lihue oliarus planthopper	*Oliarus lihue*	Cixiidae	HI.
2—Barber's Point oliarus planthopper	*Oliarus myoporicola*	Cixiidae	HI.
2—Priolan oliarus planthopper	*Oliarus priola*	Cixiidae	HI.
2—Mt. Tantalus short-wing fern planthopper	*Nesorestias filicicola*	Delphacidae	HI.
2—Iao Valley nesosydne planthopper	*Nesosydne acuta*	Dphacidae	HI.
2—Bridewell's nesosydne planthopper	*Nesosydne bridwelli*	Dphacidae	HI.
2—Nahiku nesosydne planthopper	*Nesosydne cyrtandrae*	Dphacidae	HI.
2—Glenwood nesosydne planthopper	*Nesosydne cyrtandricola*	Dphacidae	HI.
2—Kusche's nesosydne planthopper	*Nesosydne kuschei*	Dphacidae	HI.
2—Diamond Head nesosydne planthopper	*Nesosydne leahi*	Dphacidae	HI.
2—Long-footed nesosydne planthopper	*Nesosydne longipes*	Dphacidae	HI.
2—Keanae nesosydne planthopper	*Nesosydne sulcata*	Dphacidae	HI.
LACEWINGS AND ALLIES (Insects, Order Neuroptera)			
2—Haleakala nesothauman spongillafly	*Nesothauma haleakalae*	Hemerobiidae	HI.
2—Cookes's pseudopsectran spongillafly	*Pseudopsectra cookeorum*	Hemerobiidae	HI.
2—Lobe-wing pseudopsectran spongillafly	*Pseudopsectra lobipennis*	Hemerobiidae	HI.
2—Swezey's pseudopsectran spongillafly	*Pseudopsectra swezeyi*	Hemerobiidae	HI.
2—Usinger's pseudopsectran spongillafly	*Pseudopsectra usingeri*	Hemerobiidae	HI.
2—(No common Name)	*Oliarces clara*	Ithonidae	CA.
2—Molokai antlion	*Eidoleon perjurus*	Myrmeleontidae	HI.
BEETLES (Insects, Order Coleoptera)			
2—Piko anobiid beetle	*Holcobius pikoensis*	Anobiidae	HI.
2—Antioch Dunes anthicid beetle	*Anthicus antiochensis*	Anthicidae	CA.
2—Sacrameto anthicid beetle	*Anthicus sacramento*	Anthicidae	CA.
2—Beller's ground beetle	*Agonum belleri*	Carabidae	WA.
2—Schaum's Blue Ridge ground beetle	*Sphaeroderus schaumi shenandoah*	Carabidae	VA.
3C—Mojave rabbitbrush longhorn beetle	*Crossidius mojavensis mojavensis*	Cerambycidae	CA.
2—Six-banded longhorn beetle	*Dryobius sexnotatus*	Cerambycidae	MD, MS, OH, PA, AL*, AR*, IN*, KS*, KY*, LA*, MI*, MO*, TN*, VA*.
2—Rude's longhorn beetle	*Necydalis rudei*	Cerambycidae	CA.
2—Hawaiian *Plagithymysus* longhorn beetles	*Plagithymysus* (Genus, about 43 spp.)	Cerambycidae	HI.
2*—Bog idol leaf beetle	*Donacia idola*	Chrysomelidae	WA*.
2—Idaho dunes tiger beetle	*Cicindela arenicola*	Cicindelidae	ID.
2*—Cazier's tiger beetle	*Cicindela cazieri*	Cicindelidae	TX*.
2*—Smyth's tiger beetle	*Cicindela chlorocephala smythi*	Cicindelidae	TX*.
2—Columbia River tiger beetle	*Cicindela columbica*	Cicindelidae	ID, WA, OR*.
2—Northeastern beach tiger beetle	*Cicindela dorsalis dorsalis*	Cicindelidae	MA, MD, NY, NJ, RI, PA*.
2*—Oblivious tiger beetle	*Cicindela latesignata obliviosa*	Cicindelidae	CA*.
2—Coral Pink Dunes tiger beetle	*Cicindela limbata albissima*	Cicindelidae	UT.
2—Cobblestone tiger beetle	*Cicindela marginipennis*	Cicindelidae	NH, VT, NY, NJ, OH, PA, WV*, MS.
2—Los Olmos tiger beetle	*Cicindela nevadica olmosa*	Cicindelidae	TX, NM, Mexico?.
2*—Subtropical blue-black tiger beetle	*Circindela nigrocoerula subtropica*	Cicindelidae	TX*.
2*—Neojuvenile tiger beetle	*Cicindela obsoleta neojuvenalis*	Cicindelidae	TX*.
2—Puritan tiger beetle	*Cicindela puritana*	Cicindelidae	MD, CT? MA*, NH*, NH*, VT.
2—Greenest tiger beetle	*Cicindela tranquebarica viridissima*	Cicindelidae	CA.
2—Oahu nesiotes weevil	*Deinocossonus nesiotes*	Curculionidae	HI.
3C—Antioch Dune weevil	*Dysticheus rotundicollis*	Curculionidae	CA.
2—Oahu heteramphus fern weevil	*Heteramphus filicum*	Curculionidae	HI.
2—Nelson's miloderes weevil	*Miloderes nelsoni*	Curculionidae	CA.
2—Rulien's miloderes weevil	*Miloderes rulieni*	Curculionidae	NV.
2—Gifford's nesotocus weevil	*Nesotocus giffordi*	Curculionidae	HI.
2*—Kauai nesotocus weevil	*Nesotocus kauaiensis*	Curculionidae	HI*.
2—Munro's nesotocus weevil	*Nesotocus munroi*	Curculionidae	HI.
2—Lange's El Segundo Dune weevil	*Onchobarus langei*	Curculionidae	CA.
2—Windward Chain *Oodemas* weevils	*Oodemas*, 4 spp.	Curculionidae	HI.
2—Blackburn's pentarthrum weevil	*Pentarthrum blackburni*	Curculionidae	HI.

Category and common name	Scientific name	Family	Distribution
2—Obscure pentarthrum weevil	*Pentarthrum obscura*	Curculionidae	HI.
2—Hawaiian *Rhyncogonus* snout beetles	*Rhyncogonus* (Genus, 23 spp.)	Curculionidae	HI.
2—Nihoa stenotrupis weevil	*Stenotrupis pritchardiae*	Curculionidae	HI.
2*—Brown-tassel trigonoscuta weevil	*Trigonoscuta brunnotesselata*	Curculionidae	CA*.
2—Dorothy's El Segundo Dune weevil	*Trigonoscuta dorothea dorothea*	Curculionidae	CA.
1—Doyen's trigonoscuta dune weevil	*Trigonoscuta doyeni*	Curculionidae	CA.
3A—Fort Ross trigonoscuta weevil	*Trigonoscuta rossi*	Curculionidae	CA*.
3A—Yorba Linda trigonoscuta weevil	*Trigonoscuta yorbalindae*	Curculionidae	CA*.
2—Death Valley agabus diving beetle	*Agabus rumppi*	Dytiscidae	CA, NV?
2*—Fig seed diving beetle	*Desmopachria cenchramis*	Dytiscidae	FL*.
2—Texas cave dytiscid beetle	*Haideoporus texanus*	Dytiscidae	TX.
2*—Elusive hydroporus diving beetle	*Hydroporus elusivus*	Dytiscidae	NH*.
2—Folkerts' hydroporus diving beetle	*Hydroporus folkertsi*	Dytiscidae	AL.
2—Wooly hydroporus diving beetle	*Hydroporus hirsutus*	Dytiscidae	CA.
2—Leech's skyline diving beetle	*Hydroporus leechi*	Dytiscidae	CA.
2—Simple hydroporus diving beetle	*Hydroporus simplex*	Dytiscidae	CA.
2—Spangler's hydroporus diving beetle	*Hydroporus spangleri*	Dytiscidae	UT.
2*—Sulphur Springs hydroporus diving beetle	*Hydroporus sulphurius*	Dytiscidae	AR*.
2—Utah hydroporus diving beetle	*Hydroporus utahensis*	Dytiscidae	UT.
3A—Mono Lake hygrotus diving beetle	*Hygrotus artus*	Dytiscidae	CA*.
2—Curved-foot hygrotus diving beetle	*Hygrotus curvipes*	Dytiscidae	CA.
2—Narrow-foot hygrotus diving beetle	*Hygrotus diversipes*	Dytiscidae	WY.
2—Travertine band-thigh diving beetle	*Hygrotus fontinalis*	Dytiscidae	CA.
2—Sylvan hygrotus diving beetle	*Hygrotus sylvanus*	Dytiscidae	MN, NY*.
2—Hatch's click beetle	*Eanus hatchi*	Elateridae	WA, Canada?
2—Hawaiian *Eopenthes* click beetles	*Eopenthes* (Genus, 17 spp.)	Elateridae	HI.
2—Necker *Itodacnus* click beetles	*Itodacnus*, 2 spp.	Elateridae	HI.
2—Wawona riffle beetle	*Atractelmis wawona*	Elmidae	CA.
2—Parker's cylloepus riffle beetle	*Cylloepus parkeri*	Elmidae	AZ.
2—Brownish dubiraphian riffle beetle	*Dubiraphia brunnescens*	Elmidae	CA.
2—Giuliani's dubiraphian riffle beetle	*Dubiraphia giulianii*	Elmidae	CA.
2—Little dubiraphian riffle beetle	*Dubiraphia parva*	Elmidae	OK, LA.
2—Robust dubiraphian riffle beetle	*Dubiraphia robusta*	Elmidae	WI.
2—Dubiraphian riffle beetle (undescribed)	*Dubiraphia* sp. (undescribed)	Elmidae	ME.
2—Stephan's heterelmis riffle beetle	*Heterelmis stephani*	Elmidae	AZ.
2—Marron's San Carlos riffle bettle	*Huleechius marroni carolus*	Elmidae	AZ.
2—Brown's microcylloepus riffle beetle	*Microcylloepus browni*	Elmidae	MT.
2—Brown's optioservus riffle beetle	*Optioservus browni*	Elmidae	AR.
2—Pinnacles optioservus riffle beetle	*Optioservus canus*	Elmidae	CA.
2—Scott optioservus riffle beetle	*Optioservus phaeus*	Elmidae	KS.
2—Devil's Hole warm spring riffle beetle	*Stenelmis calida calida*	Elmidae	NV.
2—Moapa warm springs riffle beetle	*Stenelmis calida moapa*	Elmidae	NV.
2—Douglas stenelmis riffle beetle	*Stenelmis douglasensis*	Elmidae	MI.
2—Gammon's stenelmis riffle beetle	*Stenelmis gammoni*	Elmidae	NC.
2*—Warm spring zaitzevan riffle beetle	*Zaitzeva thermae*	Elmidae	MT*.
2—Beer's false water penny beetle	*Acneus beeri*	Eubriidae	OR.
2—Burnell's false water penny beetle	*Acneus burnelli*	Eubriidae	OR
2—Stark's false water penny beetle	*Alabameubria starki*	Eubriidae	AL.
2—Variegated false water penny beetle	*Dicranopselaphus variegatus*	Eubriidae	IL.
2*—Dohrn's elegant eucnemid beetle	*Paleoxenus dohrni*	Eucnemidae	CA*.
2—Red Hills unique whirligig betle	*Spanglerogyrus albiventris*	Gyrinidae	AL.
2—Hungerford's crawling water beetle	*Brychius hungerfordi*	Haliplidae	MI.
2*—Disjunct crawling water beetle	*Haliplus nitens*	Haliplidae	TX*, Canada*.
2—Maureen's gymnocthebius minute moss beetle	*Gymnocthebius maureenae*	Hydraenidae	MS.
2—Maureen's hydraenan minute moss beetle	*Hydraena maureenae*	Hydraenidae	VA.
2—Animas limnebius minute moss beetle	*Limnebius aridus*	Hydraenidae	NM.
2—Texas limnebius minute moss beetle	*Limnebius texanus*	Hydraenidae	TX.
2—Utah Limnebius minute moss beetle	*Limnebius utahensis*	Hydraenidae	UT.
2—Wing-shoulder minute moss beetle	*Ochthebius crassalus*	Hydraenidae	CA.
2—Indiana ochthebius minute moss beetle	*Ochthebius putnamensis*	Hydraenidae	IN.
2—Wilbur Springs minute moss beetle	*Ochthebius reticulatus*	Hydraenidae	CA.
2—Leech's chaetarthria water scavenger beetle	*Chaetarthria leechi*	Hydrophilidae	CA.
2—Utah chaetarthrian water scavenger beetle	*Chaetarthria utahensis*	Hydrophilidae	UT.
2—Arizona cymbiodytan water scavenger beetle	*Cymbiodyta arizonica*	Hydrophilidae	AZ.
2—Ricksecker's water scavenger beetle	*Hydrochara rickseckeri*	Hydrophilidae	CA.
2—Seclusive water scavenger beetle	*Paracymus seclusus*	Hydrophilidae	MS.
2—Florida intertidal firefly	*Micronaspis floridana*	Lampyridae	FL.
2—Everglades brownwing firefly	*Photuris brunnipennis floridana*	Lampyridae	FL.
2—Turtle Mound firefly	*Photuris* sp. (undescribed)	Lampyrida	FL.
2—Blind cave leiodid beetle	*Glacicavicola bathysciodes*	Leiodidae	ID.
2—Kauai flightless stag beetle	*Apterocyclus honoluluensis*	Lucanidae	HI.
2—Hopping's blister beetle	*Lytta hoppingi*	Meloidae	CA.
2*—Mojave Desert blister beetle	*Lytta inseperata*	Meloidae	CA*.
2—Anthony blister beetle	*Lytta mirifica*	Meloidae	NM*, Mexico.
2*—Moestan blister beetle	*Lytta moesta*	Meloidae	CA*.
2—Molestan blister beetle	*Lytta molesta*	Meloidae	CA.
2—Morrison's blister beetle	*Lytta morrisoni*	Meloidae	CA.
2—Hawaiian proterhinid beetles	*Proterhinus* (Genus, 72 spp.)	Proterhinidae	HI.
2—Arizona water penny beetle	*Psephenus arizonensis*	Psephenidae	AZ.
2—White Mountains water penny beetle	*Psephenus montanus*	Psephenidae	AZ.
2—Ciervo aegialian scarab beetle	*Aegialia concinna*	Scarabaeidae	CA.
2—Crescent-Dune aegialian scarab beetle	*Aegialia crescenta*	Scarabaeidae	NV.
2—Hardy's aegialian scarab beetle	*Aegialia hardyi*	Scarabaeidae	NV.
2—Large aegialian scarab beetle	*Aegialia magnifica*	Scarabaeidae	NV.
2*—Exiguous anomala scarab beetle	*Anomala exigua*	Scarabaeidae	FL*.
2—Archbold anomala scarab beetle	*Anomala eximia*	Scarabaeidae	FL.
2*—Tibial anomala scarab beetle	*Anomala tibialis*	Scarabaeidae	TX*.
2—Ford's aphodius scarab beetle	*Aphodius fordi*	Scarabaeidae	GA.
2—Aphodius tortoise commensal scarab beetle	*Aphodius troglodytes*	Scarabaeidae	FL, SC.
2—Big Pine Key ataenius dung beetle	*Ataenius superficialis*	Scarabaeidae	FL.
2—Woodruff's ataenius dung beetle	*Ataenius woodruffi*	Scarabaeidae	FL.
2—Copris tortoise commensal scarab beetle	*Copris gopheri*	Scarabaeidae	FL.
2*—Miami roundhead scarab beetle	*Cyclocephala miamiensis*	Scarabaeidae	FL*.
2—Kelso Dune glaresis scarab beetle	*Glaresis arenata*	Scarabaeidae	CA.

Category and common name	Scientific name	Family	Distribution
2—Spiny Florida sandhill scarab beetle	*Gronocarus multispinosus*	Scarabaeidae	FL.
2—White sand bear scarab beetle	*Lichnanthe albopilosa*	Scarabaeidae	CA.
2—Pacific sand bear scarab beetle	*Lichnanthe ursina*	Scarabaeidae	CA.
2—Scrub Island burrowing scarab beetle	*Mycotrupes pedester*	Scarabaeidae	FL.
2—Onthophagus tortoise commensal scarab beetle	*Onthophagus polyphemi*	Scarabaeidae	SC, GA, FL, AL, MS.
2—Ocala burrowing scarab beetle	*Peltotrupes youngi*	Scarabaeidae	FL.
2—Wooly Gulf dune scarab beetle	*Polylamina pubescens*	Scarabaeidae	FL.
2—Saline Valley snow-front scarab beetle	*Polyphylla anteronivea*	Scarabaeidae	CA.
2—Spotted Warner Valley Dunes scarab beetle	*Polyphylla avittata*	Scarabaeidae	UT.
2—Barbate polyphyllan scarab beetle	*Polyphylla barbata*	Scarabaeidae	CA.
2—Atascodero polyphyllan scarab beetle	*Polyphylla nubila*	Scarabaeidae	CA.
2—Andrews' dune scarab beetle	*Pseudocotalpa andrewsi*	Scarabaeidae	CA, Mexico.
2—Giuliani's dune scarab beetle	*Pseudocotalpa giulianii*	Scarabaeidae	NV.
2—Frost's spring serican scarab beetle	*Serica frosti*	Scarabaeidae	FL.
2*—Tantula serican scarab beetle	*Serica tantula*	Scarabaeidae	FL.
2—Scrub palmetto flower scarab beetle	*Trigonopelastes floridana*	Scarabaeidae	FL.
2—Caracara commensal scarab beetle	*Trox howelli*	Scarabaeidae	FL.
2—American burying beetle	*Nicrophorus americanus*	Silphidae	KY, IN, MI, MO, RI, Canada, AL*, AR*, CT*, DC*, FL*, GA*, IA*, IL*, KS*, LA*, MD*, MA*, MN*, MS*, NH*, NJ*, NY*, NC*, OH*, PA*, SC*, SD*, TN*, TX*, VA*, WI*.
2—Black lordithon rove beetle	*Lordithon niger*	Staphylinidae	MO, Canada, AR*, CT*, DC*, GA*, IL*, KS*, KY*, MI*, NY*, NC*, OH*, PA*, TX*, VA*, WV*.
2—Globose dune beetle	*Coelus globosus*	Tenebrionidae	CA, Mexico.
2—San Joaquin dune beetle	*Coelus gracilis*	Tenebrionidae	CA.
FLIES (Insects, Order Diptera)			
2—Mary Alice's small-headed fly	*Eulonchus marialiciae*	Acroceridae	NC.
3A—Valley mydas fly	*Raphiomydas trochilus*	Apioceratidae	CA*.
2*—Antioch cophuran robberfly	*Cophura hurdi*	Asilidae	CA*.
2—Antioch efferian robberfly	*Efferia antiochi*	Asilidae	CA.
2—Hurd's metapogon robberfly	*Metapogon hurdi*	Asilidae	CA.
2—Nihoa two-spotted asteiid fly	*Bryania bipunctata*	Asteiidae	HI.
3A—Ko'olau spurwing long-legged fly	*Campsicnemus mirabilis*	Dolichopodidae	HI*.
3A—Lanai pomace fly	*Drosophila lanaiensis*	Drosophilidae	HI*.
3A—Hawaiian chersodromian dance fly	*Chersodromia hawaiiensis*	Empididae	HI*.
2—Wilber Springs shore fly	*Paracoenia calida*	Ephydridae	CA.
2—Sugarfoot moth fly	*Nemopalpus nearcticus*	Psychodidae	FL.
2—Delong's mixogaster flower fly	*Mixogaster delongi*	Syrphidae	FL.
2*—Ross's apatalestes tabanid fly	*Apatalestes rossi*	Tabanidae	CA*.
2—Florida asaphomyian tabanid fly	*Asaphomyia floridensis*	Tabanidae	FL.
2*—Texas asaphomyian tabanid fly	*Asaphomyia texanus*	Tabanidae	TX*.
2—Belkin's dune tabanid fly	*Brennania belkini*	Tabanidae	CA, Mexico.
2—Brown merycomyian tabanid fly	*Merycomyia brunnea*	Tabanidae	FL.
3A—Volutine stonemyian tabanid fly	*Stonemyia volutina*	Tabanidae	CA*.
BUTTERFLIES AND MOTHS (Insects, Order Lepidoptera)			
3A—Chestnut ermine moth	*Argyresthia castaneela*	Argyresthiidae	NH* VT*.
2—Green heterocrossan carposinid moth	*Heterocrossa viridis*	Carposinidae	HI.
3A—Chestnut case-bearer moth	*Coleophora leucochrysella*	Coleophoridae	PA*.
2—Ioxanthan looper moth	*Fletcherana ioxantha*	Geometridae	HI.
3A—Kona giant looper moth	*Scotorythra megalophylla*	Geometridae	HI*.
3A—Ko'olau giant looper moth	*Scotorythra nesiotes*	Geometridae	HI*.
3A—Hawaiian hopseed looper moth	*Scotorythra paratactis*	Geometridae	HI*.
3A—'Ola'a peppered looper moth	*Tritocleis microphylla*	Geometridae	HI*.
2—Necker petrochroan leaf miner moth	*Petrochroa neckerensis*	Gracilariidae	HI*.
2—Dakota skipper butterfly	*Hesperia dacotae*	Hesperiidae	MN, IA, SD, ND, IL*, Canda.
1—North Platte montane skipper butterfly	*Hesperia leonardus (=pawnee) montana*	Hesperiidae	CO.
2—Salt marsh skipper butterfly	*Panoquina panoquinoides errans*	Hesperiidae	CA, Mexico.
2—Rare skipper	*Problema bulenta*	Hesperiidae	MD, VA, NC. SC. GA.
2—Wandering skipper	*Pseudocopaeodes eunus eunus*	Hesperiidae	CA, NV?, AZ?, Mexico?.
2—Atala butterfly	*Eumaeus atala florida*	Lycaenidae	FL.
2—Comstock's blue butterfly	*Euphilotes (=Shijimiaeoides =Philotes) battoides comstocki.*	Lycaenidae	CA.
2—Langston's blue butterfly	*Euphilotes (=Shijimiaeoides) langstoni langstoni*	Lycaenidae	CA.
2—Mattoni's blue butterfly	*Euphilotes (=Shijimiaeoides) rita mattonii*	Lycaenidae	NV.
3A—Xerces blue butterfly	*Glaucopsyche xerces*	Lycaenidae	CA*.
2—Miami blue butterfly	*Hemiargus thomasi bethune-bakeri*	Lycaenidae	FL.
2—Moro Bay blue butterfly	*Icaricia icarioides moroensis*	Lycaenidae	CA.
3A—Pheres blue butterfly	*Icaricia icarioides pheres*	Lycaenidae	CA*.
3C—Bog elfin butterfly	*Incisalia (=Callophrys =Mitoura) lanoraieenisis*	Lycaenidae	CA.
3C—Wind's elfin butterfly	*Incisalia (=Callophrys =Mitora) mossi windi*	Lycaenidae	CA.
3C—Doudoroff's elfin butterfly	*Incisalia (=Callophrys =Mitoura) mossi doudoroffi*	Lycaenidae	CA.
2—Karner blue butterfly	*Lycaeides melissa samuelis*	Lycaenidae	NY, NH, IN, MI, WI, MA*, PA*.
3C—Clouded tailed copper butterfly	*Lycaena arota nubila*	Lycaenidae	CA.
2—Clayton's copper butterfly	*Lycaena dorcas claytoni*	Lycaenidae	ME.
2—Hermes copper butterfly	*Lycaena hermes*	Lycaenidae	CA, MEX.
3C—Hessel's hairstreak butterfly	*Mitoura (=Callophrys) hesseli*	Lycaenidae	NH, MA, NJ, MD, VA, NC, GA, FL.
2—Boharts' blue butterfly	*Philotiella (=Zizeeria) speciosa bohartorum*	Lycaenidae	CA.
2—San Emigdio blue butterfly	*Plebulina (=Plebejus) emigdionis*	Lycaenidae	CA.
2—Bartram's hairstreak butterfly	*Strymon acis bartrami*	Lycaenidae	FL.
3C—Hawaiian hairstreak butterfly	*Vaga blackburni*	Lycaenidae	HI.
3C—Kendall's yucca skipper butterfly	*Magathymus coloradensis kendalli*	Magathymidae	TX.
2—Maculated manfreda skipper butterfly	*Stallingsia maculosus*	Magathymidae	TX.
3A—American chestnut nepticulid moth	*Ectodemia castaneae*	Nepticulidae	MD*.
3A—Phleophagan chestnut nepticulid moth	*Ectodemia phleophaga*	Nepticulidae	MD*.
2—Albarufan dagger moth	*Acronicta albarufa*	Noctuidae	MA*, GA*, NJ*, CT*, NY*, PA*, OH*, CO*, NM*, Canada*.
3A—"Poko" noctuid moth	*Agrotis crinigera*	Noctuidae	HI*.
3A—Midway agrotis noctuid moth	*Agrotis fasciata*	Noctuidae	HI*.
3A—Kerr's agrotis noctuid moth	*Agrotis kerri*	Noctuidae	HI*.
3A—Laysan agrotis noctuid moth	*Agrotis laysanensis*	Noctuidae	HI*.
3A—Procellaris agrotis noctuid moth	*Agrotis procellaris*	Noctuidae	HI*.

Category and common name	Scientific name	Family	Distribution
2—Marbled underwing moth	*Catocala marmorata*	Noctuidae	NC, SC, VT*, NY*, NJ*, PA*, VA*, KY*, IN*, IL*, MO*.
2—Precious underwing moth	*Catocala pretiosa*	Noctuidae	NJ, NH*, CT*, MA*, NY*, PA*, MD*, VA*, TN*.
2—Hebard's noctuid moth	*Erythroecia hebardi*	Noctuidae	NJ, VA.
3A—Confused helicoverpan noctuid moth	*Helicoverpa confusa*	Noctuidae	HI*.
3A—Minute helicoverpan noctuid moth	*Helcoverpa minuta*	Noctuidae	HI*.
3A—Laysan dropseed noctuid moth	*Hypena laysanensis*	Noctuidae	HI*.
3A—Hilo hypenan noctuid moth	*Hypena newelli*	Noctuidae	HI*.
3A—Lovegrass noctuid moth	*Hypena plagiota*	Noctuidae	HI*.
3A—Kaholuamano noctuid moth	*Hypena senicula*	Noctuidae	HI*.
2—Lemmer's noctuid moth	*Lithophane lemmeri*	Noctuidae	CT, NJ, NC, SC.
2—Ceromatic noctuid moth	*Pyreferra ceromatica*	Noctuidae	FL, SC, ME, AL, IN, Canada.
2—Florida leafwing butterfly	*Anaea floridalis*	Nymphalidae	FL.
1—Uncompahgre fritillary butterfly	*Boloria acrocnema*	Nymphalidae	CO.
3C—Alamosa satyr butterfly	*Cercyonis meadi alamosa*	Nymphalidae	CO.
3A—Sthenele wood nymph butterfly	*Cercyonis sthenele sthenele*	Nymphalidae	CA*.
1—Bay checkerspot butterfly	*Euphydryas editha bayensis*	Nymphalidae	CA.
2—Mono checkerspot butterfly	*Euphydryas editha monoensis*	Nymphalidae	CA/NV.
3C—Wright's checkerspot butterfly	*Euphydryas editha wrigthi*	Nymphalidae	CA.
3C—Obsolete viceroy butterfly	*Limenitis archippus obsoletus*	Nymphalidae	AZ, CA, NM, NV, Mexico.
3C—Mitchell satyr butterfly	*Neonympha (=Euptychia) mitchelli*	Nymphalidae	MI, NJ, OH*.
3C—Chryxus arctic butterfly	*Oeneis chryxus valerata*	Nymphalidae	WA.
2—Tawny crescent butterfly	*Phyciodes batesi*	Nymphalidae	NC, VA, NY, OH, MI, WI, ND, SD, CO, MN, Canada, GA*, WV*, PA*, NJ*.
3C—Minute checkerspot butterfly	*Poladryas minuta*	Nymphalidae	TX.
2—Smoky eyed brown butterfly	*Satyrodes eurydice fumosa*	Nymphalidae	IL, IA, NE, CO*.
3C—Unsilvered fritillary butterfly	*Speyeria adiaste adiaste*	Nymphalidae	CA.
3A—Atossa fritillary butterfly	*Speyeria adiaste atossa*	Nymphalidae	CA*.
3C—Clemence's fritillary butterfly	*Speyeria adiaste clemencei*	Nymphalidae	CA.
2—Calippe silverspot butterfly	*Speyeria callipe callipe*	Nymphalidae	CA.
3C—Tehachapi Mountain silverspot butterfly	*Speyeria egleis tehachapina*	Nymphalidae	CA.
2*—Hydaspe fritillary butterfly	*Speyeria hydaspe conquista*	Nymphalidae	CO, NM.
2—Regal fritillary butterfly	*Speyeria idalia*	Nymphalidae	ME*, NH*, MA, RI, NY, NJ, MD, DE, VA, NC*, WV, PA, OH, IN, MI, IL, MO, MN, WI, IA, OK, KS, NE, SD, ND, CO, MT, Canada.
3C—Apache silverspot butterfly	*Speyeria nokomis apacheana*	Nymphalidae	CA/NV.
2—Blue silverspot butterfly	*Speyeria nokomis caerulescens*	Nymphalidae	AZ*, Mexico.
3B—Blue-black silverspot butterfly	*Speyeria nokomis nigrocaerulea*	Nymphalidae	AZ, NM.
3C—Mountain silverspot butterfly	*Speyeria nokomis nitocris*	Nymphalidae	AZ, NM, CO.
2—Great basin silverspot butterfly	*Speyeria nokomis nokomis*	Nymphalidae	CO, UT.
2—Behren's silverspot butterfly	*Speyeria zerene behrensii*	Nymphalidae	CA.
2—Myrtle's silverspot butterfly	*Speyeria zerene myrtleae*	Nymphalidae	CA.
2—Henne's eucosman moth	*Eucosma hennei*	Olethreutidae	CA.
2—San Francisco tree lupine moth	*Grapholita edwardsiana*	Olethreutidae	CA.
3A—Strohbeen's parnassian butterfly	*Parnassius clodius strohbeeni*	Papilionidae	CA*.
2—Busk's gall moth	*Carolella busckana*	Phaloniidae	CA.
3C—Catalina orange tip butterfly	*Anthocharis cethura cataline*	Pieridae	CA.
2—Andrew's marble butterfly	*Euchloe hyantis andrewsi*	Pieridae	CA.
3C—Helios yellow butterfly	*Eurema dina helios*	Pieridae	PR, Caribbean.
2*—Molokai sedge hedyleptan moth	*Hedylepta anastrepta*	Pyralidae	HI*.
2—Kohala Mountain sedge hedyleptan moth	*Hedylepta anastreptoides*	Pyralidae	HI.
2*—'Ohe hedyleptan moth	*Hedylepta asaphombra*	Pyralidae	HI*.
3A—Oahu swamp hedyleptan moth	*Hedylepta epicentra*	Pyralidae	HI*.
2*—Ola'a banana hedeleptan moth	*Hedylepta euryprora*	Pyralidae	HI*.
2*—Fullaway's banana hedyleptan moth	*Hedylepta fullawayi*	Pyralidae	HI*.
2*—Giffard's 'ohe hedyleptan moth	*Hedylepta giffardi*	Pyralidae	HI*.
2*—Kilauea pa'iniu hedyleptan moth	*Hedylepta iridias*	Pyralidae	HI*.
3A—Laysan hedyleptan moth	*Hedylepta laysanensis*	Pyralidae	HI*.
2*—Meyrick's banana hedyleptan moth	*Hedylepta meyricki*	Pyralidae	HI*.
2*—Hawaiian bean leafroller moth	*Hedylepta monogona*	Pyralidae	HI*.
2*—Maui banana hedyleptan moth	*Hedylepta musicola*	Pyralidae	HI*.
2*—Hawaiian lo'ulu hedeleptan moth	*Hedylepta pritchardii*	Pyralidae	HI*.
3A—Telegraphic hedyleptan moth	*Hedylepta telegrapha*	Pyralidae	HI*.
2—Blue margaronian moth	*Margaronia cyanomichla*	Pyralidae	HI.
2—Green margaronian moth	*Margaronia exaula*	Pyralidae	HI.
2—'Ohenaupaka oeobian moth	*Oeobia dryadopa*	Pyralidae	HI.
2*—Chestnut clearwing moth	*Synanthedon castaneae*	Sesiidae	VA*, PA*, SC*, ME*, MS*, NY*.
1—Weist's sphinx moth	*Euproserpinus wiesti*	Sphingidae	CO, NM*.
3A—Blackburn's sphinx moth	*Manduca blackburni*	Sphingidae	HI*.
3A—Fabulous green sphinx of Kauai	*Tinostoma smaragditis*	Sphingidae	HI*.
3A—Chestnut leaf miner moth	*Tischeria perplexa*	Tischeriidae	VA*.
2—'Ohe'ohe leaf roller moth	*Spheterista oheoheana*	Tortricidae	HI.
2—Green-banded 'ohe'ohe leaf-roller moth	*Spheterista pterotropiana*	Tortricidae	HI.
2—Wailupo leaf-roller moth	*Spheterista reynoldsiana*	Tortricidae	HI.
CADDISFLIES (Insects, Order Trichoptera)			
2—Mt. Hood primitive brachycentrid caddisfly	*Eobrachycentrus gelidae*	Brachycentridae	OR.
2—Artesian agapetus caddisfly	*Agapetus artesus*	Glossosomatidae	MO.
2*—Denning's agapetus caddisfly	*Agapetus denningi*	Glossosomatidae	OR*.
2—Arkansas agapetus caddisfly	*Agapetus medicus*	Glossosomatidae	AR.
2—San Marcos protoptilan caddisfly	*Protoptila arca*	Glossosomatidae	TX.
2—Flint's cheumatopsyche caddisfly	*Cheumatopsyche flinti*	Hydropsychidae	TX.
2—Helma's cheumatopsyche caddisfly	*Cheumatopsyche helma*	Hydropsychidae	TN.
2—Vannote's cheumatopsyche caddisfly	*Cheumatopsyche vannotei*	Hydropsychidae	PA.
2—California diplectronan caddisfly	*Diplectrona californica*	Hydropsychidae	CA.
2—Schuh's homoplectran caddisfly	*Homoplectra schuhi*	Hydropsychidae	OR.
2—Buffalo Springs caddisfly	*Hydropsyche etnieri*	Hydropsychidae	TN.
2—Abellan hydropsyche caddisfly	*Hydropsyche reiseni*	Hydropsychidae	OR.
2—Reisen's hydropsyche caddisfly	*Hydropsyche reiseni*	Hydropsychidae	OK.
2*—King's Creek parapsyche caddisfly	*Parapsyche extensa*	Hydropsychidae	CA*.
2—Knoxville hydroptilan micro caddisfly	*Hydroptila decia*	Hydroptilidae	TN.
2—Kite's neotrichian micro caddisfly	*Neotrichia kitae*	Hydroptilidae	MO.
2—Alsea ochrotrichian micro caddisfly	*Ochrotrichia alsea*	Hydroptilidae	OR.
2—Contorted ochrotrichian micro caddisfly	*Ochrotrichia contorta*	Hydroptilidae	MO, AR.

Category and common name	Scientific name	Family	Distribution
2—Deschutes ochrotrichian micro caddisfly	*Ochrotrichia phenosa*	Hydroptilidae	OR*, CA.
2—Provost's ochrotrichian micro caddisfly	*Ochrotrichia provosti*	Hydroptilidae	FL.
2—Vertrees's ochrotrichian micro caddisfly	*Ochrotrichia vertreesi*	Hydroptilidae	OR, CA.
2—Florida oxyethiran micro caddisfly	*Oxyethira florida*	Hydroptilidae	FL.
2—Fischer's lepidostoman caddisfly	*Lepidostoma fischeri*	Lepidostomatidae	OR.
2—Goeden's lepidostoman caddisfly	*Lepidostoma goedeni*	Lepidostomatidae	OR.
2—Vertrees's ceraclean caddisfly	*Ceraclea (=Athripsodes) vertreesi*	Leptoceridae	OR.
2*—Florida ceraclean longhorn caddisfly	*Ceraclea floridana*	Leptoceridae	FL*.
2*—Little oecetis longhorn caddisfly	*Oecetis parva*	Leptoceridae	FL*.
3A—Athens triaenodes caddisfly	*Triaenodes phalacris*	Leptoceridae	OH*.
2*—Three-tooth triaenodes caddisfly	*Triaenodes tridonta*	Leptoceridae	OK*, FL*.
2—Cascades apatanian caddisfly	*Apatania (=Radema) tavala*	Limnephilidae	OR.
2—Kings Canyon cryptochian caddisfly	*Cryptochia excella*	Lemnephilidae	CA.
2—Blue Mountains cryptochian caddisfly	*Cryptochia neosa*	Limnephilidae	OR.
2—King's Creek ecclisomyian caddisfly	*Ecclisomyia bilera*	Limnephilidae	CA.
2*—Green Springs Mountain farulan caddisfly	*Farula davisi*	Limnephilidae	OR*.
2—Mt. Hood farulan caddisfly	*Farula jewetti*	Limnephilidae	OR.
2—Tombstone Prairie farulan caddisfly	*Farula reaperi*	Limnephilidae	OR.
2—Missouri Glyphopsyche caddisfly	*Glyphopsyche missouri*	Limnephilidae	MO.
2—Klamath limnephilan caddisfly	*Limnephilus alconura*	Limnephilidae	OR.
2—Fort Dick limnephilus caddisfly	*Limnephilus atercus*	Limnephilidae	CA, OR.
2—Columbia gorge neothremman caddisfly	*Neothremma andersoni*	Limnephilidae	OR.
2—Tombstone Prairie oligophlebodes caddisfly	*Oligophlebodes mostbento*	Limnephilidae	OR.
2*—Clatsop philocascan caddisfly	*Philocasca oron*	Limnephilidae	OR*.
3B—(No common name)	*Psilotreta hansoni*	Odontoceridae	MA.
2—Oregon dolophilodes caddisfly	*Dolophilodes (=Sortosa) oregona*	Philoptamidae	OR.
2—Carlson's polycentropus caddisfly	*Polycentropus carlsoni*	Polycentropodidae	SC.
2—Nearctic paduniellan caddisfly	*Paduniella nearctica*	Psychomyidae	AR.
2—Siskiyou caddisfly	*Tinodes siskiyou*	Psychomyiidae	OR.
2—Alexander's rhyacophilan caddisfly	*Rhyacophila alexanderi*	Rhyacophilidae	MT.
3A—Castle Lake rhyacophilan caddisfly	*Rhyacophila amabilis*	Rhyacophilidae	CA*.
2—Obrien rhyacophilan caddisfly	*Rhyacophila colonus*	Rhyacophilidae	OR.
2—Fender's rhyacophilan caddisfly	*Rhyacophila fenderi*	Rhyacophilidae	OR.
2—Haddock's rhyacophilan caddisfly	*Rhyacophila haddocki*	Rhyacophilidae	OR.
2—Castle Crags rhyacophilan caddisfly	*Rhyacophila lineata*	Rhyacophilidae	CA.
2—Bilobed rhyacophilan caddisfly	*Rhyacophila mosana*	Rhyacophilidae	CA.
2—Spiny rhyacophilan caddisfly	*Rhyacophila spinata*	Rhyacophilidae	CA.
2—One-spot rhyacophilan caddisfly	*Rhyacophila unipunctata*	Rhyacophilidae	OR.
2—Zigzag blackwater caddisfly	*Agarodes ziczac*	Sericostomatidae	FL.
ANTS, BEES AND WASPS (Insects, Order Hymenoptera)			
2*—Yellow-banded andrenid bee	*Perdita hirticeps luteocincta*	Andrenidae	CA*.
2—Antioch andrenid bee	*Perdita scitula antiochensis*	Andrenidae	CA.
2—Nihoa sclerodermus wasp	*Sclerodermus nihoaensis*	Bethylidae	HI.
2—Nihoa eupelmus wasp	*Eupelmus nihoaensis*	Eupelmidae	HI.
2*—Assimulans yellow-faced bee	*Nesoprosophis assimulans*	Hylaeidae	HI*.
2*—Andrenoid yellow-faced bee	*Nesoprosopis andrenoides*	Hylaeidae	HI*.
3A—Lanai yellow-faced bee	*Nesoprosopis angustula*	Hylaeidae	HI*.
2—Anomalous yellow-faced bee	*Nesoprosopis anomala*	Hylaeidae	HI.
2*—Anthricinan yellow-faced bee	*Nesoprosopis anthricina*	Hylaeidae	HI*.
3A—Blackburn's yellow-faced bee	*Nesoprosopis blackburni*	Hylaeidae	HI*.
2*—Bluewing yellow-faced bee	*Nesoprosopis caeruleipennis*	Hylaeidae	HI*.
2*—Chlorostictan yellow-faced bee	*Nesoprosopis chlorosticata*	Hylaeidae	HI*.
2*—Comes yellow-faced bee	*Nesoprosopis comes*	Hylaeidae	HI*.
2*—Conehead yellow-faced bee	*Nesoprosopis coniceps*	Hylaeidae	HI*.
3A—Connected yellow-faced bee	*Nesoprosopis connectens*	Hylaeidae	HI*.
2*—Crabronoid yellow-faced bee	*Nesoprosopis crabronoides*	Hylaeidae	HI*.
2*—Difficult yellow-faced bee	*Nesoprosopis difficilis*	Hylaeidae	HI*.
2*—Dimidiatan yellow-faced bee	*Nesoprosopis dimidiata*	Hylaeidae	HI*.
3A—Erythrodeme yellow-faced bee	*Nesoprosopis erythrodemas*	Hylaeidae	HI*.
2*—Easy yellow-faced bee	*Nesoprosopis facilis*	Hylaeidae	HI*.
2*—Fern yellow-faced bee	*Nesoprosopis filicum*	Hylaeidae	HI*.
3A—Finitiman yellow-faced bee	*Nesoprosopis finitima*	Hylaeidae	HI*.
2—Very yellow-faced bee	*Nesoprosopis flavifrons*	Hylaeidae	HI.
2*—Yellow-foot yellow-faced bee	*Nesoprosopis flavipes*	Hylaeidae	HI*.
2—Darkwing yellow-faced bee	*Nesoprosopis fuscipennis*	Hylaeidae	HI.
2*—Shadowfoot darkwing yellow-faced bee	*Nesoprosopis fuscipennis obscuripes*	Hylaeidae	HI*.
2*—Haleakala yellow-faced bee	*Nesoprosopis haleakalae*	Hylaeidae	HI*.
3A—Hilaris yellow-faced bee	*Nesoprosopis hilaris*	Hylaeidae	HI*.
2*—Hirsute yellow-faced bee	*Nesoprosopis hirsutula*	Hylaeidae	HI*.
3A—Monocolor yellow-faced bee	*Nesoprosopis homeochroma*	Hylaeidae	HI*.
2*—Hostile yellow-faced bee	*Nesoprosopis hostilis*	Hylaeidae	HI*.
2*—Hulan yellow-faced bee	*Nesoprosopis hula*	Hylaeidae	HI*.
2*—Insignis yellow-faced bee	*Nesoprosopis insiginis*	Hylaeidae	HI*.
2*—Kauai yellow-faced bee	*Nesoprosopis kauaiensis*	Hylaeidae	HI*.
2*—Koa yellow-faced bee	*Nesoprosopis koae*	Hylaeidae	HI*.
2*—Kona yellow-faced bee	*Nesoprosopis kona*	Hylaeidae	HI*.
2*—Laetan yellow-faced bee	*Nesoprosopis laeta*	Hylaeidae	HI*.
3A—Broadhead yellow-faced bee	*Nesoprosopis laticeps*	Hylaeidae	HI*.
2*—Longhead yellow-faced bee	*Nesoprosopis longiceps*	Hylaeidae	HI*.
3A —Maui yellow-faced bee	*Nesoprosopis mauiensis*	Hylaeidae	HI*.
3A—Melanothix yellow-faced bee	*Nesoprosopis melanothrix*	Hylaeidae	HI*.
3A—Mutatan yellow-faced bee	*Nesoprosopis mutata*	Hylaeidae	HI*.
3A—Molokai yellow-faced bee	*Nesoprosopis neglecta*	Hylaeidae	HI*.
3A—Snowy yellow-faced bee	*Nesoprosopis nivalis*	Hylaeidae	HI*.
2*—Obscuratan yellow-faced bee	*Nesoprosopis obscurata*	Hylaeidae	HI*.
2*—Ombrias yellow-faced bee	*Nesoprosopis ombrias*	Hylaeidae	HI*.
3A—Pele yellow-faced bee	*Nesoprosopis pele*	Hylaeidae	HI*.
2—Perkin's yellow-faced bee	*Nesoprosopis perkinsiana*	Hylaeidae	HI*.
3A—Perspicuan yellow-faced bee	*Nesoprosopis perspicua*	Hylaeidae	HI*.
3A—Psammobian yellow-faced bee	*Nesoprosopis psammobia*	Hylaeidae	HI*.
2*—Furry yellow-faced bee	*Nesoprosopis pubescens*	Hylaeidae	HI*.
2*—Redtail yellow-faced bee	*Nesoprosopis rubrocaudatus*	Hylaeidae	HI*.

Category and common name	Scientific name	Family	Distribution
3A—Rugulose yellow-faced bee	*Nesoprosopis rugulosa*	Hylaeidae	HI*.
2*—Satellus yellow-faced bee	*Nesoprosopis satellus*	Hylaeidae	HI*.
2*—Bristlefront yellow-faced bee	*Nesoprosopis setosifrons*	Hylaeidae	HI*.
2*—Simple yellow-faced bee	*Nesoprosopis simplex*	Hylaeidae	HI*.
2*—Specular yellow-faced bee	*Nesoprosopis specularis*	Hylaeidae	HI*.
2*—Sphecodoid yellow-faced bee	*Nasoprosopis sphecodoides*	Hylaeidae	HI*.
2*—Unique yellow-faced bee	*Nesoprosopis unica*	Hylaeidae	HI*.
2*—Vicinan yellow-faced bee	*Nesoprosopis vicina*	Hylaeidae	HI*.
2—Volatile yellow-faced bee	*Nesoprosopis volatilis*	Hylaeidae	HI*.
2—Anticoh mutilid wasp	*Myrmosula (Myrmosa) pacifica*	Multillidae	CA.
2—Hawaiian deinomimesan sphecid wasp	*Deinomimesa hawaiiensis*	Shpecidae	HI.
2—Puna deinomimesan sphecid wasp	*Deinomimesa punae*	Sphecidae	HI.
2—Giffard's ectemnius sphecid wasp	*Ectemnius (=Nesocrabo) giffardi*	Sphecidae	HI.
2—Short-foot ectemnius sphecid wasp	*Ectemnius (=Oreocrabro) curtipes*	Sphecidae	HI.
2—Haleakala ecteminus sphecid wasp	*Ectemnius (=Oreocrabro) haleakalae*	Sphecidae	HI.
2—Brown cross ectemnius sphecid wasp	*Ectemnius (=Orocrabro) fulvicrus*	Sphecidae	HI.
2—Bidecoratus sphecid wasp	*Ectemnius nesocrabo bidecoratus*	Sphecidae	HI.
2—Redheaded sphecid wasp	*Eucerceris ruficeps*	Sphecidae	CA*, NV.
2—Kauai nesomimesan sphecid wasp	*Nesomimesa kauaiensis*	Sphecidae	HI.
2—Shade-winged nesomimesan sphecid wasp	*Nesomimesa sciopteryx*	Sphecidae	HI.
2*—Antioch sphecid wasp	*Philanthus nasalis*	Sphecidae	CA*.
2—Niihau odynerus vespid wasp	*Odynerus niihauensis*	Vespidae	HI.
2—Soror odynerus vespid wasp	*Odynerus soror*	Vespidae	HI.
SNAILS (Molluscs, Class Gastropoda)			
2—(No common name)	*Valvata utahensis* Call, 1884	Valvatidae	ID, UT:
3B—Newcomb's littorine snail	*Algamorda newcombiana* Hemphill, 1877	Littorinidae	CA, WA, OR.
2—St. George snail	*Amnicola deserta* Pilsbry, 1916	Hydrobiidae	UT.
2—(No common name)	*Aphaostracon asthenes* Thompson, 1968	Hydrobiidae	FL.
2—(No common name)	*Aphaostracon monas* (Pilsbry, 1899)	Hydrobiidae	FL.
2—Compact hydrobe snail	*Aphaostracon pycnus* Thompson, 1968	Hydrobiidae	FL.
2—Fenney Spring hydrobe snail	*Aphaostracon xynoelictus* Thompson, 1968	Hydrobiidae	FL.
2—Helicoid spring snail	*Cincinnatia helicogyra* Thompson, 1968	Hydrobiidae	FL.
2—(No common name)	*Cincinnatia mica* Thompson, 1968	Hydrobiidae	FL.
2—(No common name)	*Cincinnatia monroensis* (Dall, 1885)	Hydrobiidae	FL.
2—(No common name)	*Cincinnatia parva* Thompson, 1968	Hydrobiidae	FL.
2—Ponderous spring snail	*Cincinnatia ponderosa* Thompson, 1968	Hydrobiidae	FL.
2—Seminole Spring snail	*Cincinnatia vanhyningi* (Vanatta, 1934)	Hydrobiidae	FL.
2—Wekiwa Spring snail	*Cincinnatia wekiwae* Thompson, 1968	Hydrobiidae	FL.
2—Phantom cave snail	*Cochliopa texana* Pilsbry, 1935	Hydrobiidae	TX.
2—Muddy Valley turban snail	*Fluminicola avernalis* Pilsbry, 1935	Hydrobiidae	NV.
1—Point of Rocks Spring snail	*Fluminicola erythropoma* Pilsbry, 1899	Hydrobiidae	NV.
2—Pahranagat Valley turban snail	*Fluminicola merriami* Pilsbry and Belcher, 1892	Hydrobiidae	NV.
2—Longstreet Spring snail	*"Fluminicola"* sp.	Hydrobiidae	NV.
1—Median-gland Nevada spring snail	*"Fluminicola"* sp.	Hydrobiidae	NV.
1—Large-gland Nevada spring snail	*"Fluminicola"* sp.	Hydrobiidae	NV.
3C—(No common name)	*Fontelicella micrococcus* (Pilsbry, 1893)	Hydrobiidae	NV.
1—Socorro spring snail	*Fontelicella (=Amnicola) neomexicana* (Pilsbry, 1916).	Hydrobiidae	NM.
2—Elk Island snail	*Fontelicella robusta* Walker, 1908	Hydrobiidae	WY.
1—Chupadera spring snail	*Fontelicella* sp.	Hydrobiidae	NM.
1—Roswell spring snail	*Fontelicella* sp.	Hydrobiidae	NM.
2—Great Columbia River spire snail	*Lithoglyphus columbianus* (Pilsbry, 1899)	Hydrobiidae	ID, OR, WA.
2—(No common name)	*Marstonia agarhecta* Thompson, 1977	Hydrobiidae	GA.
2—(No common name)	*Marstonia castor* Thompson, 1977	Hydrobiidae	GA.
2—Obese Marstonia snail	*Marstonia ogmoraphe* Thompson, 1977	Hydrobiidae	TN.
2—Thick-shelled Marstonia snail	*Marstonia pachyta* Thompson, 1977	Hydrobiidae	AL.
2—(No common name)	*Somatogyrus catanotus* Thompson, 1974	Hydrobiidae	GA.
2—(No common name)	*Somatogyrus parvulus* Tryon, 1865	Hydrobiidae	TN.
2—(No common name)	*Somatogyrus tenax* Thompson, 1969	Hydrobiidae	GA.
2—(No common name)	*Stiobia nana* Thompson, 1978	Hydrobiidae	AL.
1—Virile Amargosa snail	Genus and species undescribed	Hydrobiidae	NV.
1—Bliss Rapids snail	Genus and species undescribed	Hydrobiidae	ID.
1—Bruneau Hot Spring snail	Genus and species undescribed	Hydrobiidae	ID.
2—Cheatum's snail	*Tryonia cheatumi* (Pilsbry, 1935)	Littoridinidae	TX.
2—White River snail	*Tryonia clathrata* Stimpson, 1865	Littoridinidae	NV.
2—California brackish water snail	*Tryonia imitator* (Pilsbry, 1899)	Littoridinidae	CA.
1—Point of Rocks Tryonia snail	*Tryonia* sp.	Littoridinidae	NV.
1—Sportinggoods Tryonia snail	*Tryonia* sp.	Littoridinidae	NV.
1—Small solid Tryonia snail	*Tryonia* sp.	Littoridinidae	NV.
1—Minute slender Tryonia snail	*Tryonia* sp.	Littoridinidae	NV.
2—Amargosa Tryonia snail	*Tryonia* sp.	Littoridinidae	NV.
1—Alamosa spring snail	*Tryonia* sp.	Littoridinidae	NM.
2—Badwater snail	*Assiminea infima* Berry, 1947	Assimineidae	CA.
1—Pecos assiminea snail	*Assiminea* sp.	Assimineidae	NM, TX.
2—Anthony's river snail	*Athearnia anthonyi* (Redfield, 1854)	Pleuroceridae	TN.
2—Crass River snail	*Athearnia crassa* (Haldeman, 1841)	Pleuroceridae	GA, TN.
2—Albany snail	*Goniobasis albanyensis* Lea, 1864	Pleuroceridae	AL, FL, GA.
3B—Indiana river snail	*Goniobasis, semicarinata indianensis* Pilsbry, 1903	Pleuroceridae	IN.
1—Spiny river snail	*Io fluvialis* (Say, 1834)	Pleuroceridae	TN, VA.
2—Mainstream river snail	*Leptoxis praerosa* (Say, 1824)	Pleuroceridae	TN.
3B—Umbilicate river snail	*Leptoxis subglobosa umbilicata* (Weatherby, 1876)	Pleuroceridae	TN.
2—Armigerous river snail	*Lithasia armigera* (Say, 1821)	Pleuroceridae	KY.
2—Dutton's river snail	*Lithasia duttoniana* (Lea, 1841)	Pleuroceridae	TN.
2—Geniculate River snail	*Lithasia geniculata* Haldeman, 1840	Pleuroceridae	TN.
2—Jay's river snail	*Lithasia jayana* (Lea, 1841)	Pleuroceridae	TN.
2—Elk River file snail	*Lithasia lima* (Conrad, 1834)	Pleuroceridae	TN, AL.
3C—Small geniculate river snail	*Lithasia pinguis* (Lea, 1852)	Pleuroceridae	TN.
2—Rugged river snail	*Lithasia salebrosa* (Conrad, 1834)	Pleuroceridae	TN.
2—Verrucose file snail	*Lithasia verrucosa* (Rafinesque, 1820)	Pleuroceridae	TN
2—Giant Columbia River limpet	*Fisherola nuttalli* (Haldeman, 1841)	Lancidae	ID, OR, WA.
2—Utah band snail	*Lymnaea kingii* Meek, 1877 (=*Stagnicola utahensis* Call, 1844).	Lymnaeidae	UT.
2—Fish Springs pond snail	*Stagnicola pilsbryi* (Hemphill, 1890)	Lymnaeidae	UT.

Category and common name	Scientific name	Family	Distribution
2—Cape Fear ramshorn snail	Helisoma magnificum (Pilsbry, 1903)	Planorbidae	NC.
2—Jackson Lake snail	Helisoma (=Carinifex) jacksonense Henderson, 1932.	Planorbidae	WY.
2—Greenfield ramshorn snail	Taphius eucosmius eucosmius Bartsch, 1908	Planorbidae	NC.
1—New Mexico ramshorn snail	Genus and Species undescribed	Planorbidae	NM.
2—Wyoming cave snail	Physa spelunca (Turner & Clench, 1974)	Physidae	WY.
2—Utah bubble snail	Physa utahensis Clench, 1925	Physidae	UT.
3B—Diamond-Y pond snail	Physa virgata bottomeri Clench, 1924	Physidae	TX.
2—Zion Canyon snail	Physa zionis Pilsbry, 1905	Physidae	UT.
1—Snake River physa snail	Physa sp.	Physidae	ID.
2—Fish Lake snail	Stenophysa (=Aplexa) microstriata (Chamberlain & Berry, 1930).	Physidae	UT.
2—Short Samoan tree snail	Samoana abbreviata (Mousson, 1869)	Partulidae	American Samoa.
3A—(No common name)	Carelia (all species)	Amastridae	HI.
2—San Clemente Island blunt-top snail	Sterkia clementina (Sterki, 1890)	Pupillidae	CA.
2—(No common name)	Vertigo alabamensis Clapp, 1915	Pupillidae	AL.
2—(No common name)	Vertigo hebardi Vanatta, 1912	Pupillidae	FL.
2—Kanab amber snail	Oxyloma haydeni kanabensis Pilsbry, 1948	Succineidae	UT.
3C—Florida tree snail	Liguus fasciatus (Muller, 1774)	Bulimulidae	FL.
2—(No common name)	Helicodiscus diadema Grimm, 1967	Helicodiscidae	VA.
2—(No common name)	Helicodiscus hexodon Hubricht, 1966	Helicodiscidae	TN.
2—(No common name)	Discus marmorensis (H. B. Baker, 1932)	Discidae	ID.
1—Slug snail	Binneya notabilis Cooper, 1863	Arionidae	CA.
2—(No common name)	Glyphyalinia pecki Hubricht, 1966	Zonitidae	AL.
2—(No common name)	Paravitrea clappi (Pilsbry, 1898)	Zonitidae	NC, TN.
2—Mt. Matafao different snail	Diastole matafaoi Baker, 1938	Helicarionidae	American Samoa.
2—Yate's snail	Ammonitella yatesi Cooper, 1868	Ammonitellidae	CA.
2—(No common name)	Ashmunella pasonis (Drake, 1951)	Polygyridae	TX.
2—Archer's toothed land snail	Mesodon archeri Pilsbry, 1940	Polygyridae	TN.
2—Clench's middle-toothed land snail	Mesodon clenchi (Rehder, 1932)	Polygyridae	AR.
2—(No common name)	Mesodon clausus trossulus Hubricht, 1966	Polygyridae	AL.
3C—Jones' middle-toothed land snail	Mesodon jonesianus (Archer, 1938)	Polygyridae	NC.
2—Magazine Mountain middle-toothed snail	Mesodon magazinensis (Pilsbry and Ferris, 1906)	Polygyridae	AR.
2—(No common name)	Polygyra hippocrepis (Pheiffer, 1848)	Polygyridae	TX.
2—Strange many-whorled land snail	Polygyra peregrina Rehder, 1932	Polygyridae	AR.
2—(No common name)	Stenotrema leai cheatumi Fullington, 1974	Polygyridae	TX.
2—(No common name)	Stenotrema hubrichti Pilsbry, 1940	Polygyridae	IL.
2—Pilsbry's narrow-apertured land snail	Stenotrema pilsbryi (Ferris, 1900)	Polygyridae	AR, OK.
2—(No common name)	Triodopsis mullani magnidentata (Pilsbry, 1940)	Polygyridae	ID.
2—Western three-toothed land snail	Triodopsis occidentalis (Pilsbry and Ferris, 1907)	Polygyridae	AR.
1—Karok Indian snail	Vespericola karokorum Talmage, 1962	Polygyridae	CA.
2—(No common name)	Oreohelix avalonensis Pilsbry, 1905	Oreohelicidae	CA.
2—Carinated striate banded mountain snail	Oreohelix strigosa goniogyra Pilsbry, 1933	Oreohelicidae	ID.
2—Idaho banded mountain snail	Oreohelix idahoensis idahoensis Newcomb, 1866	Oreohelicidae	ID.
2—(No common name)	Oreohelix jugalis jugalis (Hemphill, 1980)	Oreohelicidae	ID.
2—Vortex banded mountain snail	Oreohelix jugalis vortex Berry, 1932	Oreohelicidae	ID.
2—Walton's banded mountain snail	Oreohelix waltoni Solem, 1975	Oreohelicidae	ID.
2—Coalville mountain snail	Oreohelix peripherica weberiana Pilsbry, 1939	Oreohelicidae	UT.
2—Allyn Smith's banded snail	Helminthoglypta allynsmithi Pilsbry, 1939	Helminthoglyptidae	CA.
3B—Cape Mendocino snail	Helminthoglypta arrosa mattolensis A. G. Smith, 1938.	Helminthoglyptidae	CA.
3B—Dented Peninsula snail	Helminthoglypta arrosa miwoka (Bartsch, 1919)	Helminthoglyptidae	CA.
2—(No common name)	Helminthoglypta arrosa pomoensis A. G. Smith, 1938.	Helminthoglyptidae	CA.
2—(No common name)	Helminthoglypta arrosa williamsi A. G. Smith, 1938	Helminthoglyptidae	CA.
2—(No common name)	Helminthoglypta callistoderma (Pilsbry & Ferris, 1918).	Helminthoglyptidae	CA.
2—(No common name)	Helminthoglypta mohaveana Berry, 1927	Helminthoglyptidae	CA.
2—Nicklin's peninsula snail	Helminthoglypta nickliniana awania (Bartsch, 1919)	Helminthoglyptidae	CA.
2—(No common name)	Helminthoglypta nickliniana bridgesi (Newcomb, 1861).	Helminthoglyptidae	CA.
2—(No common name)	Helminthoglypta sequoicola consors (Berry, 1938)	Helminthoglyptidae	CA.
2—(No common name)	Helminthoglypta traski coelata (Bartsch, 1916)	Helminthoglyptidae	CA.
1—Banded dune snail	Helminthoglypta walkeriana (Hemphill, 1911)	Helminthoglyptidae	CA.
1—Concentrated snail	Micrarionta facta (Newcomb, 1864)	Helminthoglyptidae	CA.
2—Fraternal snail	Micrarionta feralis (Hemphill, 1901)	Helminthoglyptidae	CA.
2—Gabb's snail	Micrarionta gabbi (Newcomb, 1864)	Helminthoglyptidae	CA.
3B—Cathedral snail	Micrarionta indioensis cathedralis Willet, 1930	Helminthoglyptidae	CA.
2—(No common name)	Micrarionta rowelli bakerensis Pilsbry and Lowe, 1934.	Helminthoglyptidae	CA.
2—California McCoy snail	Micrarionta rowelli mccoiana Willet, 1935	Helminthoglyptidae	CA.
2—(No common name)	Micrarionta immaculata Willet, 1937	Helminthoglyptidae	CA.
3C—Horseshoe snail	Micrarionta intercisa (W. G. Binney, 1857)	Helminthoglyptidae	CA.
2—Colorado desert snail	Micrarionta morongoana Berry, 1929	Helminthoglyptidae	CA.
2—Prickly pear snail	Micrarionta opuntia Roth, 1975	Helminthoglyptidae	CA.
3C—Wreathed island snail	Micrarionta redimita (W. G. Binney, 1858)	Helminthoglyptidae	CA.
3C—Tryon's snail	Micrarionta tryoni (Newcomb, 1864)	Helminthoglyptidae	CA.
2—(No common name)	Monadenia circumcarinata (Stearns, 1879)	Helminthoglyptidae	CA.
2—(No common name)	Monadenia fidelis minor (W. G. Binney, 1865)	Helminthoglyptidae	OR.
2—Rocky coast snail	Monadenia fidelis pronotis Berry, 1931	Helminthoglyptidae	CA.
2—Indian Yosemite snail	Monadenia hillebrandi yosemitensis (Lowe, 1916)	Helminthoglyptidae	CA.
2—(No common name)	Monadenia mormonum buttoni (Pilsbry, 1900)	Helminthoglyptidae	CA.
2—(No common name)	Monadenia mormonum hirsuta Pilsbry, 1927	Helminthoglyptidae	CA.
2—(No common name)	Monadenia troglodytes Hanna and Smith, 1933	Helminthoglyptidae	CA.
2—Trinity bristle snail (=California northern river snail).	Monadenia setosa Talmadge, 1952	Helminthoglyptidae	CA.
2—(No common name)	Sonorella eremita Pilsbry and Ferris, 1915	Helminthoglyptidae	AZ.
2—(No common name)	Sonorella metcalfi Miller, 1976	Helminthoglyptidae	TX.
CLAMS AND MUSSELS (Molluscs, Class Bivalvia)			
2—Spectacle case pearly mussel	Cumberlandia monodonta (Say, 1829)	Margaritiferidae	IN, IL, KY, MO, NE, OH, TN, VA.
2—Alabama pearl shell	Margaritifera marrianae Johnson, 1983	Margaritiferidae	AL.
2—(No common name)	Alasmidonta arcula (Lea, 1830)	Unionidae	GA.
2—(No common name)	Alasmidonta atropurpurea (Rafinesque, 1831)	Unionidae	KY, TN.

Category and common name	Scientific name	Family	Distribution
2—Dwarf wedge mussel	*Alasmidonta heterodon* (Lea, 1830)	Unionidae	MA, MD, NC, NH, NJ, PA, VA, VT, Canada.
3A—(No common name)	*Alasmidonta mccordi* Athearn, 1964	Unionidae	GA.
2—(No common name)	*Alasmidonta raveneliana* (Lea, 1834)	Unionidae	NC, TN.
2—(No common name)	*Alasmidonta robusta* Clarke, 1981	Unionidae	NC, SC.
2—(No common name)	*Alasmidonta wrightiana* Walker, 1901	Unionidae	FL.
2—Wheeler's pearly mussel	*Arkansia wheeleri* Ortmann and Walker, 1912	Unionidae	AR, OK.
2—Virginia spiny mussel	*Fusconaia collina* (Conrad, 1837)	Unionidae	VA.
1—Tar River spiny mussel	*Elliptio (Canthyria) steinstansana* Johnson and Clarke, 1983.	Unionidae	NC.
2—Georgia spiny mussel	*Elliptio (Canthyria) spinosa* (Lea, 1836)	Unionidae	GA.
2—Savannah shore mussel	*Carunculina pulla* (Conrad, 1838)	Unionidae	GA, NC, SC.
2—Western fan-shell pearly mussel	*Cyprogenia aberti* (Conrad, 1838)	Unionidae	AR, KS, MO, OK.
2—Eastern fan-shell pearly mussel	*Cyprogenia irrorata* (Lea, 1830)	Unionidae	AL, TN, KY, VA.
2—Cape Fear spike pearly mussel	*Elliptio marsupiobesa* Fuller, 1972	Unionidae	NC.
2—Waccamaw lance pearly mussel	*Elliptio* sp.	Unionidae	NC.
3A—Recovery pearly mussel	*Elliptio nigella* (Lea, 1852)	Unionidae	GA.
2—Waccamaw spike	*Elliptio waccamawensis* (Lea, 1863)	Unionidae	NC.
3A—Arc-form pearly mussel	*Epioblasma (=Dysnomia) arcaeformis* (Lea, 1831)	Unionidae	AL, TN.
3A—(No common name)	*Epioblasma (=Dysnomia) biemarginata* (Lea, 1857)	Unionidae	AL, TN.
2—(No common name)	*Epioblasma (=Dysnomia) brevidens* (Lea, 1831)	Unionidae	AL, KY, TN, VA.
2—(No common name)	*Epioblasma (=Dysnomia) capsaeformis* (Lea, 1834)	Unionidae	AL, TN, KY, VA.
3A—Arcuate pearly mussel	*Epioblasma (=Dysnomia) flexuosa* (Rafinesque, 1820).	Unionidae	AL, TN.
3A—Acorn pearly mussel	*Epioblasma (=Dysnomia) haysiana* (Lea, 1834)	Unionidae	AL, TN, VA.
3A—Lefevre's pearly mussel	*Epioblasma (=Dysnomia) lefevrei* (Utterback, 1915)	Unionidae	AR, MO.
3A—Stones pearly mussel	*Epioblasma (=Dysnomia) lenior* (Lea, 1843)	Unionidae	AL, TN.
3A—Lewis' pearly mussel	*Epioblasma (=Dysnomia) lewisi* (Walker, 1910)	Unionidae	AL, TN, KY.
1—Penitent mussel	*Epioblasma (=Dysnomia) penita* (Conrad, 1834)	Unionidae	AL, MS.
3A—Fine-rayed pearly mussel	*Epioblasma (=Dysnomia) personata* (Say, 1829)	Unionidae	AL, TN.
3A—Nearby pearly mussel	*Epioblasma (=Dysnomia) propinqua* (Lea, 1857)	Unionidae	AL, TN.
3A—Steward's pearly mussel	*Epioblasma (=Dysnomia) stewardsoni* (Lea, 1852)	Unionidae	AL, TN.
2—Purple cat's paw pearly mussel	*Epioblasma (=Dysnomia) sulcata sulcata* (Lea, 1824).	Unionidae	AL, TN, KY.
2—Cracking pearly mussel	*Hemistena (=Lastena) lata* (Rafinesque, 1820)	Unionidae	KY, TN, VA.
2—(No common name)	*Lampsilis perovalis* (Conrad, 1834)	Unionidae	AL, MS.
2—Neosho pearly mussel	*Lampsilis rafinesqueana* Frierson, 1927	Unionidae	KS, MO, OK.
2—(No common name)	*Lampsilis streckeri* Frierson, 1927	Unionidae	AR TX.
2—(No common name)	*Lasmigona holstonia* (Lea, 1838)	Unionidae	AL, TN, VA.
2—Scale shell	*Leptodea leptodon* (Rafinesque, 1820)	Unionidae	AR, MO, OK.
2—Slab-side pearly mussel	*Lexingtonia dolabelloides* (Lea, 1840)	Unionidae	AL, TN, VA.
2—Golf stick pearly mussel	*Obovaria retusa* (Lamarck, 1819)	Unionidae	AL, TN, KY.
2—Little-winged pearly mussel	*Pegias fabula* (Lea, 1836)	Unionidae	AL, KY, VA.
2—Northern club shell	*Pleurobema clava* (Lamarck, 1819)	Unionidae	AL, IN, KY, MI, OH, TN.
1—Curtus' mussel	*Pleurobema curtum* (Lea, 1859)	Unionidae	AL, MS.
1—Marshall's mussel	*Pleurobema marshalli* Frierson, 1927	Unionidae	AL, MS.
2—(No common name)	*Pleurobema oviforme* (Conrad, 1834)	Unionidae	TN, VA.
2—(No common name)	*Pleurobema rubrum* (Rafinesque, 1820)	Unionidae	AL, KY, TN.
1—Judge Tait's mussel	*Pleurobema taitianum* (Lea, 1834)	Unionidae	AL, MS.
2—(No common name)	*Potamilus inflatus* (Lea, 1831)	Unionidae	AL, MS.
2—Rough rabbit's foot pearly mussel	*Quadrula cylindrica strigillata* (Wright, 1898)	Unionidae	AR, OH, TN, VA.
2—Rough maple leaf pearly mussel	*Quadrula fragosa* (Conrad, 1836)	Unionidae	OK.
1—Stirrup shell	*Quadrula stapes* (Lea, 1831)	Unionidae	AL, MS.
2—Salamander mussel	*Simpsoniconcha (=Simpsonaias) ambigua* (Say, 1825).	Unionidae	IA, IL, IN, KY, MI, MO, OH, TN, WI.
2—Choctaw pearly mussel (Athearn's Villosa pearly mussel).	*Villosa choctawensis* (Athearn, 1964)	Unionidae	AL, FL.
2—(No common name)	*Villosa (=Micromya) fabalis* (Lea, 1831)	Unionidae	KY, MI, OH, TN, VA.
2—Ortmann's pearly mussel	*Villosa (=Micromya) ortmanni* (Walker, 1925)	Unionidae	KY.
2—Fine-rayed purple pearly mussel	*Villosa (=Micromya) perpurpurea* (Lea, 1861	Unionidae	VA.
2—(No common name)	*Pisidium ultramontanum* Prime, 1865	Sphaeriidae	CA, OR.

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