PATHOLOGY AND TOXICOLOGY:

Modern Concepts and Techniques for Assessment of Long-Term Low-Dose Studies of Toxicity

by

Universities Associated for Research and Education in Pathology, Inc.

and

The Life Sciences Research Office,
Federation of American Societies for Experimental Biology

9650 Rockville Pike
Bethesda, Maryland 20014

The work upon which this report is based was performed pursuant to Subcontract No. FDA-71-97-UAREP with The University of Arkansas under Prime Contract No. FDA 71-97 between the University of Arkansas and the Public Health Service, Food and Drug Administration, Department of Health, Education, and Welfare.

December 1972
Universities Associated for Research and Education in Pathology, Inc. (UAREP) undertook a contract with the University of Arkansas in 1971 to study and report on the pathological assessment of long-term, low-dosage toxicity studies in animals. The study would consist of:

"1) A thorough review of all methods, procedures, techniques and apparatus currently known to be associated with the discipline of pathology in its broadest context. The primary criteria in this survey shall be an assessment of the actual or potential application of these in chronic low-level toxicity testing.

"2) Included in the above would be those items not only in common use but also those more infrequently used. This would include methods, procedures, techniques and apparatus found not only in the average pathology laboratory but also in the most advanced and sophisticated centers of excellence. Also, it would include those methods, procedures, techniques and apparatus that can be used by the average practitioner and technician, as well as those that can be used only in the hands of the few highly skilled and specially trained experts."
for Research and Education in Pathology, Inc. (UAREP) as Program Coordinator; Kenneth D. Fisher, Ph.D., Research Associate, Life Sciences Research Office (LSRO); C. Jelleff Carr, Ph.D., Director LSRO; with the special assistance of the UAREP Advisory Committee to LSRO on this Program: Benjamin F. Trump, M.D., Professor and Chairman, Department of Pathology, School of Medicine, University of Maryland (UAREP Advisory Committee Chairman); Robert A. Goyer, M.D., Professor, Department of Pathology, School of Medicine, University of North Carolina at Chapel Hill; and Ward R. Richter, D.V.M., Associate Professor, Department of Pathology, School of Medicine, University of Chicago.

The first draft was revised by Doctors Trump, Goyer and Richter to produce this document. UAREP is grateful to the many individuals who contributed so much to the collective effort. Responsibility for the content, however, belongs exclusively with UAREP.

This report is intended to be useful for people with diverse backgrounds of training and experience, as the work scope requires, from the novice to the experienced generalist. Few experts in pathology, mutagenesis, teratogenesis or carcinogenesis will find coverage of their respective specialties adequate to their needs. On the other hand, the constellation of these subjects in the firmament of toxicology may prove interesting and valuable.
TABLE OF CONTENTS

Foreword .............................................Page i
Summary............................................. 1

I. Introduction..................................... 2

II. Scope of the Study............................... 3

III. General Aspects of Pathology................. 5
    A. Role of the Pathologist...................... 5
    B. Defining Pathologic Endpoints of Toxicity...
       1. General Concepts.......................... 5
       2. Autopsy Procedures....................... 8

IV. Anatomical Pathology.......................... 14
    A. Gross Morphology............................ 14
       1. General Aspects......................... 14
       2. Special Problems......................... 15
       3. Recording the Gross Description........ 17
    B. Histopathology and Histochemistry......... 19
       1. General Comments......................... 19
       2. Specialized Areas in Histopathology.... 20
    C. Microscopy.................................. 23
       1. Light Microscopy.......................... 23
       2. Fluorescence Microscopy.................. 25
       3. Electron Microscopy....................... 26
    D. Direct Mass Fragmentography................ 31
    E. Automation of Techniques.................... 32
       1. Scanning Systems.......................... 33
       2. Automated Volume and Area Measurements.
       3. Continuous Flow Systems.................. 33
       4. Optical Correlation Systems............... 34
<table>
<thead>
<tr>
<th>V. Biochemical and Clinical Pathology</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Cellular Function Tests</td>
<td>35</td>
</tr>
<tr>
<td>1. Bioenergetics</td>
<td>35</td>
</tr>
<tr>
<td>2. Protein Metabolism</td>
<td>35</td>
</tr>
<tr>
<td>3. Nucleic Acid Metabolism</td>
<td>36</td>
</tr>
<tr>
<td>4. Transport and Secretion</td>
<td>36</td>
</tr>
<tr>
<td>5. Cell Surface Properties</td>
<td>36</td>
</tr>
<tr>
<td>6. Cell Morphology, Shape and Motility</td>
<td>37</td>
</tr>
<tr>
<td>7. Immunopathology</td>
<td>38</td>
</tr>
<tr>
<td>B. Tissue and Organ Function Tests</td>
<td>40</td>
</tr>
<tr>
<td>1. Introduction</td>
<td>40</td>
</tr>
<tr>
<td>2. Growth and Related Phenomena</td>
<td>40</td>
</tr>
<tr>
<td>3. Functional Profiles of Organ Systems</td>
<td>41</td>
</tr>
<tr>
<td>C. Automation</td>
<td>47</td>
</tr>
<tr>
<td>VI. General Aspects of Toxicological Evaluation</td>
<td>49</td>
</tr>
<tr>
<td>A. Concepts of Toxicity</td>
<td>49</td>
</tr>
<tr>
<td>B. General Experimental Parameters</td>
<td>53</td>
</tr>
<tr>
<td>1. Introduction</td>
<td>53</td>
</tr>
<tr>
<td>2. Nutritional Aspects Influencing Evaluation of General Health of the Animal</td>
<td>53</td>
</tr>
<tr>
<td>3. Parameters of Growth and Development</td>
<td>55</td>
</tr>
<tr>
<td>4. Reproductive Aspects</td>
<td>55</td>
</tr>
<tr>
<td>5. Behavioral Parameters</td>
<td>56</td>
</tr>
<tr>
<td>VII. Techniques in Identification of Mutagenicity</td>
<td>58</td>
</tr>
<tr>
<td>A. Basic Mechanisms</td>
<td>58</td>
</tr>
<tr>
<td>1. Introduction</td>
<td>58</td>
</tr>
<tr>
<td>2. Types of Genetic Alterations</td>
<td>58</td>
</tr>
<tr>
<td>3. Repair of Genetic Alterations</td>
<td>61</td>
</tr>
<tr>
<td>4. Chemical Mutagens</td>
<td>62</td>
</tr>
<tr>
<td>B. Test Systems</td>
<td>63</td>
</tr>
<tr>
<td>1. Submammalian Test Systems</td>
<td>63</td>
</tr>
<tr>
<td>2. Mammalian In Vivo Techniques</td>
<td>64</td>
</tr>
<tr>
<td>C. Correlations in Mutagenicity Test Procedures</td>
<td>75</td>
</tr>
</tbody>
</table>
VIII. Techniques for Identification of Teratogenicity
A. Basic Concepts
   1. Genotypic Factors
   2. Maternal Factors
   3. Developmental Stages
   4. Qualitative and Quantitative Aspects of Exposure
   5. Patterns of Abnormal Development
B. Test Systems
   1. Nonmammalian Systems
   2. Mammalian Test Systems

IX. Carcinogenicity Test Procedures
A. Introduction
B. Conventional Test Techniques
   1. Conceptual Aspects
   2. Statistical Limitations
   3. Practical Aspects
C. Potential Techniques
   1. Examination of Chemical Structures
   2. Biochemical Tests
   3. Interactions with Genetic Material
   4. Tissue Culture Studies
   5. Exposure of Gravid Females
D. Epidemiological Monitoring

X. Correlation of Test Systems

XI. Application of the Results of Animal Testing to Man
A. Introduction
B. Basis of Species Variations
C. Practical Considerations
XII.  Suggested Areas for Future Research.............  115
XIII. Bibliography..............................................  122
XIV. Consultants..................................................  149
A. List of Attendees.............................................  149
B. Other Consultants...........................................  153
SUMMARY

This report reviews concepts and techniques in pathology and related disciplines useful in assessing long-term, low-dose toxicity. It includes discussion of techniques for studying anatomical, biochemical, and clinical pathology, mutagenicity, teratogenicity, and carcinogenicity and approaches the serious problem of relating data obtained from the study of experimental animals to human experience. The need for improved correlations among test systems is also discussed. The report suggests that the National Center for Toxicological Research has a unique opportunity to address the need for developing techniques useful in assessment of the effects of long-term, low-dosage exposures to toxic substances. It identifies many useful indices of toxicity that appear promising, but require further refinement and evaluation. By developing methodology and a broad data base, the National Center for Toxicological Research can make a significant contribution to interpretation of toxicological studies on animals for man.
I. INTRODUCTION

The National Center for Toxicological Research (NCTR) was established by the President of the United States on January 27, 1971. The facilities of the Pine Bluff Arsenal (Jefferson, Arkansas) for biological research were diverted to the NCTR. Its primary mission is development of experimental protocols to be used as references and standards, and the development of data for the assessment of the potential hazards of chemical substances to man and his environment.

The NCTR will function as a national resource, initially funded by the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA), but open to cooperative efforts with other agencies of the Federal Government, universities, research institutes, and industry. The NCTR mission will emphasize:

a. Research on the adverse health effects of long-term exposures to low doses of selected chemical substances;

b. Research on the basic biological processes that underlie chemical toxicity in animals;

c. Research on improvement of methodology and experimental design for toxicological evaluation;

d. Research that develops a data base to facilitate the interpretation of toxicological studies on animals for man.

Initial studies at NCTR on known carcinogens include relatively short-term experiments at established toxic doses as a prelude to prolonged lifetime low-dose tests. As the NCTR facility develops, plans call for addition of other parameters of toxicity evaluation including teratogenicity, comparative pharmacology, mutagenicity, behavioral toxicology, and the interactive effects of potentially toxic substances.
II. SCOPE OF THE STUDY

Universities Associated for Research and Education in Pathology, Inc. (UAREP) was asked by the Food and Drug Administration to undertake a review of concepts and techniques in pathology useful in the assessment of long-term, low-dose toxicity studies in large numbers of animals. The Life Sciences Research Office, (LSRO), under a subcontract from UAREP, was asked to assist in developing this comprehensive review. This report reviews concepts and techniques in pathology and related disciplines that may be applicable to future requirements of the NCTR program. The study transcends the immediate needs of the NCTR and has been focused upon the long-range goals of more reliable and predictable toxicological evaluation by efficient utilization of experimental techniques. It emphasizes, by an overview of conventional and novel techniques in pathology and related disciplines, experimental approaches to more critical evaluation of the effects of prolonged exposures to low concentrations of natural and man-made substances.

The scope of this study includes the evaluation of concepts and techniques in pathology as well as recent advances in biomedical technology, bioengineering, analytical chemistry, instrumentation, and other disciplines that may be useful to future NCTR research programs.

The primary goals of this study are to provide:

a. A review of relevant concepts and techniques associated with the discipline of pathology. Emphasis has been placed on the potential application of these methods to assessing pathological changes following the administration of low concentrations of suspected toxic substances;

b. A review of potentially useful conventional or novel concepts, instrumentation, and techniques; and

c. Recommendations for future research in pathology applicable to the studies to be conducted at the NCTR.
In this study, pathology is defined as that scientific discipline embodying the concepts and techniques employed in the study of diseases, their essential nature, their causes and development, and the associated structural, chemical, and functional changes. The techniques in pathology that have been emphasized in this study include:

a. Studies of structural abnormalities by manual or automated methods employing light, phase-contrast, fluorescence, and electron microscopy, cytochemistry, histochemistry, or physicochemical analyses of macromolecular structure;

b. Studies of chemical changes in body fluids and tissues by conventional or novel analytical methods, cell fractionation, the use of radioisotopes and cellular metabolic exchange; and

c. Investigation of metabolic and functional changes by in vivo studies of subcellular, cellular and organ systems.

This broad definition of the scope of pathology was used by the consultants in the development of agenda topics for the ad hoc review group meetings. The report reflects the opinions expressed by the consultants in these discussions on concepts and techniques that are applicable to early identification of long-term, low-dosage effects of chemical substances. The report also denotes the emphasis that the ad hoc review group members and the other consultants placed upon the advantages and limitations of potentially useful methods that may provide a better basis for this type of toxicological evaluation. Lastly, the report emphasizes the concern of the study consultants for the limitations of knowledge in correlating various methods, and interpreting the significance of experimental animal studies for human toxicity.
III. GENERAL ASPECTS OF PATHOLOGY

A. ROLE OF THE PATHOLOGIST

The pathologist must participate in the planning and design of experimental studies as well as in the assessment of changes occurring in test animals during experimentation. The pathologist is an essential and indispensable participant in any toxicological research program; his role extends beyond mere technical service. Pathology is not limited to description of morphological changes. The pathologist can provide expertise in the development of dynamic explanations of toxic effects of substances in biochemical and biophysical terms. Cooperation and exchange of ideas among various scientific disciplines is absolutely essential to critical evaluation of toxicity.

B. DEFINING PATHOLOGIC ENDPOINTS OF TOXICITY

1. General Concepts

Structure and function are interrelated in all biological organisms; disease alters structure and function to reduce an organism's ability to adapt to its environment. Pathology is the study of functional and morphological changes occurring in disease. However, variations in structure and function are a gradual continuum from normal through adaptive changes and disease to death. It is difficult to define those conditions which represent disease, especially when it is essential to identify small deflections from normal as in a study of long-term, low level toxicity.

Traditionally, pathology has relied on the study of morphological change at ever improving degrees of resolution. Because structure and function are so inseparably related, it has been possible to draw conclusions or make predictions of altered function from observable structural change. Morphological alterations remain the primary criteria for diagnosis, but recent advances in biochemistry and clinical pathology have made functional endpoints valuable parameters in analysis of pathogenesis of disease and in evaluation of chemical toxicity (Slater, 1972).
The effects of a chemical compound on the cell result first in changes at the molecular level. As exposure to the compound or the metabolites continues, measurable biochemical and morphological changes become evident at the subcellular level. The point at which subcellular and cellular changes become evident depends on the resolution of the technique used. Definition of an ultrastructural or biochemical change in a cell or tissue as adaptive or pathological is difficult when that change is considered only as an isolated entity. Under certain conditions a response may be adaptive, under other conditions the response may be pathological. An adaptive mechanism may protect a cell from irreversible injury. On the other hand, the energy directed to the adaptive response may impair the ability of the cell to perform other functions such as transport or synthesis. Cells die when their capacity to compensate for the injury is exceeded. The point of no return is as difficult to define at the cellular level as it is at the level of the entire organism.

Reversibility of a cellular lesion is not an absolute characteristic response to a condition or compound. The degree to which reversible changes occur will depend on the duration of exposure, dosage, species, and individual physiological variability. For example, a rat kidney deprived of its blood supply for 20 minutes will recover from ischemia and normal cellular values of adenosine triphosphate (ATP) and lactic acid will return. However, if the ischemia continues for a total of 30 minutes, the kidney never fully recovers and the ATP levels never return to normal (Vogt and Farber, 1968). There are many similar examples where a toxic substance may or may not produce reversible changes depending on dosage and duration of exposure.

The ultrastructural changes observed in cell death are merely a progression and an accentuation of those described in cell injury (Scarpelli and Trump, 1971). Injury to a cell may overwhelm the cell's adaptive mechanisms and result in death of the cell. Cell death, in and of itself, cannot be defined as absolutely beneficial or totally harmful to the total organism unless the relation of the cell to the organism is known. For example, loss of cells from the intestinal villi is a normal process; death of tumor cells would benefit the organism.

If cell injury is less extreme, there may be time for repair of adaptive mechanisms of the cell to reestablish and main-
tain cellular function although at an altered steady state and/or diminished functional capacity. Examples of adaptation include: hypertrophy, hyperplasia, giant cell formation, atrophy, autophagocytosis, pigmentation, calcification, fatty metamorphosis, lysosomal overloading, metamorphosis, and induction of microsomal enzymes. These and other mechanisms are more fully described in the reviews of Scarpelli and Trump (1971), and Robbins and Angell 1971).

Certain adaptive changes may be advantageous to the organism. A classic example is the induction of liver microsomal enzymes by administration of barbiturates initially at low doses and subsequently at higher doses. In time, the organism can tolerate far higher doses of barbiturates than an untreated organism because of induction of hepatic microsomal enzymes. In this instance, the enzyme induction is an adaptive effect beneficial to the organism. However, if the treated animal is exposed to carbon tetrachloride (CCL4) at a normally nontoxic dose, the enzymes of the hepatic endoplasmic reticulum rapidly convert CCL4 to the more toxic CCL3 and Cl. The more efficient conversion of CCL4 in the treated animal results in adverse effects from administration of a normally nontoxic dose of CCL4. Thus, an adaptive change may be protective to the organism on exposure to one compound and harmful when exposed to another compound.

Various techniques are used to describe adaptive changes, moderate injury or severe damage subsequently resulting in cellular necrosis. The cell or organ may have an altered steady state and/or diminished functional reserve capacity, but these changes may be necessary to keep the organism alive. Interpretation of morphological, biochemical, and physiological changes in cells or tissues cannot be made in isolation and must consider the entire organism and its environment.

Finally, variation in structure and function which occurs among individual members of control groups becomes an increasingly severe problem as experiments are designed to detect smaller and smaller changes, over the life time of the animal, which may be initial steps in the pathological process. In addition, it is also essential to know the incidence of various pathological lesions in the control population. Thus large numbers of animals are required to define the control population.
2. Autopsy Procedures

a. Preliminary Considerations

The autopsy* is often the single most critical or determinative procedure in toxicological evaluation. Thus, the design of the autopsy procedures should be developed during the initial phases of a toxicological research project. The autopsy is an information gathering process at the morphological, biochemical, and functional level. It must be planned in detail. The "complete" and adequate autopsy should be sufficiently detailed to assess the general health of the animal, to detect lesions which have occurred spontaneously, and to differentiate them from lesions related to the substance being tested. The inclusion of nontreated controls will permit assessment of those lesions which are spontaneous and those changes caused by low-dose effects; inclusion of high-dose controls will provide assurance that experimental animals are sensitive to the material being tested.

In the absence of toxicity data, pilot experiments using small numbers of animals, usually of inbred strains, at high dosages of the compound frequently are used to obtain a starting point for further pathological evaluations. In trial experiments of this type, the autopsy should be extremely detailed, as lesions may show unusual distribution or localization. Virtually all tissues from a limited number of experimental animals should be examined in pilot studies to identify important manifestations of toxicity. For example, Montesano and Saffiotti (1968) have reported that diethylnitrosamine produces multiple types of tumors in the nasal cavity of the hamster. Occurrence of this type of tumor would have been overlooked if the autopsy had not included complete examination of the nasal cavities.

*In this report, the terms, "autopsy" and "necropsy" are used interchangeably.
There is no definable autopsy which applies to all experiments. The term "complete autopsy" appears many times in the pathology literature; however, specifically which organs or tissues are included in the "complete autopsy" will vary widely with investigator and experiment. For example, previous knowledge of the metabolism or of the primary or secondary target organs most frequently affected by the compound, will influence the type of autopsy performed. Obviously, when testing substances known to affect specific tissues careful dissection and histopathologic evaluation of these areas are required.

The autopsy includes gross morphological examination. The general appearance of the animal, such as hair, skin, and total body weight, are usually recorded at the time of autopsy. The weight and volume of tissues and organs which are readily dissected can be determined readily. Ease of dissection of particular organs will vary with animal species. Organs easily dissected in most species include: pituitary, brain, thyroids, lungs, heart, liver, spleen, kidneys, adrenals, eyes and stomach.

Recording of organ weights is essential. Increased organ weight can be the result of hyperplasia, hypertrophy or tissue infiltration by lipids or other materials. The actual changes may be evident only after histological examination. Organ weights are frequently used as a base for chemical analysis as are measurements of protein, DNA and RNA.

While a complete autopsy for all experiments cannot be defined, certain tissues are traditionally included in most autopsies. These tissues normally include:

- adrenals
- aorta
- bone and bone marrow
- brain - longitudinal and cross section
- colon and cecum
- esophagus
- eyes
- ear
- fat
- heart
- kidneys
- liver
- lungs
lymph nodes, peripheral, thoracic and abdominal mammary gland
nasal cavities
ovaries
pancreas.
parathyroids
peripheral nerve
pituitary
prostate and seminal vesicles
salivary gland
skeletal muscle, diaphragm or psoas
skin
small intestine - 3 sections: duodenum, ileum and jejunum
spinal cord
spleen
stomach, glandular and nonglandular
testes and epididymis
thymus
thyroid
tongue
tonsil
trachea/bronchii
urinary bladder
uterus
ureter

When a target organ has been defined in a suitable species, either through preliminary experimentation or from human epidemiological data, a complete autopsy emphasizes but is not limited to the single target organ. Specific autopsy procedures are required for mutagenic and teratogenic studies.

The method of killing may influence organ weight, and other experimental observations. If the animal has been exsanguinated, the weight of organs generally containing large blood volumes, such as the liver or spleen, will be markedly affected. When organs are weighed, it is important that this be done quickly to avoid drying and autolysis prior to fixation. The question of specific methods to be used in killing must be left to the discretion of the experimental pathologist as it may vary with experimental protocols. For example, if a cardiac lesion is suspected, exsanguination by heart puncture would be avoided, as morphological evidence of the lesions may be lost. Use of barbiturates may render respiratory studies on mitochondria inaccurate. The sequence in which organs are removed may be critical if certain enzymic measurements are to be made accurately. These examples illustrate the need
for careful preliminary planning and subsequent cooperation of all personnel involved in the toxicity experiment.

b. Potential Problem Areas

1. Spontaneous Disease. Pathological changes may appear in control animals. It is essential that the incidence of these changes in the control animals be accurately known. The occurrence of spontaneous disease even in well controlled animal colonies may affect the reaction of the animal to the compound under test, particularly in long-term studies.

2. Aging. Compounding factors in long-term or lifetime studies are the changes associated with aging. The effects of aging may influence the animal's reaction to the experimental compound. Therefore, the control animals must also be adequately examined to determine those changes associated with aging alone.

3. Duration of the Experiment. The stage in the experiment at which the animal ought to be autopsied is especially relevant in prolonged test situations. The investigator must decide on relevant times to detect those pathologic changes most germane to the test situation and the known toxicity of the substance under study. Reference has been made to these issues in various sections of this report.

Certain lesions such as neoplasms may require long periods of time to develop. Some investigators favor termination of an experiment after a given number of months; however, many believe far greater information on tumorigenesis is obtained by allowing the animal to complete its life span. While it may be argued the animal should be allowed to die naturally, the problems of cannibalism if the animals are not singly caged and tissue autolysis would be prohibitive to either a complete autopsy or adequate histology. A third alternative is sacrifice of moribund animals. Whatever experimental protocol is selected, consistency in subsequent experiments should be maintained.

4. Early Death of Experimental Animals. The quality of an autopsy performed on an animal found dead clearly depends on the degree of autolysis which has taken place. Because of a number of variables, it is not possible to make precise statements of the length of time after death that autolysis will occur. In many cases,
the time of death can only be estimated. In general, the smaller the species, the shorter the time period before adequate autopsy is impossible. If a dead animal is found and a parapathologist or pathologist is not available for performance of the necropsy, it may be desirable to train animal caretakers to open the visceral cavity, excise and section major organs, place them in formalin, and then store the remaining carcass in the refrigerator. Placing an intact animal in a freezer will produce ice crystals in the tissues which are likely to result in artifacts in histology. If an animal is found dead during the course of the experiment, a notation of the death, time, date and condition of the animal must be made. Animals found dead during the course of the experiment must be recorded and not simply discarded.

Differences in personal judgment as to what physical appearance constitutes a moribund animal make it desirable to rotate animal handlers from one animal room to another to avoid biasing results. Electronic monitoring of body heat, heart rate, or activity of animals are potential techniques to detect the moribund animal. Actual use of these detection systems is only in the developmental stage. In practice, most laboratories with large numbers of animals have animal handlers check cages several times a day on a 7-day per week basis to detect animals near death.

5. **Tissue Handling.** Proper handling is essential for biochemical studies. Analyses of tissues for many heavy metals can become totally meaningless if the tissues have been contaminated either by instruments during removal of the tissue from the animal or through storage of tissues in formalin or in improper containers such as certain plastics. Similarly, proper fixation of tissues through choice of the best fixative, proper amounts of time in the fixative, and adequate washing of tissues can eliminate artifacts in histology and enhance meaningful interpretation of slides.

c. **Management of the Autopsy Procedures**

The autopsy begins with observations of organs and tissues by qualified pathologists as described in Section IV.

Based on the experience of investigators handling large numbers of small animals, the following general process has evolved. The actual gross autopsy is performed by individuals who have been specially trained in necropsy of the species of animal under study during an intensive period of instruction. This
training stresses recognition of gross abnormalities.

While the specially trained technician may do the actual necropsy, a senior pathologist should supervise the work. In low dose toxicity studies, if an unanticipated or unusual abnormality is recognized by the technician, the animal should then be removed from the standard autopsy routine for examination by the senior pathologist.

Photographic equipment should be readily available. Photographs are labeled with information identifying the experimental animal.

The senior pathologist should be available to answer questions and to do spot checks, especially in early stages of the study, and to ascertain the technician's ability to distinguish the normal from the abnormal. The number of competent animal necropsy technicians who can be adequately supervised by a pathologist is variable and depends on the training, skill, experience, and responsibility of the technicians and on similar characteristics of the pathologists.

In performing animal autopsies under a scheme as described above, auxiliary personnel in addition to the pathologist and the animal necropsy technicians can be of great value in relieving these personnel of time-consuming logistic tasks. Such tasks as labeling of tissues, killing of animals, delivering fresh bottles of fixative, removal of tissue from autopsy site can do a great deal to relieve the more highly trained personnel from these tasks thereby increasing overall efficiency of the laboratory. It is emphasized that, irrespective of any later use of computers in handling of data, a hard copy of the autopsy findings including descriptions of gross and histological changes should be maintained. Auxiliary personnel can be used to record the necropsy observations on record sheets; or the necropsy technician can dictate for later transcribing. The latter method would, however, offer greater opportunity for accidental mislabeling, confusion of results or loss of records.

This entire discussion should be considered illustrative of the importance of details in the autopsy. In the final analysis, the specific steps and techniques of necropsy must be planned and executed under the direction of the senior pathologist.
IV. ANATOMICAL PATHOLOGY

A. GROSS MORPHOLOGY

1. General Aspects

The single most important step in the examination of tissues is the gross autopsy. The prosector is the only person who can observe the tissues undisturbed in situ. His oral, written, or photographic records will reflect personal bias, but he will make the critical decisions on selection of organs and tissues for subsequent examinations. The prosector may elect to preserve the remnants of the carcass; however, the preserving fixative will differ from the fixative in which specimens for histology are immersed. Such decisions affect the flexibility of subsequent examination of tissues.

The "assembly-line" approach to multiple animal examinations makes difficult observation of the relationships of one organ to another and the synthesis of an accurate description of disease as depicted by constellations of related lesions in several tissues. This problem can be approached by rigorous training, standardization of evaluating normal versus abnormal, and emphasis on accurate record-keeping.

If a tissue is recognized as grossly abnormal, then detail of the gross morphology should be more extensive. For example, if a gross tumor is identified, it should be carefully examined as to size, anatomic location, local invasiveness, and extension beyond the affected organ. A dissection of the regional lymph nodes and a search for hematogenous metastases should be made. Similar degree of careful evaluation of other types of lesions should be performed. Similarly, autolysis of an individual organ or tissue, as opposed to generalized autolysis, can only be confirmed by microscopic examination of that organ tissue, as certain pathological conditions will produce changes grossly similar to autolysis.

Identification of visible lesions is one purpose of the autopsy. In many experimental situations if a tissue appears
normal on gross examination, no further evaluation, e.g., histology, of the tissue is performed. Histologic studies should be performed on tissues which appear grossly normal (Thompson and Hunt, 1963). If a morphological lesion is detected in normal appearing tissue of one animal through routine histological monitoring, then that organ from all animals in the experiment should be examined microscopically. Any tissue or organ which appears grossly abnormal should be subjected to histologic study.

The number of apparently normal organs to be sectioned for histological examinations is a function of experimental design. One may elect to perform a pilot experiment with a comparatively small number of animals each of which is subjected to detailed and rigorous examination of the principal organs and tissues. Others prefer random sampling from larger experimental groups to determine the need for histological and other special studies. In any case, the unexpected usually materializes but is accompanied by changes in microscopic appearance that can be recognized by the sophisticated practitioner.

Gross morphology is useful as a guide to sectioning for histopathology and can increase the likelihood of detection of certain lesions and reduce the number of histology sections required. Because only a limited number of sections of an organ may be taken for histology, the location of the lesion by gross morphology becomes extremely helpful. For example, location of tumors in the distended urinary bladder by use of a dissecting microscope has proved to be a reliable technique. These tumors are then confirmed by histologic examination (Levin and Richter, 1971). Tumors in the pancreas of certain species can be detected grossly and sections taken from that particular region. Whole mounting, clearing, and gross observation of breast tissues is a useful procedure in detecting mammary tumors in laboratory animals.

Preservation at autopsy of a full set of organs and tissues regardless of the number ultimately examined histologically is recommended.

2. Special Problems

When evaluating a large series of animals, certain lesions may occur in control as well as experimental animals. For
example, pulmonary congestion secondary to the presence of pneumonia is endemic in many rodent colonies. When these rodents are in prolonged experiments, control animals show a strikingly high incidence of this lesion. In colonies of specific pathogen-free animals, this condition would not be expected to occur; but if murine pneumonia or similar diseases do appear, severe mortality can be anticipated. Other problems such as renal granularity, hepatic congestion or infiltration with fatty or fibrous material may be encountered. Such changes could be related to nutritional factors in the diet or due to the aging processes. However, their presence and incidence in control animals should be established. It should be noted that compounds being tested for toxicity may increase the incidence or severity of such lesions above that found in control groups or cause them to appear at an earlier age.

Frequently, the skeletal system, bone marrow, central nervous system and endocrine systems are examined inadequately. Lesions of the eye have been noted in pharmacologic evaluations. Drugs such as chloroquine known to cause corneal deposits and retinopathy in animals and man (Saunders, 1967) (Meier-Ruge, 1965).

The diagnosis of neoplasia may not be difficult, but determination of the malignant characteristics can involve pathologists in endless controversy. The histologic criteria of cancer in man are reflected in some but not all neoplasms of experimental animals. A significant number of rodent and canine neoplasms can and do metastasize if allowed to develop that potential over long periods. The metastases mimic the structural characteristics of the parent lesion, and neither may fulfill the criteria expected in cancers that affect man. Nevertheless, they are clearly malignant because of their biologic propensities.

The experimentalist has another important tool at hand to fortify his diagnosis of malignant neoplasia - transplantation. Normal tissues and benign neoplasms may persist but do not grow progressively when transplanted to genetically appropriate members of the same species; malignant neoplasms do. This favors work within inbred strains of experimental animals when cancer is the expected endpoint and limits the practical available species to mice, rats, and guinea pigs. It is possible to demonstrate transplantability of malignant neoplasms among randomly bred members of species, but the cost is high. The pioneers in cancer research
conventionally transplanted fragments of a spontaneous neoplasm to as many as 100 subjects in order to get two or three that would grow progressively. Perpetuation beyond the first transplant generation was the exception, not the rule, and large numbers of hosts were required to obtain small numbers of progressively growing tumors. The failure to grow progressively under such circumstances is fairly well understood today as an expression of immunological intolerance to foreign antigens resulting from biochemical individuality.

Hepatic tumors in mice follow exposure to a variety of carcinogens. The precise relationships between morphology and degree of malignancy have not been established in these animals. Because of the proposed use of mice in the NCTR study, and because of the common spontaneous occurrence of these neoplasms, it would be desirable to sponsor research on this problem, which would require detailed and long-term studies in mice with high quality morphological studies, repeated biopsies, and transplantation studies. Though such studies have been partially completed in rats (Reuber 1966, Firminger 1955) they have not been conducted as extensively in mice (Reuber 1971, Andervont and Dunn, 1952).

If a neoplasm is to be transplanted this must be contemplated and adequate preparations made at the time of the gross autopsy.

The autopsy rooms should be suitably equipped with apparatus and reagents for special morphological and biochemical studies. A list would include fixatives for electron microscopic study, deep freeze, liquid nitrogen containers, perfusion apparatus, and homogenizers.

3. Recording the Gross Description

There are numerous examples of autopsy forms for recording observations during the gross morphological examination. It is anticipated that NCTR will develop a standardized form that meets the needs of the NCTR program.

The importance of completeness of records and efficiency of note-taking is recognized. It may be desirable to have observations written or recorded by someone other than the prosector. Details of the gross examination can be recorded in prose and later transferred to typed record cards. The need for consistency in
coding systems used on animals, autopsy, and tissues for histology is self-evident. A rigid protocol must be developed for each experiment appropriate to its conditions and objectives.

The NCTR program will generate quantities of data that will probably require computerized storage and retrieval for analyses. Several industrial laboratories, the Armed Forces Institute of Pathology, National Cancer Institute, and the Oak Ridge National Laboratory have experience with systems of computer language for automated data collection. A systematic nomenclature of pathology (SNOP) has been developed and has proved useful in recording pathological information. It can readily be adapted to animal lesions.
B. HISTOPATHOLOGY AND HISTOCHEMISTRY

1. General Comments

Hematoxylin and eosin stains, which were developed as early as 1904, (Mayer, 1904) are still the preferred method for routine differentiation of nuclei and cytoplasm. While histopathologic staining reactions depend on the chemical composition of the structures demonstrated, the primary purpose of the stain is for morphological information and the chemical basis is often unknown. Besides the routine H & E stain, there are numerous special histologic and histochemical techniques used to differentiate cellular constituents, which are described in standard reference texts such as Luna, Pearson 1968, Lillie 1965, and Barka & Anderson 1963.

It is necessary in most research projects to correlate morphologic findings with other research data, therefore a large histology volume can be expected. In order to handle this volume efficiently, as well as, providing all the special histologic techniques it is necessary to have a centralized histology laboratory. However, specialized functions to be performed within such a centralized laboratory require organizational considerations to maximize the utmost in efficiency. There is a maximum size for a histopathology laboratory which can not be defined explicitly because the capabilities of supervisors in managing subordinate personnel are the primary determining factors and geographic realities should be considered in developing a comprehensive plan. This laboratory should be equipped with all the equipment necessary to do both routine and special histology, and should be staffed by appropriate technical personnel. An estimate of the work capacity and the cost of such a unit will be given below.

For an estimated volume of 100,000 slides, plus special procedures, annually the technical staff required would be 8 histology technicians, 1 supervisory technician, and a laboratory director who is familiar with both production histology and all special histologic techniques, ie (special staining, histochemistry, immunology, autoradiography, and special embedding techniques). The equipment needs for such a laboratory would include; 9 rotary microtomes, 1 sliding microtome, 2 light microscopes 1 microscope with light, phase, polarizing, and flourescent capabilities, 37 metal knives, 6 - two level automatic tissue processors, 5 automatic knife sharpeners, 2 bone decalcifiers, 2 cryostats, 4 ovens, 4 slide dryers, 2 pH meters and two fume hoods.
Based on present costs, equipping this unit initially would cost approximately $200,000 to $250,000. The annual operating cost excluding salaries, would be approximately $25,000 per year. The cost in a large system for producing a stained histologic slide would be approximately $1.50 each for most routine and special stains.

The centralized laboratory can take advantage of all the automated techniques available to produce the maximum output at a minimum cost. Equipment for automating tissue processing, H & E staining, and knife sharpening is commercially available.

2. Specialized Areas in Histopathology

a. Histochemistry

Histochemistry deals with chemical composition including enzymes contained within cells or tissues. These techniques are valuable as early indicators of cellular change. Histochemistry is helpful in determining the pathways of cellular changes due to experimental conditions. Staining reactions such as acid phosphatase, glucose - 6 - phosphatase, 5' - nucleotidase, and lactic dehydrogenase can be used to detect initial changes in intermediary metabolism within specific cell organelles. Such alterations may be clues to early manifestations of cellular toxicity or carcinogenesis. Similarly, histopathological identification of lipid material or glycogen may indicate early changes in cell metabolism.

In recent years heavy metals (Ericsson and Trump, 1965) and other electron dense materials (Graham and Karnovsky, 1966) have been used as capturing agents in enzyme substrates in order to localize enzyme sites for electron microscopy. This has made it possible to study, with high resolution, early intracellular enzyme changes due to cell injury.

Care must be taken in all steps of the procedures. Correct fixation (Janigan, 1965; Holt et al., 1960) precision in making solutions, incubation times, tissue handling and proper controls are important in histochemistry. It is therefore necessary to have reliable well trained technical help in this area and proper supervision.

b. Autoradiography

The principal aim in autoradiography is to localize, with the aid of a photographic emulsion, the precise position of radioactive atoms that have been introduced into a specimen. In
common with other cytochemical techniques, it is possible, with the aid of autoradiographs to localize known biochemical reactions to specific intracellular sites without disrupting the cells. Autoradiography has the special merit, however, of allowing the localization to be made quantitatively. It also can be used to localize reactions for which there are no specific cytochemical staining methods. Autoradiography has the added advantage in that it allows new synthetic products to be distinguished from accumulated stores within tissues.

Autoradiography is useful in determining distribution and localization of a potentially toxic substance and its metabolites. This may aid in identifying target organs, tissues, or cells for further action of chemical substances, especially at the cellular level. Labeled compounds such as tritiated thymidine are useful in studying hyperplasia, regeneration, cell cycle times, and related cellular dynamic phenomena related to DNA synthesis. Other compounds may be incorporated only if altered metabolic pathways have developed or may not be incorporated if pathways have been blocked by the toxic substance.

Advances have been made recently in several areas of radiographic technic. It is possible now through technics developed for diffusible substances (Roth and Stumpf, 1969) to study with autoradiography many more substances in their relation to tissue than was before possible. Other workers (Salpeter and pachmann, 1964; Hay and Revel, 1963; Garo and Van Tübingen, 1962) have made it possible to study localization within organelles by adapting this technic to electron microscopy. Data on the efficiency of various emulsion and developer combinations (Vrensen, 1964) and resolution measurements (Salpeter et al., 1969) have made it possible to quantify the amount of radioactive material within a labeled specimen.

c. Immunofluorescence

Proteins can be labelled by chemical combination with fluorescent dyes, without material effect on the biological or immunological properties of the proteins. They may then be visualized in microscopical preparations by fluorescence microscopy. Proteins labelled with fluorescent dyes and injected into animals can yield useful information about the precise distribution and metabolism of proteins in experimental physiology and pathology. This technique has important applications for the localization and quantitation of antibody buildup due to the administration of toxic
substances. Because of the loss of organ specific antigens in malignancy the distinction between benign tumors and premalignant lesions can perhaps be made with this technique (Nairn et al., 1960).

By substituting ferritin (McLean and Singer, 1970) and peroxidase (Moriarity and Halmi, 1972) for the fluorescent dye we are now able to perform the immunologic techniques at the electron microscope level.

In order to perform this technic with precision, technical personnel as well as a professional with a background in immunology are needed.

d. Special Sectioning and Embedding Techniques

The "film strip" technique is economical and efficient for handling large numbers of serial sections. The techniques involve use of 35 or 70mm cellulose acetate film strips as the mounting base rather than glass slides for histology sections (Wilson and Pickett, 1970; Pickett et al., 1964; Pickett and Sommer, 1960). The sections from an embedded specimen are mounted on 35 or 70mm flexible film and covered with a hardening plastic rather than a conventional glass cover slip. This plastic mounting has proven to be as permanent as glass slides. The procedure has been semi-automated during the past few years and is considered especially suitable for laboratories doing large scale serial sectioning of tissues. The principal advantages of the technique are: savings in time and money by the semiautomation of the technique, lower cost of materials while preserving quality of staining, and a substantial reduction in the space required for storage. This method is best for relatively large structures; smaller ones are better handled by classic embryologic techniques.

The need for higher resolution in histologic sections has caused the development of plastic embedding material (Ashley and Feder, 1966; Leduc et al., 1963) and microtomes for sectioning larger tissues. Paraaffin embedding causes denaturization and shrinkage which is eliminated by the use of plastics. Tissues such as lymph node, placenta, appendix, bone, skin and other difficult to section material may be readily cut in plastic because of the rigid nature of the embedding media.

The development of the Sorvall JB-4 microtome has made it possible to cut these plastic blocks at thickness of 1/2 to 6 microns. These sections give far superior resolution, with no compression, thinner sections, and less embedding - damaged tissue.
C. MICROSCOPY

1. Light Microscopy

Transmission light microscopy has been traditionally used for examination of tissue sections. Transmission light microscopy depends on differences in the absorption of light by various cellular structures to reveal morphological characteristics. Because these differences are decreased with the thin sections needed for microscopy the specimens are stained to counteract the reduction in absorption. Staining has the additional advantage of identifying specific chemical groups. In empirical methods such as hematoxylin and eosin, although the exact chemistry is not known, the basis for separation of the cationic or anionic groups in the tissue forms the basis for recognizing principal cellular structures.

The utility of bright field microscopy is enhanced by superior histological preparations. Details of structural changes are often overlooked in poorly prepared slides. Many investigators, concerned with more quantitative laboratory techniques, fail to recall that microscopy is the technique fundamental to most of biological science.

Phase microscopy is also useful in examining living cells. Because differences in the thickness and density of living tissues will produce variations in the phase of light transmitted through these tissues, processing and staining to achieve contrast, which kills the cells, is not necessary. The phase microscope is also useful in observing unfixed, unstained tissues requiring no special specimen preparation. When performed on fixed, embedded tissues, thin sectioning is required to obtain specimens which transmit adequate amounts of light. Sections for phase microscopy are cut at a thickness of 0.5 μ for most purposes.

Phase microscopy may be performed using either bright or dark contrast. Phase microscopy can also be combined with fluorescence microscopy enabling the identification of the fluorescence. In this case, the phase is by transmitted light and the fluorescence by so-called epi-illumination.

When specimens are examined by dark field microscopy, the specimen details stand out bright against a dark back-
ground. Dark field microscopy is most commonly used for observing very thin, highly transparent specimens. It is much more successful at revealing surface detail than internal morphological characteristics. Dark field microscopy is also important in fluorescence microscopy where a dark field condenser is often used.

The phase microscope converts variations in the phase of light, normally undetectable by the human eye, into visible changes in light intensity. Phase microscopy is useful for studying detailed morphology of extremely thin, transparent specimens. These structures may be visible by conventional bright field or dark field microscopy; however, fine detailed morphology is not revealed with these latter techniques. An example of the use of phase microscopy is given by Thomassen et al., (1961) in the study of a transparent, colorless material deposited in the tissues of patients or of dogs treated with a silicone antifoaming agent used in pump-oxygenators during extracorporeal circulation. These deposits would not have been detected by conventional microscopic examination.

Interference microscopy is related to phase microscopy. However, the various systems available commercially permit estimation of the degree of phase retardation and thereby estimation of the dry mass of objects if the specific refractive increment is known. Interference microscopy also gives color contrast when white light is used. This can be useful for observing living specimens and has the additional advantage that the halos around particles such as mitochondria characteristic of phase microscopy are absent. A recent modification of this, termed Nomarski optics, has proven useful in screening for effects in unfixed living cells (Allen et al., 1969).

The polarizing microscope depends on the ability of certain substances to be doubly refractive which implies an ordered structure. Many biological materials possess this capacity to a limited extent and require sophisticated optics to detect the fine differences in structure. For example, the proteins amyloid and collagen are birefringent and small amounts of birefringence can be detected in the areas of membrane accumulation or packing, such as myelin, areas of packed rough endoplasmic reticulum and muscle. Here organic material is crystalline and tissues are highly birefringent. The polarizing microscope is useful in screening for the presence of crystals and with proper utilization the nature of crystals can be identified.
2. Fluorescence Microscopy

This technique is basically a histochemical technique because it provides information on the location of a chemical within the cell. Fluorescent microscopy can utilize naturally occurring substances (autofluorescence), dyes or fluorochromes (e.g., acridine orange) or fluorochrome tagged antibodies.

Some endogenous substances possessing alternating double bonds in their structure will fluoresce when exposed to the proper wavelength of light (autofluorescence).

The color of light emitted is characteristic for a given compound; quantification is possible because specific compounds will give maximum fluorescence at certain wavelengths of excitation. One example of autofluorescence in animal tissues is ceroid or aging pigment which emits a yellowish fluorescence in lysosomes, presumably due to oxidation products of degenerating membrane material. This may be useful as a sensitive indicator of cellular injury.

Some fluorochromes, such as acridine orange, which bind to lysosomes, can be introduced into cells. Changes in lysosomal structure may be indicated by differences in fluorochrome binding, which, in turn, are evidence of cell damage. Acridine orange staining can also be combined with automated methods for cell identification (Melamed et al., 1972 a,b; Adams and Kamentsky, 1971).

Greater specificity can be obtained with the fluorescent antibody method (Coons, 1958). Using fluorescent tagged antigens which retain their specificity for the antibody, accurate and precise identification of certain compounds within the cell is possible. The cell sites which bind the particular antigen are reacted with an antigen first, followed by addition of the fluorescent-tagged antibody specific for that antigen. Fluorescein-isothiocyanate is a frequently used dye for antibody labeling (Rygaard and Olsen, 1969).

Fluorescence microscopy can detect much lower concentrations of compounds than conventional cytochemical dye techniques. Disadvantages include the lack of natural fluorescence of many compounds; the inability to label some compounds with fluorescent tags; and the relative nonspecificity of attachment of the
fluorochrome. Fluorescent antibody techniques add specificity but are useful only for compounds with antigenic properties.

Fluorescence microscopy has been used chiefly as a qualitative technique for the location of specific molecules within the cell. Recently developed microspectrofluorometers permit quantification (Gurkin and Kallet, 1971).

The use of interference filters has been reported to improve immunofluorescent microscopy (Rygaard and Olsen, 1969). The main advantages of the interference filters are selective, bright, specific immunofluorescence, low or absent autofluorescence, and high contrast between fluorescence and background tissue. Much greater detail is possible with the filter because background fluorescence is eliminated.

Examples of the use of fluorescent microscopy include assay of oncogenic virus (Aaronson and Todaro, 1970) and rapid sorting of cells by immunological properties. Fluorescence has also been used in gross morphology to identify location of stored materials.

Time lapse cinematography is a useful method for detecting effect of compounds of living cells usually growing in vitro. Cells usually viewed with interference or phase microscopes are photographed at time intervals to detect slow intracellular movement and transformation that would be otherwise undetected during review of the recordings. This provides a rather sensitive way of observing directly the effects of injurious compounds on intracellular movement and organization.

3. **Electron Microscopy**

The electron microscope has found great applicability in the evaluation of normal and abnormal subcellular structures during the past twenty years. It is, for example, possible to determine if a cell has been lethally or sublethally injured and to estimate the degree of reversibility as well as the approximate duration following the onset of injury. Early changes that are difficult or impossible to detect by light microscopy are readily observed. For example, by light microscopy the effects of lethal ischemic injury in the myocardium are not observable until 8 or 12
hours after injury, whereas the earliest changes are detectable by electron microscopy within five minutes and irreversible change within one hour (Scarpelli and Trump, 1971).

The detection of sublethal change, for example, the hypertrophy of the smooth endoplasmic reticulum in the liver following administration of enzyme-inducing drugs, which is difficult or impossible to detect by light microscopy, is easily observable by electron microscopy after several days of drug administration. Having established the nature of change with the use of electron microscopy, it is often possible to screen for effects by observing 0.5 μ stained sections of well fixed, plastic embedded tissue by high resolution light microscopy.

In terms of evaluating the effects of injury on cells and correlating altered structure and function, the use of thin sections of fixed, embedded tissue observed by transmission electron microscopy is desirable for most purposes. The techniques involved in producing such thin sections have become standard and involve fixation in one or more chemicals such as glutaraldehyde, followed by osmium tetroxide post fixation, followed by dehydration and embedding in resins. Epoxy resins are most frequently used. Thin sections, approximately 50-100 μ, may be cut with various types of ultramicrotomes using glass or diamond knives and collected on small copper grids for insertion into the electron microscopes. Often one or more stains such as uranyl acetate and lead citrate are utilized to enhance contrast of intracellular organelles. Observations are made usually at magnifications between 5,000 and 50,000 and recorded on plates or film for later study.

To perform these tasks most efficiently, a centralized electron microscopy laboratory containing electron microscopes and associated equipment, staffed by appropriate professional and technical personnel, is most desirable. To estimate the capacity and cost of such a unit, the following may be helpful.

Serving the unit would be a well-trained pathologist experienced in cell biology, electron microscopy, and related techniques. Supporting professionals would include three to five associate investigators and a technical staff. The technical staff would include a supervisory technician, an electronics technician for microscope maintenance, two photographers, and four electron microscopy technicians for thin sectioning and processing. This
system would include three electron microscopes each with service contracts. Associated equipment would include film and developing facilities with nitrogen burst agitation, large film drying cabinets, five ultramicrotomes, freeze-etching equipment, shadow caster, 15 diamond knives, three enlargers with automatic exposure devices and automatic processing equipment, large print washer and print dryer.

Based on 1972 prices, initial expenses for equipping such an electron microscopy facility would be approximately $500,000 to $600,000. The annual operating cost, excluding professional and technical salaries, would be on the order of $100,000 per year. The output of such a unit would be approximately 200 to 300 pictures per day on a five day/week basis. A trained investigator using an electron microscope can examine approximately four specimens in one-half day. This would mean, for example, that the effects of an agent on the livers of four animals could be examined in a half day. The investigators would spend their time using the microscope and interpreting results. All other procedures would be carried out by the technical staff. In such a large system the approximate cost of producing thick and thin sections and all photography from one specimen, for example from a liver, would be approximately $50.

The scanning electron microscope differs most significantly from transmission, light, or electron microscopy in that micrographs with informative three-dimensional qualities are produced. The scanning electron microscope is capable of a wide range of magnifications which overlap those of the light microscope up to that of the transmission electron microscope. Ultrathin (0.05 μ) sections needed for transmission electron microscopy are not required for scanning electron microscopy. In general, sample preparation is substantially simpler and, because the method is new, a number of different techniques are currently employed.

While scanning electron microscopy has proven to be an extremely useful technique for revealing previously unstudied topography of the cell surface, little information is obtained on intracellular structures, although new methods appear to provide means of obtaining such information. Techniques for obtaining surface structure and subsequently sectioning and viewing the underlying internal structures are now being developed. Little information of the type obtainable by differential staining is available, as such staining techniques are not yet available.
High voltage electron microscopy employs large accelerating voltages up to 1,000 kilovolts or more, enabling visualization of thicker specimens because of the increased penetration of the electrons. The increased power of resolution is due to the shorter wave length (Fisher, 1972). Its biological applications have been explored to a very limited extent, but include the suggestion of visualizing living cells in thin chambers that could be penetrated by the beam.

The electron microscope may be used as an analytical instrument by measuring the X-rays generated in the specimen by the electrons that are passing through it. The high resolution morphology of thin specimens may be combined with simultaneous elemental analysis on the same areas of the specimen. There are several scanning or transmission electron microscope systems commercially available which combine X-ray microanalyses of elements with conventional electron microscopy.

X-ray microanalyzer electron microscopes record the X-ray emission given off by the constituent elements of the specimen. Each element, when bombarded by high energy electrons gives off X-rays of characteristic wave length and energy. The specimen may be scanned and the emitted X-rays passed to spectrometers which sort them into different wavelengths or energies. The amount of emission due to a given element can be measured. Alternatively, the spectrometer can be set to detect only the radiation characteristic of a particular element. This observation may be correlated with an electron micrograph of the particular area under study. Spots as small as 0.20 nm in diameter may be examined.

X-ray spectrometry will measure elements above the mass number of 12 (carbon) in amounts down to $10^{-14}g$ but is even more sensitive for heavier elements such as calcium, sulfur, iron and gold.

A number of applications of this instrument are immediately apparent. The elemental content of a cell organelle such as a lysosome can be determined directly from an ultra thin section. The proceedings of a recent symposium on biologic application of combined high resolution electron microscopy and X-ray microanalysis have been published (AEI Scientific Apparatus Ltd., 1972).
D. DIRECT MASS FRAGMENTOGRAPHY

The detection of extremely low levels of biological compounds is significant in histochemical techniques. Recent innovations in technology have extended the limits of detection of a wide variety of chemical substances to parts per billion. The method of direct mass fragmentography (DMF) has been used experimentally in several laboratories and is under development by instrument companies (Green, 1969, 1972). DMF is similar to combined gas-liquid chromatography (GLC) with mass spectrometry (mass fragmentography) for the detection of mass spectral fragmentations, except that the GLC inlet system is omitted - hence the term direct mass fragmentography. The record of the instrument can be analyzed for specific compounds and a computer search can be made for any compound that will pass through the semi-permeable membranes of the molecular separator.

DMF offers many possibilities in the overall program of NCTR in addition to the applications in pathological techniques. The method has been used to measure quantitatively a large number of drugs and their metabolites in biological fluids and to detect alcohol vapors from the skin of human subjects after ingestion of moderate doses of alcohol. It is ideally suited to detect volatile substances in the exhalation of animals and man at extremely low levels.

A method of this type could be used to measure the concentrations of chemicals and their metabolites in tissue sections selected by microscopy to provide a microanalysis of a discrete region of the tissue or cluster of cells.
Freeze etching or freeze cleaving provides a different view of membrane structures including surfaces and internal aspects of intracellular membranes. It is the only method presently available to us for studying large areas of opposing cell membranes. Cell junctions, which are thought to be responsible for intercellular electronic coupling, are demonstrated well with this technique. Cellular change can cause changes in the junctions (Berkeley Scientific Laboratories, Inc., 1971), (communication between cells) which can be shown morphologically with this technique. It is based on the use of frozen specimens which may or may not be fixed prior to freezing. The process of freezing produces fissures or fractures (cleavages) in the cell. The frozen specimen is cleaved under high vacuum and a replica is made by depositing platinum and carbon onto the frozen cleaved surface. Following removal of the replica from the cleaved tissue, the replica is visualized in the electron microscope (Koehler, 1968).

The use of negative staining has found wide application in visualization of subcellular particulates and in screening for certain types of organelle effects such as membrane alterations, as well as identification of microbiologic agents. Specimens, usually in suspension, are mixed with a suitable negative stain, a salt of a heavy metal such as phosphotungstic acid (PTA), and spread on grids and visualized in the electron microscope. Because of the thin nature of the specimen in many cases, the full resolution power of the electron microscope can be utilized.

Through the use of the diffraction image in electron microscopy, it is possible to identify crystalline or paracrystalline material or to examine spacings in periodic structures. Both high angle and low angle diffraction methods can be used. The low angle is used for repeating structures of a high spacing of the order of 10 nm in proteins. Wide angle diffraction is used for the identification of inorganic crystalline deposits. So-called limited area diffraction enables diffraction information from limited areas of the specimen to be obtained. Through the use of electron diffraction, it is possible to identify inorganic crystalline aggregates; for example, calcium phosphate minerals such as hydroxyapatite in tissues (Parsons, 1968).
E. AUTOMATION OF TECHNIQUES

Automated concepts and techniques in morphology involve two major areas: (a) preparation techniques for morphological study, and 2) automated methods of cellular, including image, analysis.

It seems evident that classic morphological pathology urgently needs methods for automated image processing including pattern recognition, partly because of the need to minimize professional time involved, but also to assure consistency and surpass current detection limits. Moreover, many studies involving correlations between structural, biochemical, or functional information are urgently in need of methods for quantifying images in a way that can be handled by data processing techniques. Recent automated procedure methods for image scanning in light microscopy suggest that these methods, together with image or pattern recognition programs, may ultimately permit the detection of cell alterations that escape visual recognition.

In many cases, automated morphological and morphometrical methods have advantages in terms of depth of understanding that may exceed considerations of reduced professional personnel time, and indeed in many instances the professional time may well be substantially increased. Such advantages also include acquisition of quantitative information, more sensitive methods of detecting alteration, better reproducibility between measurements and the capability for computer based image processing and correlation.

Presently, available types of automated morphological methods can be considered in four categories: (1) scanning systems depending upon measurements of optical density; (2) methods for area and shape determination; (3) continuous flow analytical systems based on scattering fluorescent or absorbence signals or continuous flow systems based upon conductivity measurements; and (4) optical correlation systems. All these systems require further development before their use in long-term toxicological testing could be adopted as routine procedures.
1. **Scanning Systems**

In these systems the image, which may be a drawing, photograph or the direct image as seen grossly or microscopically, is scanned by moving the specimen over a fixed beam or by moving the beam over a fixed specimen in the so-called flying spot systems. Several commercial instruments are available (e.g., APAMOS Program). Such scanning systems provide digitized representations of the optical density characteristics of the specimen at specified scan intervals. Digitized representations are then susceptible to a variety of analytical systems involving computers such as the Taxonomic Intracellular Analytic System (TICAS) (Wied and Bahr, 1970) and the Spectre II (Stein et al., 1969). Such systems have found applicability in chromosome analysis, in hematology, and potentially in exfoliative cytology. There is a wider field of applicability for these techniques based on selective stains for particular cell components and employment of other types of microscopy such as interference and electron microscopy.

2. **Automated Volume and Area Measurements**

Instruments for this task employ various television systems, such as the plumbicon tube, for scanning the image which is then subjected to modification including intensification or contrast enhancement. This permits rapid measurements of surface area/volume ratios and shapes. Contrast may be conferred on specific parts of the specimen by staining for specific enzymes such as acid phosphatase as a marker of lysosomes. Such instruments are available commercially and three well-known examples include the Vickers Quantimet; the Zeiss Videomat; and the Leitz Classimat. Such approaches are especially useful for rapid acquisition of morphological or chemical data in modes facilitating computerized data processing and analysis (Wied and Bahr, 1970).

3. **Continuous Flow Systems**

In these systems, cells in suspension are observed or measured while flowing through narrow channels within the instrument. For example, the Coulter Counter measures the number of cells and their individual volume by conductivity measurements with on-line computation of frequency distribution histograms for volume and number. Other instruments are available commercially in which the principle of flow systems serves morphological and biochemical
identification. Typically, they depend upon measurements of scattering, fluorescence, or absorbance (Drake et al., 1972; Melamed et al., 1972 a & b). For example, in one system devised for automated differential counts of peripheral blood leukocytes, various enzymatic methods are included giving specific reactions with each of the cell types. Reactions occur in separate channels to provide enumeration of the individual types of leukocytes by absorption measurements. In some systems measuring absorbance, it is possible to rapidly estimate numbers of dead cells by staining cell suspensions with supravital dyes such as trypan blue. Such methods may, in the future, be adaptable to solid tissues after preliminary steps including cell dissociation, and converting the solid organ to a cell suspension. These techniques may have potential in screening injured and dead cells (Adams and Melamed 1972; Hulett et al., 1970).

4. Optical Correlation Systems

Such a system is under development and is designed to permit separation of normal and abnormal histological sections of routine type (Bond et al., 1973). The system, in brief, is based on the use of the coherent light source from a laser which, after collumination, is passed through the specimen and through an objective lens. At the back focal plane of the lens, the so-called filter is created by photographing the hologram resulting from interference of a reference beam with the diffraction image of the lens. By viewing additional specimens using the filter thus produced, it is possible to compare the similarity by measurement of the intensity at the image plane and comparing it with that from the control. Preliminary results indicate that the system is effective in separating certain types of pathological patterns in abnormal livers from those seen in normal liver. The system appears to have the advantage that relatively large areas can be examined without the need of scanning, and may ultimately serve as a suitable screening procedure if various interfering factors, such as staining intensity, section thickness, and other types of artifacts, can be controlled.
V. BIOCHEMICAL AND CLINICAL PATHOLOGY

A. CELLULAR FUNCTION TESTS

Significant cellular functional changes can be demonstrated following exposure to injurious agents by examination of the biochemical and associated cell energy exchange mechanisms. It is usually possible to correlate this information with the morphological changes observed by electron or light microscopy and histochemistry.

1. Bioenergetics

Alterations of mitochondrial function are sensitive indicators of cell damage and in some instances appear to constitute the limiting factor in cell survival following exposure to certain types of injurious agents. Mitochondrial function can be assayed by first isolating mitochondria through any one of the standard differential centrifugation techniques followed by examining the mitochondria for respiration, phosphorylating ability, and so-called respiratory control (Hackenbrock, 1967, 1968). These methods can be performed accurately and are reproducible: They involve use of polarographic methods for determining oxygen consumption, isotopic determination of phosphate incorporation, and manometric techniques.

Other parameters of mitochondrial function that can be used as screening tests include rate of swelling measured by light scattering change in isosmotic media, rates of ion accumulation (e.g., calcium accumulation) and rates of contraction (Greenawalt et al., 1964). These tests may be correlated with morphologic changes in mitochondria in samples of the mitochondria from the assay system or from analysis of mitochondria in situ in ultrathin sections of tissue.

2. Protein Metabolism

Estimates of rates of protein synthesis can be made in vivo and in vitro by measuring the rate of incorporation of labeled amino acids into either whole cell protein or into microsomal protein. The effects on microsomal function can be studied in vitro incubating microsomes together with suitable cofactors and labeled amino acids and determining incorporation. It is relatively easy to separate smooth and rough microsomes, to estimate free and bound polysomes, and to study the differential effects on each type of subcellular structure. Studies of cell total protein content may be useful and can be done at the gross chemical level or by cytophotometry and specific protein staining.
3. Nucleic Acid Metabolism

Toxic compounds often influence nucleic acid metabolism. This may be measured by noting the effects on DNA or RNA synthesis or by incorporation into DNA or RNA of labeled thymidine or uridine, respectively. Total DNA and RNA can also be measured in the tissue as a whole or on individual cells using cytophotometry. Overall effects can also be measured. Cell cycle analysis may permit elucidation of differential effects on phases of the cell cycle which may allow estimation of effects on cell turnover.

In the case of protein, RNA and DNA synthesis, it must be stressed that the uptake of the precursor isotope administered, as measured crudely by the total acid soluble radioactivity, must be determined in order to rule out significant changes in rates of uptake under different pathological circumstances. This can be a major drawback in the interpretation of any data of macromolecular synthesis, especially when comparing a control and experimental group. The measurement of radioactivity in the macromolecule is not sufficient even as a function of time to give a valid index of overall rates of synthesis.

4. Transport and Secretion

Measurements of active transport are sensitive indicators of damage, especially in some organs such as the kidney where this function is of prime importance. This is assayed in the kidney by incubating slices of renal cortex with labeled transportable compounds such as 14C labeled para-aminophippurate. Transportability for sodium and potassium in the kidney or other tissues is often determined by first leaching the slices in ice-cold Ringer's solution which permits sodium to enter and potassium to leak out, returning the slices to warm (37°C) media and measuring the effects on sodium extrusion and potassium accumulation (Judah et al., 1964; Tosteson, 1964).

5. Cell Surface Properties

Many injurious chemicals produce cell surface effects that may impair cell behavior or survival. In other instances, they are intimately related to malignant transformations (Benjamin and Burger, 1970; Ben-Bassat et al., 1961). Properties of the cell surface can be studied by detection of altered surface antigens using immunofluorescent microscopy, measurement of surface charges, analysis of growth patterns in culture, and measurement of agglutinin binding sites such as those for concanavallin A. The effects on sodium transport systems at the surface, an important homeostatic
factor in cells, can be assayed by measuring the effect of the compound on the sodium-potassium stimulated, ouabain sensitive ATPase in whole cell homogenates or in fractions of the cell surface (Ginn et al., 1968).

Lysosomal structure and function, as well as amounts and distributions of lysosomal enzymes, are often sensitive indicators of cell damage (De Duve and Wattiaux, 1966). Lysosomal enzymes are predominantly acid hydrolases confined to membrane bound granules in which the enzymes are protected from substrate entry. Thus, in homogenates of normal cells the total acid phosphatase activity is low unless "activation" is instituted by procedures such as sonication, or freezing/thawing, etc., which damage the membrane and permit substrate access. It is usual to classify activities of lysosomal enzymes as total, free and unsedimentable activities. Total activity refers to the activity after complete activation; the free activity is the activity of the untreated homogenate; and the unsedimentable activity is the activity of a 100,000g x 30 minute supernatant. Damage to tissues is often followed by an increase in free and unsedimentable activity. Free activity is an index of increased permeability of the particles as well as to released enzyme while unsedimentable activity measures enzyme present in the soluble phase.

6. Cell Morphology, Shape and Motility

Cell shape and motility are associated with the activity of microtubules and microfilaments in the cytoplasm as modified by calcium ions and cyclic adenosine monophosphate (cyclic-AMP). The structural integrity of these filaments and tubules can be estimated by electron microscopy or by phase contrast time-lapse observations of normal motility characteristics of the cells. Compounds such as colchicine and the Vinca alkaloids affect the microtubule protein (tubulin) causing changes in these movements including interruption of the mitotic cycle at metaphase. A variety of cell phenomena are also affected by the compound cytochalasin which interferes with cell filaments (Zigmond and Hirsch, 1972). For example, secretion in many cases is linked to microfilament action. By selective use of cytochalasin with or without colchicine or the Vinca alkaloids, it is possible to explore the role of these components in the action of a new situation or compound.

Characteristics of the cell surface or other structure can also be probed by the use of both immunofluorescence and peroxidase labeled antibodies as well as by the use of electron dense probes which bind to the cell surface including lanthanum (Garant, 1972; Hashimoto, 1971; Shea, 1971), ferritin and ruthenium red (Luft, 1971a and b).
7. **Immunopathology**

Simple chemical substances can function as haptens and participate in the initiation of an immune or allergic response. Substances with related chemical structures that undergo similar metabolic alterations often possess similar antigenic properties because of a common hapten. Combined with proteins or as antigens themselves, haptens are useful as diagnostic indicators but because of the lack of absolute specificity in hapten antibody reactions, cross sensitization among related chemicals may occur. These immunological phenomena are well recognized from studies of drug reactions. Many drugs function as haptens and induce sensitivity or the allergic response (Davies, 1970).

Therapeutic drugs containing such groups as thiol, sulphonic acid, aldehyde, quinone or activated halogens are known to function as haptens (Davies, 1964). It is possible that environmental contaminants containing similar groups may induce formation of haptens and antigens. At the present time this is not known; however, specific antigens are known to be present in animals and man that may be ultimately utilized as markers for some cancers.

In certain animal and human carcinomas, circulating and cell bound antigens have been found which are not present in normal tissues of animals of comparative age. Certain of these auto-transplantation antigens may trigger the immune response. For example, Abelev (1965) described an \( \alpha_1 \)-fetal protein which occurs in the serum of many individuals with primary liver carcinoma. Similarly, Gold and Freedman (1965) have shown that a specific antigen is present in patients with carcinoma of the colon (see p 102). Assaying for embryonic antigens may be a useful technique for detection of certain cancers, provided the tumor has reached sufficient mass to release antigens into the blood or lymph. While these findings stem from observations of clinical cancer, the field is new, should be watched and exploited to the extent that similar changes occur in experimental species (Baldwin et al., 1972; Deckers et al., 1972; Woo and Cater, 1972).

Similarly, circulating and cell bound antigens or antibodies that are produced or induced in response to trace quantities of a substance with haptenic properties may be useful tools in identification of the potentially toxic hapten. Currently, antigen-antibody screening techniques, although relatively simple, are often difficult to interpret because of lack of knowledge as to the history of sensitization of the subject, individual differences in response, cross sensitivity and other factors. Use of these as screening techniques would be complex and, at present, would require extensive effort to correlate and interpret results.
In recent years, two areas of research in immunopathology have received increased attention. These include the development and study of the mechanisms of action of immunosuppressive drugs and the investigation of autoimmune diseases such as glomerulonephritis. The techniques and methodology developed in these two specialties may have some applicability to the problem of identifying immunologic aspects of adverse effects from long-term, low-dosage exposures.
B. TISSUE AND ORGAN FUNCTION TESTS

1. Introduction

Tests of this type may be general but highly sensitive as are those described under growth, or more specific although less sensitive as are the tests described under organ function. Abnormalities detected by these tests are secondary responses to basic cellular damage and indicate failure of the whole animal to compensate completely for injury sustained from a chemical. It is highly desirable, but often difficult, to correlate changes in function, as demonstrated by chemical techniques, with altered morphology.

2. Growth and Related Phenomena

a. Whole Body Weight

The rate of gain of whole body weight in growing animals and consumption of food and water are general but extremely sensitive parameters of toxicity. Reduced consumption of food or water, or failure to grow at the rate observed in control animals, are often the first expression of response to a toxic compound. However, these changes do not indicate the organ systems involved in the response to the toxic substance. Detection of organ specificity may be accomplished by clinical, chemical or morphological techniques.

The use of subnormal growth as an indicator of toxicity has been used for many years. It is essential that the normal or control group be comparable in genetic background to the test group. Percentile comparisons of experimental and control groups in animal colonies are useful in detection of growth impairment.

Many investigators have used weight loss in determining the maximum tolerated dose of a compound. This is defined as the dose of a compound that can be given either orally or parenterally without causing death during a six week period of administration.
A loss of 10 to 20% of body weight during this period is considered indicative of toxicity and, if this occurs, additional experiments are conducted to elucidate the origin of the toxic response. For some compounds, there is a very fine demarcation between a 10-20% weight loss and mortality; thus, 10-20% loss of weight may be at times too extreme a criterion for toxicity. Determination of food consumption and weight gain are techniques likely to be useful to NCTR in assessment of response to low doses of chemicals.

b. Organ Weight

Total organ weight is a highly sensitive and rapidly determined indicator of toxic response of a tissue to a chemical which would be of value to NCTR. Hypertrophy or hyperplasia can be confirmed histologically. Extreme changes in weight of an organ are almost always indicative of an abnormal tissue, and such organs should always be studied histologically. As noted previously (see p weight determinations are frequently very useful for expressing the results of chemical analysis.

3. Functional Profiles of Organ Systems

a. Liver Function Tests

Liver function tests based on chemical determinations that have been developed for use in clinical medicine include (Breen and Schenker, 1971):

Bilirubin (total serum bilirubin, serum bilirubin fractionation, urine bilirubin, urinary urobilinogen, and fecal bilirubin)

Bromsulphthalein retention

Serum enzymes (alkaline phosphatase group enzymes 5'-nucleotidase, glutamic-oxaloacetic transaminase, lactate dehydrogenase, glutamic dehydrogenase)

Prothrombin time

Serum proteins (albumins, globulins)

Tissue antibodies

Australia antigen

α₁-fetoprotein
Of the available tests applicable to small laboratory animals, serum enzyme assays have received the most investigation (Grice, 1972). The release of intracellular enzymes into blood and body fluids is associated with alterations in cellular membrane permeability and subsequent cellular necrosis (Dinnan et al., 1962). To correlate altered serum enzyme levels and morphological changes, the time course of these changes must be taken into account since cells may lose their ability to regulate the loss of enzymes long before they are judged necrotic by light microscopy. The maximal rise in serum enzymes may occur substantially before cell damage is evident by light microscopy.

Serum transaminases (SGOT, SGPT), lactate dehydrogenases (LDH), and alkaline phosphatase determinations have proven useful in detection of hepatotoxicity. Determination of isoenzyme patterns in addition to determination of serum levels of certain enzymes is considered highly desirable in assessing the organ affected by a compound (Nerenberg and Pogojeff, 1969). The isoenzyme patterns of LDH have been used to detect liver and renal damage (Cornish et al., 1970). For example, in hepatic injury, LDH-5 is primarily increased; while in renal toxicity, LDH-1 and -2 are elevated to the greatest degree. Grice (1972) reports in a study of rats exposed to a number of hepatotoxins (carbon tetrachloride, mercuric chloride, thioacetamide, and diethanolamine) that morphologic changes are generally present at dosage levels considerably below those necessary to induce detectable serum enzyme alterations. However, morphologic change may be limited to a small area of the liver. Hepatic damage induced by a toxin could be repaired by the time of autopsy and not detectable morphologically. Serum enzymes, while less sensitive to changes in small numbers of cells, could be monitored over a time period substantially prior to autopsy and thus permit detection of changes prior to regeneration of new hepatocytes.

Schein et al. (1970), in evaluating prediction of human toxicity of chemical compounds by animal tests using the dog and monkey, reported liver toxicity was never underpredicted by their experimental models. They considered serum alkaline phosphatase and serum transaminase determinations to be predictive of human liver toxicity. In these studies, bromsulphalein retention determinations added little information not provided by the serum enzyme determinations and were a source of false positive predictions.

Recently, the measurement of serum levels of gamma-glutamyl transpeptidase (GGT) has been recommended as a highly sensitive indicator of hepatic dysfunction (Lum and Gambino, 1972). GGT levels are considered valuable in detecting hepatic metastases
of nonhepatic primary carcinomas (Orlowski, 1963; Rutenberg et al., 1963; Lum and Gambino, 1972). Serum GGT is also elevated in acute pancreatitis but is not diagnostic of pancreatitis because concomitant liver disease occurs in many patients.

Serum enzyme determinations appear to be techniques which may be applied to large numbers of small animals if the methodology for a specific enzyme determination can be automated. A large number of enzymatic reactions can be monitored by automated techniques. The usefulness of multienzyme determinations in providing data on patterns of metabolism is worthy of further study (Knox, 1972).

b. Renal Function Tests

The kidney, like the liver, is frequently affected by chemical compounds; excretion of chemical compounds or their metabolites may be a factor in susceptibility. In evaluating the use of the dog and monkey in prediction of renal toxicity, Schein et al. (1970) consider the animal kidney to be more sensitive than is the human kidney to drug effects based on the relative frequency of false negative and false positive findings.

Histopathological assessment of renal damage is a highly sensitive technique. However, there are different opinions (Grice, 1972; Raab, 1972) on the relative sensitivity of morphological and clinical chemistry techniques. This difference is likely related to the type of nephrotoxicity produced. A consideration favoring use of clinical chemistry in screening for renal toxicity is the reversibility with time of certain renal lesions because of the regenerative capacity of the kidney. There are a number of tests of renal function which may be used to determine general renal toxicity and to determine the location of the lesion within the organ. For example, the inulin clearance test, readily performed in small rodents, is a measurement of glomerular function. Determination of the maximal ability of the kidney to secrete glucose or para-aminohippurate measures renal tubular function (Kassirer, 1971).

Techniques should be able to detect damage to proximal convoluted tubules since this is the site of most chemically induced renal lesions. Other techniques which appear most useful for assessing renal toxicity in large numbers of small animals include determination of blood urea nitrogen (BUN), microscopic examination of urine, determination of certain urinary enzymes, and determination of urinary protein, glucose, ketones and amino acids.
Determination of BUN is a commonly used procedure for assessment of renal toxicity in humans and has value for assessment of renal toxicity in small animals. Simplified chemical methodology has been developed by Dugle and Free (1960) in which BUN can be determined on a single drop of blood and be performed in about one minute per determination. Similar BUN values were found in all species studied, which included mouse, guinea pig, rat, and dog. Tests for BUN have been automated and are included in most automated clinical chemistry systems.

Microscopic examination of urine is performed on the sediment obtained from centrifugation of urine. This test most often involves simply microscopic examination of urine for red blood cells, white blood cells, epithelial cells, and casts. The determination may be quantitated on urine samples collected over several hours (Goodale and Widmann, 1969), and has been recommended for assessment of renal toxicity in small animals (Balazs et al., 1963).

Measurement of certain enzymes in the urine is a means of assessing renal damage. Changes in urinary enzyme levels may be a reflection of damage to tissues other than the glomerulus or renal tubules and for this reason it is desirable to measure serum enzyme levels as well as urine enzyme levels.

Urinary lactate dehydrogenase has been extensively studied in humans and has been found to be elevated in a large number of renal and urologic diseases. If a normal level of lactate dehydrogenase is present, renal disease is unlikely; but renal damage is not absolutely ruled out as its presence is indicative of disease in the urogenital system (Raab, 1972). Urinary alkaline phosphatase determinations appear of value for assessment of chemically induced kidney damage since all disease states which are accompanied by necrosis, decomposition or desquamation of renal tubular cells or by disturbances of glomerular filtration provoke increased alkaline phosphatase activity in the urine (Raab, 1972). Renal and bladder tumors are more frequently accompanied by elevated activities of urinary alkaline phosphatase than is the case for other disease (Dubach and Padlina, 1966).

For animals, the decrease of alkaline phosphatase activity of kidney tissues after renal damage can be correlated with increased urinary alkaline phosphatase activity (Raab, 1967; Pollak et al., 1960; Alibert et al., 1963). These two urinary enzymes are good indicators of renal toxicity but they do not provide differential information on the type of renal disease. However, their use in screening for renal damage would appear valuable.
Rapid determination of urinary glucose, protein, and ketones may be accomplished by testing with paper tapes marketed by a number of companies. While these changes are by no means specific for renal disease, they are very rapidly performed, may be associated with renal disease, and their alteration is generally indicative of metabolic abnormalities in some organ system of the animal.

Use of serum or urinary enzyme determinations is a technique for assessment of renal toxicity which can be applied to small animals. If chemical methodology for these methods is automated, these techniques can be readily applied to large numbers of animals.

c. Hematopoietic System Analyses

Routine evaluation of the hematopoietic system is desirable in assessment of the toxicity of a compound. Because of the rapid rate of synthesis, the cellular components of the hematopoietic system may readily demonstrate toxicity of a chemical. Routine evaluations include hematocrit; hemoglobin; enumeration of red blood cells, white blood cells and platelets; and a differential white blood cell count. All of these determinations have been automated and commercial equipment is available.

d. Cardiac Function Tests

Assessment of cardiac toxicity of a compound is highly important because of the inability of cardiac muscle fibers to regenerate following damage. In clinical medicine, a number of sophisticated techniques are available to determine cardiac status while the patient is alive. The most commonly used test is electrocardiography which measures electrical conductivity of the surface of heart muscle. This technique has been applied to experimental animals, particularly the dog, and can be adapted for use with small rodents (Grice, 1972). Schein et al. (1970) stress the need for an improved model for the monitoring of cardiac effects of toxic compounds and recommend use of a battery of physiological parameters including blood pressure, cardiac output, electrocardiogram, and pulmonary X-rays. Because of the irreversibility of the cardiac lesion, evaluation of histological sections of heart muscle obtained at autopsy should assess damage due to toxic effects of a compound.

Ischemic damage to cells of cardiac muscle will result in release of enzymes from the muscle to the blood stream. The ability to detect enzymatic changes will vary directly with the number of cells affected, and the maximal release of enzymes from damaged cardiac muscle cells follows a different time course for various enzymes. In the human, creatine phosphokinase is released earliest
with a maximum at about 8 hours after damage, and the level of this enzyme will be influenced by skeletal muscle activity. Serum glutamic-oxaloacetate transaminase shows a maximum at 24 hours, and lactate dehydrogenase shows the highest levels at 48 hours. These enzymes are considered useful aids in diagnosis of myocardial infarction in the human and would seem of value in assessment of cardiac damage in the experimental animal. However, because of the irreversibility of cardiac lesions, histological examination of tissues should provide adequate information.

e. **Neurological Toxicity**

Assessment of peripheral or central nervous system neurological toxicity depends almost entirely on clinical determination of the presence or absence of various reflexes. Techniques of detecting neurotoxicity by clinical chemical determinations are, at present, very limited. Histological evaluation and behavioral testing appear to be more useful.

f. **Skeletal Muscle Toxicity Tests**

Necrosis of skeletal muscle may be detected by determination of the enzymes creatine phosphokinase and aldolase in the serum. The level of creatine phosphokinase will be influenced by level of skeletal muscle activity, so appropriate controls must be utilized.

g. **Measurement Techniques**

Use of fluorometry rather than spectrophotometry will increase the sensitivity of enzymatic analyses in a number of tissues. Since the same enzymes are released from several organs – for example, lactate dehydrogenase from cardiac muscle, liver or kidney – the use of isoenzymes has been recommended as a means of determining which organ is the origin of the enzymes. This procedure is most frequently done by gel electrophoresis.
C. AUTOMATION

There is little doubt that use of automated equipment for chemical analysis has greatly increased the efficiency and production capacity of the chemical laboratory. Continuous flow analyzers dominate the field although a number of early instruments such as the Robot Chemist (Berkeley Scientific Laboratories, Inc., 1971) were produced. The Technicon Corporation, with the Autoanalyzer and later the Sequential Multiple Analyzer (SMA), has led the field of automated clinical chemistry equipment.

This report does not cover detailed aspects of technical information on automated clinical chemistry equipment because two recent reviews provide this information in depth. "A Study of Automated Clinical Laboratory Systems", Berkeley Scientific Laboratories, Inc., 1971; and "The Mechanization, Automation and Increased Effectiveness of the Clinical Laboratory", by the Automation in the Medical Laboratory Sciences Review Committee of the National Institute of General Medical Sciences, 1971, should be consulted for this information.

Since the publication of these reports, several developments in automated clinical pathology equipment have occurred that are of importance to NCTR. A recent new development in the commercial automated analyzer field is the GeMSAEC fast analyzer (Electro-Nucleonics, Inc.). The GeMSAEC differs from conventional continuous flow analyzers in that it employs a multiple cuvet rotor into which the measured samples and reagent solutions are simultaneously moved by centrifugal force from a transfer disc placed in the center of the rotor (Moss et al., 1971). This system will do photometric measurements of either absorbence or fluorescence. The system is ideally designed for use with a small on-line computer. The GeMSAEC is available commercially but is still undergoing technical refinement. The GeMSAEC can be used most profitably for very rapid single analyses of large numbers of samples, especially when small sample size is a factor. Recently, previously developed equipment has been modified for use with small samples (approximately 50 µl). For example, the SMA 12/60 (Technicon Corporation) has been modified to use smaller samples which are frequently encountered in studies of experimental animals. Damon Engineering Corporation markets a system called the Ortho-Damon which is specifically intended for doing large numbers of tests with small volume samples. Several companies (Gilford Instrument Laboratories, Inc.) are marketing new automated pipetting equipment which can
accurately and reproducibly measure 50 µl samples. Micromedics Laboratories produces an automatic pipetting instrument which will measure 10 µl samples reproducibly and accurately. In hematology, Technical Instruments Corporation has offered its most recent model, Hemalog D, which performs a totally automated differential leukocyte count.

The mention of specific instruments or manufacturers does not imply superiority over other products that are available. They are included as examples of types of rapid automated analyzers that will be required for biochemical and clinical tests of body fluids and tissues in experiments with large numbers of animals. The relative merits of commercially available automated laboratory instruments were reviewed by Moss et al. (1971). The NCTR should undertake or support evaluation of automated laboratory equipment most suited to its specific needs.
VI. GENERAL ASPECTS OF TOXICOLOGICAL EVALUATION

A. CONCEPTS OF TOXICITY

Toxicologists have developed numerous test systems to evaluate the effects of specific substances. These techniques are based upon knowledge of (a) the chemical and physical properties of the substance, (b) the amount, frequency and patterns of exposure, (c) the chemical and metabolic products in living systems after use, and (d) the types of injury produced in the test organism. From these data, the relationship between dosage and response can be defined and dose-response curves can be used to predict toxicity over a limited range of exposures beyond those determined experimentally. This prediction is based upon knowledge of the mathematical relationships between dosage and response. Theoretically, if all metabolic aspects of the interactions between a substance and a given organism were known, there would be a high degree of confidence in predicting the safety or harmfulness of the substance for the organism.

Classical toxicological techniques involve administration of graded doses of substances to several species of animals followed by administration to man. Initial studies usually focus upon establishing acute toxicity in relatively limited experimental time periods. Many types of test systems for evaluating toxicity have been developed, utilizing both observation of adverse effects on subcellular metabolic processes, cell and tissue morphology, tissue and organ function, and the correlations among various test systems. The most frequently tested toxicological endpoints are irreversible cellular injury with attendant functional or morphological alteration, the induction of neoplasia, effects upon genetic material, or alteration of normal development.

The classical toxicological approach is admittedly empirical, but historically it has been accepted as useful in evaluating potentially toxic substances. However, accurate identification of subtle effects due to prolonged exposure, perhaps for many years, to low levels of environmental contaminants or food additives, requires a significant commitment, beyond that of the classical approach.
The study of effects of prolonged exposures to low dosages of potentially toxic substances poses difficult problems. Long-term studies are time consuming, expensive, and may provide inconclusive results. It is difficult to relate the effects of exposure to the material under study because of intervening events in the life of the experimental subject. The delayed appearance of genetic alterations is difficult to assess because time is required for accumulation of data from several generations of the test organism. Lastly, when toxic effects are found in experimental animals, at given dosages, it is necessary to interpret the significance of the animal data in terms of human toxicity. For many reasons, this extrapolation from animal to man is difficult and potentially deceptive.

One of the most controversial subjects in toxicological evaluation is the concept of a "threshold" of toxicity. Exposure to a sufficiently large quantity of any substance, will produce injury; however, the corollary, that exposure to minute amounts of the substance is harmless, is disputed. Some toxicologists assume that there is a threshold below which no biological effects are elicited; however, this conceptual dispute is not directly relevant to the purposes of this report. The review discussions are oriented to the requirement for experimental methods that produce measurable effects, useful as tests of predictive value.

Normally, dose-response relationships are established in experimental animal models using dosages and responses that are essentially linear; the no-effect dose is calculated by extrapolation of the dose-response curve back to the abscissa (zero response). In actual practice, a safety factor is often introduced that is as large as 1/100th of the dose determined by the point at which the extrapolated curve intersects the abscissa. While this practice is accepted as empirical, it does have some statistical validity (Mantel and Bryan, 1961). They suggested that the mathematical nature of the dose-response relationship near zero dosage might be expressed as a probit curve. Then the dose which might theoretically affect one in 100,000,000 subjects at risk described as "virtually" safe was 1/100th of the dose with a 99 percent statistical assurance level and a conservative probit slope of 1 normal deviate per log. Mantel and Bryan (1961) used neoplastic responses as their data base, but their statistical approach can be usefully applied to other irreversible responses.
Assumption of other mathematical forms such as one-particle or logistic curves rather than the probit curve for expressing the "virtually" safe dose can reduce that value by several orders of magnitude (Schneiderman, 1971; FDA Advisory Committee on Protocols for Safety Evaluation, 1971; Cornfield et al., 1956). These statistical techniques, including that of Mantel and Bryan, do not address the question of a "threshold" dose, but provide a method for data analysis based upon an assumption of a "virtually" safe dose level.

There is some experimental evidence that suggests dose-response relationships may not be linear at minute dose levels. For example, Conzelman and Moulton (1972) recently completed a prolonged study of bladder tumor induction by 2-naphthylamine in beagle dogs. They observed that the total amount of carcinogen required for tumor induction was less when smaller daily doses were administered over 24-30 months than when larger daily doses were administered for 9-18 months. Conzelman and Moulton (1972) concluded that their data suggested a power of the treatment time \( t^n \) was a component of the dose-response relationship.

The determination of the dose at which a potentially toxic substance has little or no apparent biological effects is related to the nature of the effect being studied, and the accuracy and sensitivity of the experimental methods. At the present time, techniques to assess the long-term, low-dosage effects of exposures to environmental contaminants are generally considered to be incomplete and unreliable for predictive purposes. Assessment of these hazards requires accurate, reproducible, analytical methodology that will define the capacity of a substance to produce injury. Hazard, or the predictive evaluation of the probability that injury will result from association with that substance in the manner prescribed, can be assessed only when the spectrum of possible toxicity has been examined over the dosage range that approximates the prolonged exposure to minute amounts of the substance.

This problem is further complicated by incomplete knowledge of the fundamental biochemical and physiological responses of cells to foreign substances. Thus, while acute toxicity manifested by gross morphological or physiological changes are relatively well recognized, the subtle and difficult to identify accumulative alterations induced by chronic exposures to low-dosages, may require
long time periods for development. Finally, it is well recognized that toxicological evaluation in animals by itself can not insure absolute safety for man because the interpretation of data requires statistical prediction and value judgments.
B. GENERAL EXPERIMENTAL PARAMETERS

1. Introduction

Failure to thrive, less than normal weight gain, reduced food or water consumption, lower fertility, and ultimately shortened life span can be highly sensitive indicators of chemical toxicity. In many cases, the development of a specific pathological endpoint, e.g., pancreatic β-cell atrophy or adenocarcinoma may be preceded by these less specific changes in the animals. High quality animal husbandry is absolutely essential to evaluation of chemical toxicity in long-term, low-dosage studies because many variables other than the compound being tested may modify the health of the animal. These variables include the genetic strain of animals, freedom from endemic disease, and optimal nutrition.

2. Nutritional Aspects Influencing Evaluation of General Health Of the Animal

a. Diet

Biological effects of toxic compounds are influenced by the nutritional status of the experimental subject (McLean and McLean 1967).

The selection of diets for use in long-term toxicity studies must consider not only maintenance of health of the animal colony but also that the diet may be used as the vehicle for administration of the toxic compounds. In the NCTR program it is assumed that the experimental route of administration of the compound will correspond with the route of human exposure to the compound. In many cases, the exposure will be through ingestion.

Valuable guidelines for the nutrient requirements of certain species of laboratory animals have been published by the National Research Council (1972). Those recommendations are for groups of animals and are intended to meet the requirements of virtually all animals in a normal state of health, but may not apply to an individual animal. These recommendations take into account age of the animal and physiological states such as pregnancy or lactation.
The NRC report reviews the nutrient requirements of common laboratory animals such as the mouse, rat, hamster, guinea pig, cat and dog. However, it does not include other species infrequently or only recently used in nutrition research, e.g., the Japanese quail (Spivey-Fox and Harrison, 1964). If use of these types of experimental animals is contemplated, nutritional guidelines will need to be established.

Many commercial firms supplying diets attempt to deal with the problem of individual variations in the animal's requirements for nutrients by providing considerable excess above the recommended levels of nutrients, particularly for vitamins and minerals. These excesses also diminish such problems as loss of nutrients during storage and alteration of their bioavailability.

Increasing the levels of nutrients in the diet to achieve the requirement for all animals is further complicated by the problem that, although essential, many nutrients may be toxic when given in excess. There is also the further possibility of complex interrelations with the compound under test for potential toxicity.

It is impossible to make any general recommendation about the ratios by which levels of nutrients should be supplemented in the diet. However, in long-term, low-dosage experimentation, the importance of more accurate definition of nutrient requirements becomes mandatory.

The nutritional requirements of specific pathogen-free animals, anticipated at NCTR, must be defined and the interactions between the animal, the toxicity of a compound, and the level of nutrients in the diet should be studied.

Finally, if the oral route is used for administration of test compounds, the food consumption must be measured in order to determine the amount of test substance actually ingested by the animals. Thus, the homogeneous distribution of the test compound throughout the diet should be monitored during the experimental period.

b. Water

The drinking solution may be a convenient method of
administering the test compound. If used, the water consumption must be measured. However, equivalent amounts of the compound administered in the water may not produce the same effect or degree of response as when the substance is administered in the food.

There are a number of specific problems that must be addressed in the development of each experimental protocol. For example, the chemical structure may change in water solution or protracted exposure to light, e.g., decomposition or formation of dimers; altered taste of the drinking solution may affect food and water consumption; microbial contamination may occur over time; and, such complex factors as interactions with compounds in the diet during digestion and absorption can readily influence potential toxicity.

c. Problems with Specific Pathogen-Free Animals

Based on the decision to maintain the animal colony in a specific pathogen-free state, possible bacterial contamination of the animal colony by water or diet is a problem. Autoclaving of the diet has been suggested as a means of dealing with bacterial contamination. However, reduced bioavailability and destruction of some nutrients will occur. NCTR should continue to reassess this problem as they gain experience with the barrier system already designed for use.

3. Parameters of Growth and Development

Routine evaluation of the growth and development of a large group of animals receiving a test compound may be valuable predictors of possible toxicity. Monitoring the animal colony for such effects as litter size, total litter weight, sex ratio, infertility and life span are useful indicators of toxicity. These parameters have been considered in the development of NCTR research protocols.

4. Reproductive Aspects

The role of reproductive studies in safety evaluation of food additives and pesticide residues has been reviewed recently (FDA Advisory Committee on Protocols for Safety Evaluation, 1970). In this report for FDA, the Advisory Committee concluded that a suitably modified multigeneration test, patterned after the three-
generation test procedure of Fitzhugh (1968), would be the most useful approach to collecting data on the overall functioning of the reproductive system.

The NCTR program will require multigeneration testing. Guidelines for these studies are discussed in the report of the FDA Advisory Committee on Protocols for Safety Evaluation (1970).

Additional discussions of mutagenicity and teratogenicity are on p 58 and p 77, respectively.

5. Behavioral Parameters

Behavior is a well known but often ignored endpoint in toxicological testing, especially in long-term studies. Changes in behavior may result from changes in the nervous system, endocrine system, or in general metabolic capabilities. Often the behavioral change may be easier to detect than biochemical or morphologic expressions of injury and thus becomes a useful endpoint as part of a total pathologic evaluation of toxicity. For example, a gradual loss or degeneration of neurons in the central nervous system may be impossible to detect morphologically but may result in some change in behavior. Similarly, alterations in the rate of synthesis, storage, or release of neurochemical transmitters, such as acetylcholine or biogenic amines, may be difficult to detect biochemically, or the morphological changes that result might be undetected by currently available techniques; but, the basic alterations could be expressed and detected as changes in behavior.

However, the analysis of behavior of test animals is limited in value, and is not always the most sensitive endpoint because of the redundancy in the central nervous system. Many lesions, especially when focal, do not result in detectable changes in behavior.

Behavioral testing may involve such simple parameters as activity, alertness, sleep time, and eating habits; but more sophisticated tests for problem-solving ability, memory, and ability to learn have been devised for many species of animals. Primates are the most useful and can be subjected to the most critical tests.

NCTR should consider the inclusion of behavioral testing
in planning future research programs. Where possible, behavioral studies should be coordinated with other endpoints of toxicological evaluations.
VII. TECHNIQUES IN IDENTIFICATION OF MUTAGENICITY

A. BASIC MECHANISMS

1. Introduction

Advances in biochemical genetics have come primarily from the study of submammalian genetic systems. Sophisticated methodology in handling viruses, bacteria, and fungi have provided the foundations of current understanding of the chemical basis of heredity, the synthesis and transcription of deoxyribo nucleic acid (DNA), and the role of ribonucleic acid (RNA) in gene expression and protein synthesis. The study of other submammalian systems, such as Drosophila, have provided detailed information on chromosomal structure and function.

The genetic effects of radiation have been studied extensively for several decades, but the effects of environmental contaminants, food additives, and pesticides have not been investigated as thoroughly. Many geneticists would agree that chemical damage is more important than the possibility or the problem of radiation damage (Neel, 1970). While there may be no immediate danger of widespread genetic damage to man from exposures to small quantities of chemicals, Neel (1970) has concluded that the exposure of man to small amounts of these chemicals will ultimately increase human mutation rates. Because mutations are considered to be more frequently deleterious than beneficial, the increased exposure to a larger number of potential mutagens is considered detrimental to man. Therefore, there is an urgent need to determine any increases in the human mutation rate and an equally important requirement for techniques that identify potential mutagens. The following discussions emphasize techniques that are applicable in screening tests for mutagenic activity of potentially hazardous substances.

2. Types of Genetic Alterations

The concept of DNA as the primary genetic material is universally accepted. Alterations of DNA or modification of its transcription or chromosomal replication by a chemical or physical agent is recognized as harmful, if not potentially lethal. In
general, deleterious alteration of the genetic information may produce a change in the number of chromosomes per cell, recombination of existing genetic information, or an abrupt change in the genetic information; i.e., mutation (Srb, Owen, and Edgar, 1965). In general, ploidy is relatively easy to detect cytogenetically in organisms with readily visible chromosomes. Recombination is a normal phenomenon in meiotic division and in microbial reproduction involving reciprocal exchange of genetic information between two homologous DNA molecules or chromosome segments.

Freese (1971) has defined mutation as hereditary alteration that involves neither ploidy nor recombination. One contemporary definition of mutation includes an arbitrary division of defects on the basis of chemical configuration of the lesion rather than on the basis of the gene, the organism, or the phenotype expressed (Drake, 1971). According to this concept, mutations involving chromosomal aberrations such as breakage, deletions, duplications, inversions, and translocations are called macrolesions. Those mutations which usually involve one or a few DNA base pairs are termed point mutations or microlesions and include transitions, transversions, deletions, and frameshift mutations (Herriott, 1971). These terms refer to substitution of one purine for a pyrimidine or vice versa, the loss of one or more base pairs, and insertion or deletion of one or more extra bases in the replicated DNA, respectively. Frameshift mutations alter transcription from DNA to RNA because the sequence of 3 base pairs (a frame) is shifted. There is no well-defined boundary between a large frameshift mutation and a macrolesion. Similarly, the exact number of nucleotide base pairs involved in a point mutation can not be defined exactly. Nevertheless, the terms are useful. For a more complete discussion of the molecular basis of mutation, the reviews by Drake, (1971, 1970) and Freese (1971) should be consulted.

Chromosomes are composed of numerous macromolecular units in addition to DNA. The specific genetic information in the DNA molecule is arranged linearly and consists of the sequence of nucleotide base pairs in the two strands of the nucleic acid. Prior to meiotic or mitotic division, the double stranded DNA replicates itself, each strand serving as the template for a new complementary strand. The stability of the DNA molecule is remarkable, considering that it exists in a dynamic chemical system that contains numerous deleterious physical factors that could induce mutation (Table 1). Similarly, it is not surprising that macrolesions occur most frequently during mitosis and meiosis when the chromosomes are doubling and separating.
interpretation requires scientific judgment by knowledgeable experts.

3. Repair of Genetic Alterations

Once produced, mutation may not be expressed because the alteration is lethal to the cell or it is corrected. Macrolesions, if severe, usually lead to cell death or aberrations of normal cell division. Some macrolesions are corrected by further changes termed suppressor mutations (Freese, 1971). Most cells contain metabolic systems that correct errors in DNA or repair mechanisms that induce reversion of altered DNA to the normal structure.

In principle, three methods of DNA repair mechanisms exist: (a) the damage may be repaired in situ by chemical alteration; (b) the altered nucleotides replaced; or (c) the altered section of the genetic information bypassed in transcription. This third method involves recombination and may operate in the absence of cellular recognition of the DNA damage (Hanawalt, 1972).

Repair phenomena were first discovered in bacteria and most of our current understanding of these processes has come from microbial systems (Dulbecco, 1949; Kelner, 1949; Hanawalt, 1972). However, Cleaver (1968) has shown that normal human skin fibroblasts are capable of repair and replication after ultraviolet irradiation. Brutlag and Kornberg (1972) have shown that DNA polymerase I can function to correct errors in mismatched nucleotides at the terminal end of the DNA chain during synthesis.

While the techniques of biochemical genetics used in studying repair mechanisms are not directly applicable to screening for mutagenicity, theoretically, several possible applications might be useful. For example, test strains of bacteria are known that lack the excision repair system. These are 10 to 100 times more sensitive to mutagens when compared to parent strains (Ames, 1971). It is possible that clones of diploid mammalian cells with similar characteristics could be developed for use in a modified host-mediated assay (see p 71).

Perhaps the role of DNA error correction and repair is most significant by itself. It has been found that chromosome breaks and errors in DNA occur more frequently than heretofore
believed. If this is confirmed by additional observation, then the occurrence and expression of mutation may signify disruption or inhibition of the repair mechanism rather than the aberration of DNA error itself. This concept suggests that mutagens may be mutagenic because they affect the activity or efficiency of repair mechanisms. This idea is consistent with views of DNA repair as an evolutionary process. The concept has not yet produced techniques for investigating mutagenesis per se but appears to be a reasonable approach that requires further clarification.

4. Chemical Mutagens

The mechanisms of mutagenesis of many physical and chemical agents are remarkably similar. Certain mutagenic agents such as ionizing radiation and alkylating agents have been shown to produce macrolesions in mammalian test systems and to induce microlesions in several submammalian test systems. It is generally assumed that these mutagenic agents also produce microlesions in mammalian cells, but definitive test procedures demonstrating this activity are lacking.

Chemical mutagens may be active per se or they may become active when metabolically altered by enzymic or nonenzymic reactions of the host tissues or microbial flora of the host tissue. Thus, submammalian test systems may not detect mutagens that require metabolic activation in the host. Moreover, the more practical and sensitive the mutagenicity test system, the further the test organism from man phylogenetically (FDA Advisory Committee on Protocols for Safety Evaluation, 1970).

However, there is sufficient knowledge from presently available data to suggest that prediction of the potential for induction of mutagenicity based upon chemical structure is possible. Freese (1971) has recently collated the available information on the effects of various chemical groups upon DNA and chromosomes. Miller and Miller (1971) have reviewed the mutagenic activity of carcinogens; Kalter (1971) has compiled current data on mutagenic teratogens and teratogenic mutagens; and Bateman and Epstein (1971) have summarized the activity of a large number of substances in the dominant lethal assay. These reviews should be consulted for further definitive information on the relationship between chemical configuration and mutagenicity.
B. TEST SYSTEMS

1. Submammalian Test Systems

There are numerous assay systems for the detection of chemical mutagens that use microbial species as indicators (Hollaender, 1971a). The viruses, especially bacteriophages, various strains of bacteria and fungi are recognized as excellent tools in basic studies of biochemical genetics. As indicator organisms in screening for mutagenic effects of substances, microorganisms have numerous advantages including high sensitivity, ease of test procedures, simplicity and economy. For example, some viruses are chemically unique in that the DNA is naked. Viruses such as the bacteriophages have extremely short generation times, and large populations of genetically stable virus particles are easily maintained. Various bacterial systems and certain yeasts that exist in both the haploid and diploid phase have distinct advantages in testing genetic effects of potential mutagens. Ames (1971) suggests that bacteria are the system of choice for mass screening of new compounds because of simplicity, sensitivity, and economy.

Other procedures utilizing insects such as Drosophila, plant meristems or invertebrate animals have been developed for detection of mutation. These nonmammalian systems avoid the difficulties inherent in experiments involving large numbers of mammals. In addition, submammalian tests are relatively efficient, requiring minimal expenditure for facilities and professionally trained manpower. Submammalian systems, as prescreening procedures, provide a convenient method of studying genetic effects of several dosages of a substance on large populations of several diverse species. Positive results would be indicative of a need for subsequent testing in mammalian systems; however, negative results suggesting no mutagenic effects do not obviate the necessity for subsequent mammalian testing. Despite this latter drawback, many knowledgeable scientists consider submammalian test procedures logical choices in screening for mutagenic effects of chemicals, especially when large numbers of substances must be tested (Ames, 1971; Drake, 1971).

While submammalian test systems are important in understanding the molecular mechanisms of mutagenic action, these types of tests are not directly relevant to mammals. Microbial
test systems do not reflect mammalian host metabolism which may alter certain compounds to active forms, detoxify substances that do induce microbial mutation, or may repair genetic damage in situ. Because of these major disadvantages, the use of submammalian test systems and in vitro cell cultures are considered to be ancillary procedures in the detection of mutagenicity in man (World Health Organ., 1971; FDA Advisory Committee on Protocols for Safety Evaluation, 1970). The NCTR program should be guided by the conclusions of these two reports.

2. Mammalian In Vivo Techniques

a. The Specific Locus Test

The most productive mutagen screening procedure utilizing mammalian organisms has been the specific locus technique which has been developed with the mouse (Russell, 1951). This method has found its greatest use in the assessment of genetic effects of ionizing radiation. Cattanach (1971) suggested that specific locus tests can be used in a similar manner to reflect the effects of environmental and chemical mutagens.

This technique consists of mating treated and untreated animals, usually mice of either sex, with a strain known to be homozygous for a number of recessive genes with readily visible phenotypic expression. The progeny from these matings will be normal unless a dominant mutation has occurred at one of the recessive gene loci. If such a mutation has occurred, an observable modification of the phenotype will be produced for there are a series of multiple alleles known, with graded dominance or recessiveness to one another, at several loci. Thus, the specific locus test provides a relatively simple means of estimating mutation frequencies at recessive gene loci.

Several stocks of multiple recessive tester mice have been developed (Carranach, 1971). The "T" stock developed by Russell at Oak Ridge carries seven recessive genes in the homozygous condition. A second stock developed at Harwell, the HT stock, carries six recessive genes only one of which is common to the T stock (Lyon and Morris, 1966).

The specific locus method has a number of advantages in assessing the capacity of potential mutagen to induce genetic alteration. For example, specific mutations at known chromosome loci can be observed in premeiotic germ cells of the male and in
oocytes. Similarly, preparations of spermatagonia and spermatocytes can be scored for induction of aberrant chromosomes, chromosome breakage and other phenomena. Similarly, the alteration of the characteristics of the phenotype which are indicative of mutation can be observed in offspring carried to term.

However, the specific locus test has certain disadvantages which for the present appear to preclude its use in screening for the effects of long-term, low-dosage exposures. The primary drawback is lack of animal strains homozygous for a large number of phenotypically observable recessive mutations. These are exceedingly rare. In addition, the specific locus test requires that large numbers of progeny be scored to detect statistically meaningful estimates of mutation frequency.

The test might be usefully employed following the establishment of a correlation between other more readily utilized mutagenic techniques. Because the procedure does denote alteration of specific loci on certain chromosomes, the test may be useful as a method of providing additional information on the mechanism of genetic damage produced by specific chemicals (Cattanach, 1971).

b. **Dominant Lethal Method**

The dominant lethal assay has been used successfully in measuring the mutagenic effects of radiation and the mutagenic potential of numerous substances (Bateman and Epstein, 1971). A positive test is indicative of major genetic damage that has its basis in numerical and structural chromosome aberrations (Epstein and Rohrborn, 1971). This damage is expressed as increased preimplantation losses of nonviable zygotes, early fetal deaths, and reduced fecundity in first generation progeny. Bateman and Epstein (1971) have prepared a complete review of the test procedures and the various ramifications.

The procedure involves administration of subtoxic dosages of suspected mutagens to male rodents and subsequent sequential mating of treated animals with groups of untreated females. In mice, matings are made in weeks 1–2, 3–4, and 5–8 after treatment; in rats, matings are made in weeks 1–4, 5–6, and 7–10. These three time periods in each animal represent samples of postmeiotic, meiotic, and premeiotic stages of spermatogenesis respectively. Females are examined for vaginal plugs and later sacrificed and dissected at midpregnancy, where corpora lutea and total implants are observed.
Implants are recorded as early and late fetal deaths, and living fetuses. The mutagenic effects can be expressed as an index of early fetal deaths/total implants X 100 (Epstein and Rohrborn, 1971).

The technique has been modified by Epstein and Shafner (1968) for large scale toxicity tests in mice. This procedure eliminates the time-consuming counting of vaginal plugs and scoring corpora lutea, and substitutes comparisons of pre-implantation losses in females mated with treated and control males. Epstein and Rohrborn (1971) recommend this modified assay procedure for screening studies in mice; but with rats, matings must be timed and corpora lutea counted.

However, the mutagenic index presupposes that the number of early fetal deaths is proportional to the total number of implants. Since both the number of early fetal deaths and the total implantations may vary, the mutagenic index may be misleading; for example, at relatively high sublethal doses where total implantations are reduced, few or no early fetal deaths would provide a mutagenic index which would not reflect the absence of implantation.

In a subsequent study of tris (aziridinyl) phosphine oxide (TEPA) and tris (2-methyl-1-aziridinyl) phosphine oxide (METEPA), Epstein et al., (1970) used the modified technique but measured pregnancy rate, mean early fetal deaths per pregnancy, and mean total implants per pregnancy. These three measures provided dose-related data indicative of subtoxic induction of mutagenicity. It should be noted that use of these endpoints does not require the time and manpower necessary for other endpoints such as checking for vaginal plugs or counting corpora lutea.

A more critical protocol for the dominant lethal assay has been prepared recently (Epstein et al., 1972). Based upon rigorous statistical analyses, the only experimental parameters (found to be valid) were early fetal deaths, percent pregnancy, and total implants per pregnancy expressed on a per female basis. These criteria are based upon the conclusion that reduction in total implantations is only an indirect expression of dominant lethal mutations. Reduction in total implants is not equivalent to genetically induced preimplantation losses because these may be of nongenetic origin; for example, maternal infection (Rohrborn, 1968). Thus, Bateman and Epstein (1971) indicate that the most critical parameter of dominant lethality is increased in early
fetal deaths. Because the mutagenic index is a ratio of two variable parameters, it should not be used as a measure of mutagenicity. The previously reported mutagenic activity of numerous substances requires additional confirmation.

In a recent review of 58 chemical substances tested by the dominant lethal method, Bateman and Epstein (1971) found that many substances reduced total implantations or had other antifertility effects; however, only alkylating agents, as a group, caused significantly increased early fetal deaths. Epstein et al., (1972) in a more extensive examination of 174 substances confirmed the mutagenic effects of alkylating agents and the absence of mutagenic effects in the majority of other substances.

The dominant lethal test is in one sense an extension of in vivo cytogenetic evaluation over time. It is a useful marker of genetic damage to the developing germ cells during spermatogenesis. However, the actual mechanisms of premeiotic, meiotic, and postmeiotic chromosomal alterations within the tests require additional investigations. Similarly, the occurrence of dominant lethal mutations in the oocyte is worthy of further study. In the oocyte, meiosis is completed in the mature egg and no postmeiotic cell divisions occur as in the spermatogenesis. Thus, chromosomal damage may not be selected out prior to fertilization.

Data from several studies exhibit considerable variation in the rate of mutation induced in male mice over the sequential mating period after treatment. These variations reflect the response of the male germ cell during development and could signify primary sensitivity based upon the physical status of the chromatin, selection of certain cells and deletion of others following the meiotic division, or specific metabolic factors in spermatogenesis (Bateman and Epstein, 1971). If sperm cells mature and fertilization takes place, the dominant lethal damage may be expressed in abnormal development of the zygote. Chromosomal breakage, formation of anaphase bridges, monosomy and trisomy in early cleavage have been suggested (Bateman and Epstein, 1971).

Interference with repair mechanisms is also a possibility that requires investigation. Elucidation of the mechanism of genetic damage would enhance the applicability of the dominant lethal assay to long-term, low-dose toxicity studies.
One of the major advantages of the dominant lethal test is that it can be conducted as a part of any experiment without alteration of the experimental protocol. For example, in a chronic toxicity test, treated males in the experimental and control groups can be mated with untreated female animals which are not a part of the major study. After conception, females can be sacrificed and expression of dominant lethals noted from preimplantation losses and early fetal deaths. The males are returned to the chronic toxicity test and can be used in additional dominant lethal or other toxicity studies subsequently. Progeny of the initial matings can be evaluated for sterility or translocation phenomena in further matings with control animals.

A major drawback of the dominant lethal assay in chronic studies is that the procedure is based upon induction of dominant lethal mutations. Such genetic damage is not directly applicable to man because such mutations in man are not common and would be spontaneously aborted. Another factor that may influence the application of the dominant lethal test is the possible lack of genetic expression in the generation being tested (Lavappa and Yerganian, 1971). These reservations should be viewed in relation to the fundamental purpose of identifying genetic damage. The dominant lethal assay would be used in concert with other studies such as in vivo cytogenetics, teratological tests, and other toxicologic procedures. As noted by Epstein et al., (1970), toxicologic screening tests, including the dominant lethal system, should be integrated into a protocol that evaluates numerous endpoints of toxicity in the same test animals.

c. Detection of Polygenic Effects

Experimental protocols have been developed that evaluate mutagen induced changes of a polygenic nature by comparing treated and control F1 and F2 generation progeny survival to weaning (Crenshaw and Yake, 1968) and body weight means and their variance (Soares and Crenshaw, 1970). Treated and control male mice are mated to one female each. The resultant progeny are compared with respect to (a) body weight at 3 and 4 weeks; (b) number; and (c) sex ratio of young carried to weaning.

At sexual maturity, males in the F1 generation from the treated parent cross are mated with nonsibling females from the treated parent cross. Similarly, control males are crossed with nonsibling females from the original control group. Difference in body weight variances, number of young carried to weaning, and sex ratios are compared by standardized statistical analyses.
In a study of the effects of 150 mg/kg of ethyl methanesulfonate (EMS) Soares and Crenshaw (1972) have shown that a single dose of EMS induced significant reductions in body weight variances in F\textsubscript{1} treated males and females over control F\textsubscript{1} animals. In contrast, the F\textsubscript{2} treated progeny exhibit increased variance in body weights over comparable control group values. The mean body weights in treated and control animals in both generations were not significantly different. The investigators suggest that the increased phenotypic variance in the experimental animals is the result of different frequencies of induced polygenic mutations in the heterozygous combinations in both F\textsubscript{1} and F\textsubscript{2} generations. The data also indicate a reduction in the number of females in the F\textsubscript{2} generation. The reduction averages about 10% when compared to control groups. The genetic basis is unclear, but this trait may be useful phenotypic expression of polygenic recessive mutations.

This mutagenic test system has several distinct advantages. Determination of the endpoints of genetic damage (number of young carried to weaning, body weight variance, and sex ratios) do not require autopsy of test animals. All that is required is a sufficient number of animals for statistical accuracy and maintenance of experimental and control groups for three generations. Furthermore, the frequency of dominant lethal mutations can be determined in parental females by uterine dissection after progeny are weaned (Soares, 1972). Additional genetic studies on any one or all three generations are possible after the F\textsubscript{2} generation reaches sexual maturity.

This type of mutagenicity test system could be conducted concurrently with chronic toxicity tests, carcinogenicity studies, or investigations on induction of teratogenicity using the animals for several studies. Assuming that reproductive ability, body weights, and growth are experimental parameters that will be investigated in most chronic studies (see p 53), this system of detecting polygenic recessive trait modification as well as dominant lethal mutations provides a convenient and efficient protocol for mutagenicity testing. It is suggested that further evaluation of this methodology be undertaken by NCTR.

d. Cytogenetic Studies

There is general agreement that cytogenetic techniques are useful and convenient methods for evaluation of mutagenicity (FDA Advisory Committee on Protocols for Safety Evaluation,
1970; Cohen and Hirschhorn, 1971; Hollaender, 1971b). Chromosomal alterations can be readily observed and scored directly by light microscopy. Cells from the bone marrow, testes, and regenerating liver have been used extensively in assessing the effects of chemical mutagens. Cells from the bone marrow have a high turnover rate and are extremely sensitive to mutagens; testicular tissues contain readily accessible germ cells; and regenerating liver parenchyma cells reproduce slowly, providing an opportunity to assess cumulative effects of exposure to mutagens.

Cytogenetic studies may be done in vitro using cultured human cells, or in vivo using certain cells from treated animals, usually mice or rats. In the in vitro studies, cultured cells are exposed to a potential mutagen at various concentrations for standardized times. Following the incubation period, cells are harvested, fixed, and stained for light microscopy. The stained chromosomes are scored at metaphase and anaphase for abnormalities. In vivo studies involve intraperitoneal or per os administration of the suspected mutagen to young, randomly bred male rodents. Animals are sacrificed after the incubation period, and cells of bone marrow or testes are prepared for light microscopy. Aberration in chromosomal complement are recorded in cells observed at metaphase.

The techniques of chromosomal methodologies in mutagen testing have been reviewed recently by an ad hoc Committee of the Environmental Mutagen Society and the Institute for Medical Research (Nichols, et al., 1972). This group concluded that cytogenetic testing should be employed as one of several tests for mutagenicity and should include an in vitro and an in vivo mammalian system. They considered the in vitro test system ancillary to the in vivo procedures and recommended human diploid fibroblasts and human diploid epithelial cells as the cells of choice. Scoring of both metaphase and anaphase preparations was suggested.

The committee recommended the young adult randomly bred rat as the animal of choice but suggested that mice could be used. They indicated a need to restrict in vivo testing to these two animal models in order to create a coherent body of data upon which current procedures could be evaluated and future test methods could be compared. Chromosomal abnormalities should be scored in bone marrow preparations at metaphase. These suggestions should be incorporated into the NCTR program of mutation research.

The report of the ad hoc committee (Nichols, et al., 1972) and the recent review of Cohen and Hirschhorn (1971) provide
specific details of test methodologies and protocols for dosage regimens. Both reports indicate the importance of standardization of experimental protocols, scoring, and terminology in data reporting as a basis for universal comparisons of test results. Automation of preparative procedures and use of record sheets amenable to computerized data storage, retrieval, and analysis are suggested. The committee concluded that periodic review of specific test protocols, modifications of procedures, and review of data would be valuable.

It is suggested that these definitive reviews (Nichols et al., 1972; Cohen and Hirschhorn, 1971) be utilized as the basis of the formulation of the NCTR program of cytogenetic testing.

e. **Host-mediated Assay**

This technique uses microbial mutation as an indicator of mutagenic activity but depends upon a mammalian host to mediate the microbial response. Gabridge and Legator (1969) introduced the host-mediated assay as a technique that combined *in vitro* testing of microbial sensitivity with *in vivo* metabolism of the test compound by the mammalian host.

In the assay, the compound under study is administered to the test animal, usually a rodent, and the microbial indicator is administered by a second route, usually abdominal injection. After a predetermined incubation time, the microorganism is withdrawn from the host and the number of mutations determined. The comparison between the mutagenic action of the compound on the microbial species *in vitro* and on the microbial species in the host-mediated assay is either (a) an indication of the detoxification of the compound by the mammalian host, or (b) the formation of mutagenic products by metabolism of the substance by the mammalian host.

Indicator organisms employed in this test system include a series of genetically well characterized histidine auxotrophs of *Salmonella typhimurium*, a canavanine mutant yeast and a heterokaryon of *Neurospora crassa* (Legator and Malling, 1971). *In vitro* studies on the mutagenicity of test substance in the microbial organism are run prior to, or concurrently with, the host-mediated assay. A number of substances have been shown to exhibit mutagenic properties by this test procedure (Legator and Malling, 1971). However, these experimental protocols have been developed recently and only limited experimental data on a wide range of
chemical substances have been developed.

Legator and Malling (1971) have suggested that the outstanding feature of this assay is its characterization of the ability of the host to detoxify a mutagen or metabolically alter a substance to a form which induces microbial mutation. They point out that the procedure, although indirect, may be applicable to correlation of mutagenicity and carcinogenicity in animals.

Most investigators would agree that, at the present time, the host-mediated assay is an interim procedure. The procedure may be useful in a battery of screening techniques for identification of potential mutagens; however, the applicability of this procedure in assessing mutagenicity of specific substances to long-term, low-dosage exposures requires further study. The host-mediated assay does not take into account the basic differences between the relatively naked microbial genome and the protected deoxyribonucleic acid of the nucleus in the mammalian cell. As with other mutagenic test procedures, the host-mediated assay does not reflect deoxyribonucleic acid repair mechanisms of the mammalian cell. Finally, another deficiency of the host-mediated assay is the absence of a method of evaluating induction of mutagen in the mammalian host tissues. However, this objection might be circumvented by the use of human tissue culture cells rather than microorganisms as the indicator cell type. NCTR should consider further study of modifications of the host-mediated assay.

f. Additional Methods of Mutagenicity Testing

The majority of techniques of mutagenicity testing presently in use have been developed from existing knowledge of the model system. For example, the biochemical simplicity, short generation time, and ease of handling makes bacteriophages ideal systems for investigations of biochemical genetics. Similarly, the existence of phenotypically observable recessive mutations in certain strains of mice provides a basis for the specific locus test. It is generally agreed that the procedures outlined in this report are useful, but require further improvement for enhanced utility within the scope of the NCTR mission.

In addition to these techniques, there are other procedures which may be applicable to evaluation of mutagenic potential of exposures to environmental contaminants over long time periods. The following methods are included as procedures that require further
methodological refinement to assess their usefulness in long-term, low-dosage toxicity studies.

Electrophoretic separation of proteins is a recognized method of identifying specific proteins and protein related diseases; e.g., hemoglobinopathies. Electrophoretic separation of proteins within the oocyte, zygote, specific tissues of the early embryo, or blood of progeny of treated animals might be a technique of identifying mutational damage produced by exposure to certain substances (see p 35).

Glenner et al., (1971a, 1971b, 1971c) and Banes and Glenner (1968) in a study of amyloidosis, have shown by amino acid sequence analysis and immunochemical studies that the amyloid protein fibrils which are characteristic of this disease are primarily amino terminal variable segments of the light polypeptide chain of homogeneous immunoglobins. In this instance, X-ray diffraction and immunological techniques have been used to elucidate the structure and synthesis of specific proteins associated with a specific disease process. In other situations, similar techniques might be useful in identifying changes in protein synthesis which resulted from chemically induced alterations of genetic information.

Recently, fluorescent microscopy using tagged antibodies or fluorochromes to reveal banding patterns of chromosomes has been used to identify a human triploid genome (Uchida and Lin, 1972). Wolman et al., (1972) employed similar techniques in differentiating banding patterns of chromosomes in normal and malignant cells of rat liver (see p 25).

These types of definitive chemical tests are not generally included in procedures for mutagenicity testing. However, they afford a different approach to identification of genetic changes and are worthy of further study. A number of procedures have been developed in other disciplines such as oncology and biochemistry that identify structural or functional changes in nucleic acids (see p 98 et seq.).

A WHO scientific review group (1971) has suggested additional research areas that may provide useful mutagenicity test methods. In addition to those mentioned above, they suggested pachytene mapping, denaturation-renaturation analysis of chromosomes, differential cellular susceptibility, automated chromosome analysis, and assay of body fluids for mutagenic substances showed sufficient promise and required further investigation.
Monitoring of the human population for an increase in mutation rate caused by environmental mutagens represents another approach to identification or detection of mutagenicity. There is ample precedent for monitoring for mutagenicity in the mass screening of infants for phenylketonuria.

Neel (1971) has suggested three approaches to detection of altered mutation rates in man. These include (a) the use of population characteristics such as sex ratio, birth weights, frequency of stillbirths; (b) monitoring sentinel phenotypes, e.g., epilöia, neurofibromatosis, hemophilia, and childhood progressive muscular dystrophy; and (c) the use of biochemical or chromosomal mutations as markers. The experience gained in long term study of the Japanese atomic bomb casualties is pertinent to monitoring the human population for mutagenic effects of substances in the environment. However, much more effort would be required in an extensive program in mutagenesis (Brent, 1972; Sutton, 1971; Malling, 1970; Traut, 1969; Neel, 1971).

Crow (1971) has reviewed the techniques of monitoring human population and the applicability of these techniques through the detection of environmental mutagens. He suggests that presently available systems are inadequate; however, better systems are being produced. Crow indicated that somatic tests are preferable to germinal tests despite the fact that germinal mutations are more important genetically. This opinion is based upon the fact that somatic mutations occur in individual cells and the induction of mutation then becomes measured in units of cells rather than individuals. Neel (1971) has cautioned that the relationship between somatic and germinal mutation must be established before tests of somatic mutation could be used as valid monitoring procedures. Crow (1971) proposed that somatic cytogenetic monitoring might be done by screening umbilical cord blood in the newborn infant for specific biochemical mutations and that germinal mutation tests might include cytogenetic and biochemical tests for aberrant proteins.

A program of genetic monitoring would require significant commitment of time, manpower, and fiscal support. As noted by Neel (1970), this approach is recognized as a difficult one, but the significance of the problem of environmental mutagens demands consideration of these techniques. It may be premature for NCTR to focus its resources upon this approach; however, the concept of monitoring for increased mutation rate in the human population is within the long range goals and hope of the NCTR mission.
C. CORRELATIONS IN MUTAGENICITY TEST PROCEDURES

Techniques of assessing mutagenicity have been discussed at numerous conferences in the past several years. There appears to be general agreement that testing compounds in submammalian systems, especially microbial tests, is relatively straightforward, can provide additional information of relevance to human mutagenicity, and that these procedures are ancillary to tests in mammalian systems. Because of constraints on human experimentation, efforts have focused upon development of a data base from mammalian test systems which may be extrapolated to human exposures.

As recently as 1968, one group of scientists discussing chemical mutagenesis concluded that no laboratory or clinical tests would predict with any degree of certainty mutagenic hazards in man (Heming and Brown, 1969). Most authorities are more optimistic at the present time. There is general agreement that no single test system will be adequate in detecting and evaluating the several types of genetic damage that may be produced in man and that a battery of mutagenicity tests will be required to screen and assess the effects of long-term exposures of man to low-dosages of mutagens. It is recognized that presently available methods are incomplete and require further improvements and modification and that there is a continuing need for development of new techniques that can be added to the tests currently in use.

Several recent reviews have suggested that mutagenicity testing should focus upon: (a) the dominant lethal assay; (b) in vivo cytogenetics; and (c) the host-mediated assay (de Serres, 1972; Hollaender, 1971; Legator, 1970) because these three methods are reasonably well developed. Hollaender (1971) noted that these three methods are, at present, the techniques that are sufficiently well perfected for use by a properly trained technician under the supervision of a more experienced investigator.

The discussions of the LSRQ ad hoc review group included presentation of other techniques that may be applicable to the NCTR mission. These include the method for detecting polygenic effects and biochemical techniques that focus upon structural and functional changes in DNA, RNA, and proteins. Increased emphasis on the molecular basis of genetic damage is attractive but would be more meaningful if the results of these tests were closely correlated with
induction of chromosomal aberrations, dominant lethality, or other
types of genetic damage. If the specific effects of a mutagenic
agent upon nucleic acid metabolism were known, as they are with
caffeine (Bateman and Epstein, 1971), the gross observations from
such procedures as the dominant lethal test would have additional
credibility.

It was recognized that none of the mutagenicity test systems,
with the exception of the dominant lethal test, have addressed the
possibility of synergistic mutagenic action. Additional methods of
predicting synergistic effects of potential mutagens remain to be
developed. Further application of the dominant lethal test system
to synergistic effects appears to be a logical point of departure.

The results of the several mutagenicity assays should be
compared using a larger number of known and potential mutagens,
addressing such problems as dosage regimen, animal species, and in
some cases metabolism of the substance. Rall (1972) has suggested
that mutagenic test systems should be evaluated in terms of validity,
relevance, accuracy, and economy. Where differences in mutagen-
icity as determined by the several tests occur, further investigation
of the biochemical genetics which underlie that list procedure would
be appropriate.
Within the same species of animals, different strains may show marked variation in the frequency and type of deviation produced by a teratogen (Cahen, 1966). For example, the $F_1$ hybrids from two strains of mice will show marked differences in the incidence of cleft palate following cortisone exposure depending on the strain of origin of the dam (Kalter, 1954).

NCTR should include within its teratology program collection, comparison, and interpretation of data on similarities and differences in teratological effects among animal species and strains.

2. Maternal Factors

In many cases, within one litter of animals from a maternal organism exposed to a teratogen, not all offspring will be affected, or they will individually manifest different degrees of response. Some embryos may die and be resorbed, while others survive with no evidence of defect. This differential sensitivity may reflect subtle differences in the genetic makeup of the offspring or differences in the intrauterine environment of the animal. Intra-litter differences in susceptibility reflect maternal phenotype, implantation site within the uterus, developmental age, and other extramaternal environmental factors that interact in some way with the genotype of the embryo to cause variation in the degree of deviation from normal developmental patterns (Wilson, 1972; Done, 1964). In some cases, teratogenicity may be secondary to maternal toxicity per se.

3. Developmental Stages

A basic principle of teratology is that the susceptibility of the embryo to teratogens varies with the developmental stage of the embryo at the time of exposure to the teratogenic compound. In general, there are three broad stages in the normal development of the embryo (Cahen, 1966). During the first stage the fertilized ovum becomes segmented and transformed into the blastocyst. Agents administered during this time will cause death but not malformation because differentiation into organ systems has not begun. The general resistance of the preimplantation embryo to teratogenic effects may be due to the totipotency of the embryonic cells at this early stage of development (Clegg, 1971). Another possible explanation for the failure to find teratogenic effects during this early period of development may be the absence
of a well developed placental membrane. Little is known presently regarding the actual quantity of teratogenic agents reaching the mammalian blastocyst following maternal administration. NCTR studies might address this aspect of early developmental effects of teratogens.

If the blastocyst is overwhelmed by the effects of the compound at the blastocyst stage of development, it will die. If it survives, it will not show gross malformations because differentiation has not begun. There are some exceptions to this general statement; however, these mostly involve compounds which are known to interfere or are capable of interfering with nucleic acid synthesis (Clegg, 1971).

The period of organogenesis is the second developmental stage and is referred to as the embryonic stage or gastrula. This is the time during which organogenesis or the differentiation of cells into various organs or the rudimentary precursors of these organs occurs. In the rat, organogenesis takes place from the seventh to the 14th day and, in the human, from the 14th to the 60th day. During this period of time, teratogens may produce gross malformations. Death of the embryo is not excluded at this stage if the effects of the compound are overwhelming. In many animals, grossly abnormal embryonic stages are resorbed and are never delineated as malformations. Thus, counting of resorption sites or corpora lutea is extremely important in ascertaining the toxicity of compounds producing severe effects on the gastrula or embryonic stage.

The third stage of organogenesis, the fetal stage, is the period of growth of the organism. The organ systems, for the most part, have been developed and are undergoing growth and in certain cases further cellular differentiation and maturation. Teratogenic agents in terms of producing gross malformations are no longer effective with the exception of certain segments of the urogenital system and the central nervous system (Legrand et al., 1961).

Different opinions exist about the relationship between the stage of organogenesis and the effect of the teratogen as compared to specificity of the teratogen. Cahen (1966) indicated that most drugs, if given during the same period of organogenesis, will produce the same type or same spectrum of malformations in a given species. This is based on the assumption that teratologic sequelae are a manifestation of arrested development and that a specific stage of organogenesis may be arrested by a number of different compounds if given during the same critical period. However, there are a number of well recognized exceptions to this concept. For
example, cortisone in the mouse will produce cleft palate; vitamin A will rather specifically produce central nervous system abnormalities; thalidomide and the immunosuppressant azathioprine (Imuran) will arrest development of the long bones; and certain steroids produce primarily eye and facial lesions. Recent studies reviewed by Ferm (1972) have shown highly specific teratogenic effects from administration of certain heavy metals during the early stages of organogenesis. For example, lead salts elicit a specific teratogenic response from the tail region of the golden hamster embryo (Ferm and Ferm, 1971). The NCTR research program might profitably include the search for and use of this and similar specific teratological effects.

The amount of a compound reaching the conceptus which is necessary to produce malformations will vary during the period of organogenesis. The earlier in organogenesis the embryo is exposed to the compound, the smaller the amount of compound which is needed to produce the effect. As the embryo develops, progressively larger amounts of the compound are needed to be effective. In the third period, lethality, but no malformations, may be produced by extremely large doses of the teratogen.

Different types of malformations may be produced in a particular species by administration of the same compound at different stages of organogenesis. This may be a reflection of differences in the time of development of various organ systems, resulting in differential sensitivity of one organ system as compared to another. However, as discussed above, certain compounds are specific in their teratogenic action irrespective of time of administration, provided the period of administration coincides with the teratogenically sensitive period.

Morphogenesis involves a large variety of poorly understood events which may be grouped into three general areas (Saxen, 1972). These include the rearrangement and polarization of cells and their organelles, the migration and condensation of cell groups, and controlled death of certain cellular populations. Use of mammalian tissue culture systems of embryonic cells has made possible the study of single steps in progressive embryogenesis.

Normal embryogenesis involves controlled death of certain cell populations and entire rudiments (Saxen, 1972). If this process is disrupted, anomalies such as duplication of various parts of urogenital tract or postnatal retention of parts of the fetal cardiovascular system and polydactyly may result (Moller et al., 1971; Hinchcliffe and Ede, 1967).
There are normal interactions between different groups of cells during the development of the fetus that are vulnerable to disruption by genetic and environmental factors. Thus, the effect of the teratogen may not be directly on the target or affected tissue but on the inducing tissue (Saxen, 1972).

The basic mechanisms of teratogenicity, beyond the arrested development concept, are poorly understood, and several concepts have been proposed to explain teratogenesis. Basically, development is arrested because of paucity of cells or lack of development of cells along normal lines. Thus, several mechanisms could be responsible for initial cellular injury which leads to pathogenesis (Table 2). Overall, these could be classified into two types of action, (a) production of a specific toxic compound which arrests cellular development through one of the initial cellular reaction mechanisms, or (b) competitive inhibition by the teratogen with the availability of certain nutrients or metabolites essential to the developing cell, i.e., antimetabolites, which arrest cell development (Cahen, 1966). Support for both these concepts exists, and there is no reason to presume that all teratogens function by the same mechanism.

Development of an extensive program involving use of alteration of developmental stages as toxicologic endpoints in evaluating long-term, low-dose exposures to potentially toxic substances should be firmly established on advances in our understanding of developmental biology. At the present time, progress in developmental biology is a major limitation of teratological research.

4. Qualitative and Quantitative Aspects of Exposure

A fourth basic concept holds that the compound must reach the fetus in adequate quantity at the proper time of embryonic development. In most mammalian test systems, the compound is administered to the maternal organism in order to study the effects on the fetus. Virtually no compound is completely excluded from the fetus by placental, maternal, metabolic, or physical mechanisms. The physiochemical properties of the compound and the metabolism of the material organism will influence the quantity of the teratogenically active compound reaching the fetus. Only in the duck or chick egg embryo test systems is the embryo directly exposed to the chemical substance.
a. **Dosage**

The basic factors of route of administration, dose, vehicle, and physical form will influence the quantity of the compound that is absorbed by the maternal organism. In some studies, the maternal or fetal blood level of a compound is considered the best index of quantitative effect. However, the ability of the fetus to metabolize a compound is related to the activity of enzyme systems and other factors that are poorly understood.

Fetal blood levels seldom equilibrate with maternal blood levels of a compound. Measurement of the concentration of the compound in the fetal circulation is difficult and seldom performed. Methods which seek to measure (yolk sac fluid, amniotic fluid, or) fetal blood levels of a compound indirectly using radioisotope techniques suffer from lack of specificity with the exception of the heavy metals. The development of better methods is within the scope of the NCTR mission.

In most mammalian species, the fetal circulation is not even established in the early developmental stages that are teratogenically sensitive. In later stages of gestation, the maternal tissues and placenta are thought to provide a barrier between the compound and the fetus (Northdurft, 1970; Cahen and Fave, 1970; Franz and Degenhardt, 1969). In most situations, the fetal blood levels of a compound are lower than the maternal levels. However, this is not always so, particularly for certain essential nutrients. Another consideration is that for a given blood level, the fetus may be more susceptible than the mother because of differences in metabolic pathways, detoxifying capacities, and smaller surface area.

Kalter (1972) observed that, while the equal plasma level-equal response hypothesis has been confirmed for various agents affecting physiological systems, it does not seem to apply to growth processes which are theoretically more related to surface area. Because teratogenicity, fetal growth retardation, and perhaps embryolethality can result from interference with growth processes, it is not certain whether the fetal plasma level of a compound is the most relevant factor in teratology. Such information as fetal tissue concentration of a compound may be far more important and should be collected by NCTR in the course of the teratogenicity research program.

b. **Metabolic Differences**

The maternal organism has developed a variety of detoxifying mechanisms, particularly liver microsomal enzymes.
The concentration of many of these enzymes may be increased following prolonged administration of the compound. These normal enzyme systems may provide for excretion or conversion of a teratogen into a nonteratogenic form and thus prevent toxic quantities of the compound from reaching the fetus.

The enzyme systems of the embryo and fetus are less fully developed than those of the mother, e.g., at the time of organogenesis. Thus, the portion of the substance which reaches the fetus may be metabolized differently, producing products which could be more or less teratogenic.

There are a wide variety of species differences in the metabolism of various compounds. In some cases, the compound per se is the actual teratogen; however, frequently it is a metabolic product that is teratogenic. Generally, it is considered desirable to use an animal species that metabolizes a compound in a manner similar to the human if information on human metabolism is available (Clegg, 1971). Limitations of this concept have been discussed by Kalter (1972). It is significant that the pharmacologically active metabolite is frequently not the teratologically active metabolite. In the case of thalidomide, even after 10 years of investigation, the actual metabolite causing teratogenicity has not been identified.

This type of problem should be taken into account in the development of the long range goals of the NCTR teratology program.

c. Placentation

The placenta, as with other membranes, is selective in the transfer of chemical compounds. Active and passive transport as well as facilitated diffusion occur across the placenta. Many factors such as charge, particle size, and lipid solubility influence passive transfer across the placenta. Because of active absorption, certain compounds are transferred to the fetus more readily than their physicochemical properties would suggest. Differential placental transfer of compounds possessing teratogenic activity has been suggested as one basis for species differences in susceptibility to teratogens (Carpenter and Ferm, 1969; Brent, 1964).
There are species differences in placental transfer. For this reason it is necessary to select a species with placentation similar to the human at the time of organogenesis. Unfortunately, certain types of basic information on placental physiology are not yet known. For example, the yolk sac placenta plays a dominant role in nutrition of the rodent embryo, but its importance in human development is debated (Brent, 1972). The placenta and the liver contain numerous inducible enzyme systems that may vary with species.

d. Relation Between Chemical Structure and Teratogenic Effect

Numerous types of chemical compounds have been shown to be teratogenic for man. Therefore, it is not possible to correlate teratogenic action with a particular chemical moiety (Clegg, 1971) or a specific chemical function. Teratologists have investigated the teratogenicity of the chemical analogues of well recognized teratogens such as trypan blue without particularly outstanding success. Similarly, neither glutethimide, which has been used therapeutically in humans, nor phthalimide, while chemically similar to thalidomide (≠ phthalimidoglutarimide), show teratogenic effects. As noted previously, it must be stressed that the pharmacologically active metabolite of a drug may be different from the active teratogen which may be a metabolite (Clegg, 1971; Cahen, 1966).

5. Patterns of Abnormal Development

A fifth general observation is that death, malformation, growth retardation, and functional disorders are four manifestations of deviant development which are encompassed under the broad definition of teratology. Functional disorders refer to either physiological functions or behavioral deviations. Certain functional defects are not present at birth but manifest themselves only later in life.

The four expressions of deviant development are not likely to occur at one exposure time, and one of these tends to predominate at different times during development (Brent, 1969). Teratogen-induced death may occur at any stage during the gestational period.

Malformations can only be produced at specific times during the gestation process. However, embryolethality and malformation are not mutually exclusive. As near total embryolethality is approached, the number of malformations declines because many
conceptuses die early and are resorbed before being identified as being defective. Death and malformation are not necessarily due to the same teratogenic process (Wilson, 1972). There is a decrease in both malformation and mortality rate as embryonic age at the time of teratogenic treatment increases (Kalter, 1957; Wilson, 1954). For both lethality and malformation the quantity of the compound needed to produce these effects increases later in the organogenic period. Growth retardation and functional defects generally are due to exposure to toxic substances later in gestation but may be induced by factors such as nutrient deficiency during gestation.

The manifestations of deviations from normal development increase in frequency as the dosage of a teratogenic compound is increased from no effect to totally lethal levels. Wilson (1972) points out that experimental teratologists almost without exception have assumed threshold effects. The threshold concept is considered valid for lethality, growth retardation, and gross malformation. Thresholds for embryotoxicity in terms of postnatal functional disturbances could be questioned, but only because so little is known about this process currently (Wilson, 1972).

Generally, embryolethality and malformation begin at about the same dosage, although lethality may be seen earlier in gestation. The incidence of these increases in parallel fashion until embryolethality precludes malformed survivors at term. Frequently, the dose response curve for embryotoxic effects is quite steep (Wilson, 1972). Kalter (1972) comments that a given compound may produce embryolethality, malformation, and/or growth retardation. While increased frequency of all three effects may occur at a given dose, one of the effects may occur first at doses lower than necessary to produce the others. The dose-response curves for each of these three effects can be relatively shallow, steep, or intermediate, and the shape of the curve for any one may have little or no obvious relation to those of the others (Kalter, 1972).

It is hazardous to draw conclusions on threshold effects from studies of a single compound; certain teratogens may be additive or synergistic in their effect (Ferm, 1969; Cahen, 1966). It is obvious that human exposures involve simultaneous exposure to more than one potential teratogen.
B. TEST SYSTEMS

1. Nonmammalian Systems

Several models for evaluating potential teratogenicity of compounds have been proposed, and literature in this field is extensive. The use of nonmammalian test systems antedated the mammalian because the first experimental production of fetal malformations was performed by Geoffrey Saint-Hilaire in 1832 by puncturing duck embryos through the shell (Cahen, 1966). In experimental teratology the hen egg is used to study potential teratogenic compounds and has the advantage of ease of accessibility to the developing embryo, a short incubation time of 21 days, a convenient fetal size, a known genetic background, and a well-defined sequence of morphological events (Clegg, 1971).

If such a system is used for teratology evaluation, the toxicity is evaluated from the percentage hatch at varying dosages of the chemical as compared to noninjected (control) eggs and/or eggs treated with the vehicle (solvent) at equal volumes. In specific experiments, the embryos are examined grossly for skeletal or other obvious malformations. The techniques of skeletal examination may include alizarin red staining or X-ray examination. Soft tissue defects may be evaluated by histopathology techniques.

Observations on chemical changes could be conducted using types of techniques described in a previous section (see p Physiological or behavioral defects could be evaluated by studying some of the hatched chicks during their postnatal development.

There are several disadvantages to the chick embryo system. A fundamental difference between the chick embryo and mammalian systems exists in that using the former, the embryo is exposed directly to the compound under investigation. Direct exposure of the embryo removes the influence of placental selectivity to transfer. Likewise, maternal metabolic effects, either of converting the compound to its metabolically active form or detoxification product of the compound, are nonexistent in the chick embryo. Further, physical factors of the compound such as physical size, vehicle of administration become even more significant than in the mammalian species (Walker, 1967; Williamson et al., 1963). Even chemically inert substances may be teratogenic in the chick embryo.

In addition to these metabolic considerations, a number of factors are known to affect the results of this test system
(Clegg, 1971). These include age, diet, and strain of the maternal hen (Adams, 1958); effects on fertility by internal and storage temperature between laying and incubation (Landauer, 1937); variability between time of incubation and onset of development; and vulnerability to changes in external environment (Lindsey, and Moodie, 1967; Leighton et al., 1964). This second group of factors are of lesser importance because they can be controlled by careful monitoring of experimental conditions; however, there is no method to circumvent the differences due to lack of placental influences and the interaction with maternal metabolism. Thus, the role of the chick embryo test would seem to have little direct applicability to potential teratogenicity of compounds in the human but might serve as a coarse screening mechanism. Either negative or positive results would have to be confirmed in mammalian test systems because of the importance of maternal metabolic and placental factors in altering potential teratogenicity of a compound.

Occasionally, the use of other lower nonmammalian species has been suggested. However, these are phylogenetically so far removed from mammals that their use, while advantageous in terms of accessibility and ease of manipulation of the test system, is limited. For example, Kalter and Warkany (1959) point out that the morphogenesis of cleft palate cannot be studied in the sea urchin, and the abnormal separation of the four-chambered heart cannot be observed in fish.

2. Mammalian Test Systems

The evaluation of potentially teratogenic compounds in mammalian species most frequently involves administration of the substance under study to the maternal organism and observation of the effects of the compound in the fetus or on the general performance of the fetus. A number of options exist regarding dosage, route of administration, species selection, and method of evaluation of the fetus.

a. Experimental Factors

(1) Dosage. Administration of the test compound to the maternal organism may be either as a single or as multiple doses. If a single dose is administered, the time of administration is significant. For evaluation of gross malformations, the period of organogenesis must be chosen. However, within this period, sensitivity may be limited to only a few days or even a few hours.
Multiple dosage administration is more frequently used for evaluation of teratogenicity. The multiple dose schedule may be limited to several days or may cover the lifetime of the animal. However, with extended treatment, the maternal mechanisms for detoxification or conversion of a compound into a teratogenetically active metabolite must be considered.

Clearly, the choice of treatment used in the experimental animal depends on how the human is exposed to the compound in question. Exposure to most environmental or food contaminants is likely to be continuous over a long period of time. Under these circumstances, multiple dose exposure would appear most comparable to the human situation.

(2) **Route of Exposure.** Most exposure of humans to environmental or food teratogens is through ingestion, inhalation, or dermal contact. Parenteral administration of various types, e.g., subcutaneous or intraperitoneal, while convenient is unrealistic in terms of applicability to human toxicity. The route of exposure can influence the amount of a given dose of a compound that is absorbed and excreted. Further, ingestion is the usual route of human exposure. Possible interactions with the microflora of the gastrointestinal tract may cause detoxification or conversion of an innocuous compound to an active teratogen. In addition, teratogenic agents or their products can be transferred to the embryo or fetus only after uptake by the maternal blood stream.

(3) **Species.** Tuchmann-Duplessis (1972) has provided a detailed discussion of the susceptibility of various animals to a variety of teratogens and their species-specific response. No single animal species has been found to resemble man closely in the pharmacologic response to many chemical compounds. The number of animals suggested for use in teratology is an expression of this fact. Kalter (1972) points out that the absorption, distribution, excretion, binding, interactions, and site and mechanism of action of different species are more similar to one another than are the interspecies differences in metabolism of a compound. Indeed, some (Brent, 1964, 1972) have questioned realistic resemblance of any other animal species to the human. Selection on the basis of similarity of metabolic factors becomes extremely difficult, because the basic mechanisms of teratogenicity are poorly understood.

Species are at present selected mainly on the basis of their convenience as laboratory animals. The species most frequently used are rodents, particularly the rat, mouse, hamster, and rabbit, and occasionally nonhuman primates. Primates have been
suggested as an optimal animal; in addition to cost, dramatic pharmacological differences between man and primates have been observed (Kalter, 1972).

In addition to the very real question of applicability to human teratogenicity in the selection of animal species, the species of animal finally chosen for teratogenesis experimentation at NCTR or in any other research program must show suitability and have been well defined with regard to a number of parameters including:

- high fertility under laboratory conditions;
- thorough knowledge of the type and rate of spontaneous malformations and resorption rate under the specific laboratory conditions in which the investigation is taking place;
- feasibility of confirming onset of gestation;
- knowledge of teratogenetically sensitive period in the animal.

b. **Types of Response**

1. **Embryolethality.** Evaluation of early lethality is generally accomplished by observation of resorption sites, and would also be evident by differences in the number of offspring per litter. Late embryolethality is evidenced by nonviable fetuses at term. These may be studied by alizarin red staining for skeletal defects if more specific information is required. Because nonviable fetuses have generally undergone some degree of autolysis, routine histology or gross sectioning would appear of limited value (Fern, 1965; Wilson, 1965). Determination of the lethal dose in 50% (LD<sub>50</sub>) of the fetuses and comparison of these with maternal LD<sub>50</sub>'s has been suggested as an important parameter in evaluation of compounds possessing teratogenicity. Compounds which produce total embryolethality at doses either completely nontoxic or totally toxic to the maternal organism have relatively little potential to induce malformations. However, compounds which produce little maternal effect and require high doses for embryolethality have strong potential for further teratogenic effects.

2. **Gross Malformations.** These represent the most important area of teratogenesis because of the viability of the fetus and the irreversibility and dysfunction of major defects. Extremely severe malformations may be apparent on gross examination. These observations may be supplemented by alizarin red staining of
the skeleton and by microdissection of the fetus. It is to be noted that this technique will not reveal defects in cartilaginous tissues. Internal or visceral aberrations are generally studied by the whole fetus sectioning technique developed by Wilson (1965) or by light or electron microscopy. It is necessary that a pathologist with adequate familiarity with the normal appearance of fetal tissues conduct the evaluation of the sections.

Observation of resorption sites is important in the study of a compound producing gross malformation because, if the malformations are severe, the fetus may die in utero and be resorbed before being identified as defective.

(3) Growth Retardation. Compounds administered during the third trimester of pregnancy may show teratogenic effects by interference with growth. Evaluation of this type of teratologic effect has not been investigated extensively although it is frequently considered by nutritionists in their research. The techniques are very simple. Weight of the total litter, number of fetuses and individual weights and lengths of fetuses may be obtained and will identify interference with the overall growth process. In cases where the fetus appears edematous, fetal dry weights could be obtained for a portion of the litter. Growth retardation of specific organs, which impinges upon the area of functional defects, could be ascertained by gross sectioning or histopathology of various tissues.

(4) Functional Defects. This term has previously included both behavioral and physiological malfunctions. Behavioral defects can be evaluated in the animal postnatally. This may be accomplished by a variety of techniques which are employed by experimental psychologists. These aspects are beyond the scope of this review. However, morphological and biochemical pathology techniques may be correlated with behavioral change. Excellent examples of this type of study are the evaluation of perinatal effects of lead administration to the maternal organism by Krigman et al. (1972) and effects of methylmercury (Spyker et al., 1972). Physiological defects may be evaluated postnatally by morphological and chemical pathology techniques (see p 14 and p 35).

It seems logical to conclude that teratological studies can play a significant role in evaluating the toxicity of long-term, low-dose exposure to potentially toxic substances. However, there is a need for development of additional data on specific teratogenic responses of several animal model systems and correlation of these
data with results of other toxicological test procedures. These
types of investigations will require additional emphasis on develop-
ment of methodology applicable to long-term, low-dose exposures.
NCTR should include such research in its long range goals.
IX. CARCINOGENICITY TEST PROCEDURES

A. INTRODUCTION

The discussions of the LSRO ad hoc review group on carcinogenicity testing were based, in part, upon knowledge of the recently submitted report of the Food and Drug Administration Advisory Committee on Protocols for Safety Evaluation: Panel on Carcinogenesis Report on Cancer Testing in the Safety Evaluation of Food Additives and Pesticides (1971). As noted in the introduction to the FDA Advisory Committee report, carcinogenicity is only one of the manifestations of toxicity but has assumed a dominant role in consideration of endpoints of toxicity. The FDA Advisory Committee report reviews the present status of carcinogenicity testing and is a source of information on many aspects pertinent to the study of chemical carcinogens.

Based upon a general concurrence with the contents of the FDA Advisory Committee report, the LSRO ad hoc review group discussions focused upon certain conceptual aspects of carcinogenicity testing and potential techniques for identification of early changes associated with neoplasia and malignancy. It would be redundant to repeat much of the information in the FDA Advisory Committee report and the other documents submitted to NCTR during the genesis of its research program. Because these other reports have focused, for the most part, upon carcinogenesis as the endpoint in toxicity studies, the following sections of this report should be considered as an extension of the suggestions made by other panels of experts.

Extensive literature has accumulated in the past two decades on techniques for determining the carcinogenic potential of chemicals. Several comprehensive reviews discuss methods for evaluating the carcinogenicity of various substances (Magee, 1970; Weisburger and Weisburger, 1967; World Health Organization, 1967; Clayson, 1962). They are valuable sources of information on many aspects of conventional lifetime carcinogenicity studies.
Most test methodologies are similar, regardless of the type of substance being tested. In general, the chemical is administered by an appropriate route to at least two species of susceptible animals at several dosages. The growth, development, and reproduction of test and control animals are monitored over the life span of the animals for several generations. Pathological evaluation includes gross morphology at autopsy and subsequent histopathological studies with emphasis on identification and delineation of tumor type and location. Tumorigenesis is the definitive endpoint used in most conventional lifetime studies.

The term, tumor, is used almost universally as a synonym for neoplasm (Meissner and Warren, 1971). It is in this sense that tumor or, more clearly, the morphological manifestation of disturbed growth characterized primarily by continued, abnormal, and excessive cell proliferation, has become the accepted endpoint in proof of chemical carcinogenicity. When neoplastic growth proliferates and spreads discontinuously to secondary sites (metastasis), this evidence serves to confirm the conclusion that the substance being tested induces cancer, i.e., is carcinogenic.

Neoplasia is a concept more readily described than defined. It includes growth of cells which in themselves are different from those that make up the body's normal tissues and organs. The differences may be so subtle as to escape detection by conventional microscopy or so bizarre as to leave no doubt that the lesion is malignant, cancerous, and will destroy its host. The normal constraints that regulate cell growth and/or proliferation in healthy organs and tissues are lost to greater or less degree in neoplasia. Moreover, the neoplasm may be benign, not threatening the economy of the host, or malignant, inimical to its survival.

Pathologists have studied the morphological manifestations of neoplasms in man since the dawn of cellular pathology more than 100 years ago. They have derived histological criteria of malignancy by correlating the natural history of specific anatomical types of neoplasms with changes in cellular characteristics and their interrelationships with other tissues and organs which are extremely useful in human pathology. When the same criteria apply equally well to the known course of particular neoplasms in other species, all is well and good. A significant number of neoplasms in experimental animals, on the other hand, appear to be histologically benign when interpreted in terms of human neoplasms but possess biological attributes of the malignant state. They metastasize, albeit late in the course of the disease when measured in
relation to the expected life span of the species. They may or may not be locally invasive, as are most cancers that affect man. Malignant neoplasms will grow more or less progressively when transplanted to genetically appropriate members of the same species—a quality most useful to the experimentalist if he be prepared to test the matter as the occasion arises. Malignant neoplasia is a biological phenomenon.

Neoplasia is not to be confused with metaplasia (Capell and Anderson, 1971). The metaplastic lesion usually represents a higher degree of specialization than had its progenitor. A simple columnar epithelium may become keratinized and assume squamous characteristics, and a tissue once predominantly fibroblastic that produced collagen may produce bone or cartilage. In any case, the metaplastic change is confined to limits of the antecedent tissue or cells from which it derives; it is not invasive.

A few precancerous lesions have been defined, such as osteitis deformans, oral leukoplakia, and hepatic cirrhosis. These are entities in their own right which predispose to the development of completely new malignant neoplasms, usually at the affected sites.

More controversial are a series of lesions often termed precancerous which display in man many of the cytological characteristics associated with malignancy but are limited to the normal confines of the organ component in which they arise. The uterine cervix is a case in point. Years ago "precancerous" changes were described as such. Sufficient experience has been gained to show that these lesions will become frankly invasive and acquire histologic and biologic attributes of the cancerous state. Such lesions are variously termed carcinoma in situ or intraepithelial, intraepidermoid carcinoma today and have been incorporated into the international classification of cervical carcinoma originated in the 1920's by the League of Nations as Carcinoma of the Cervix, Stage 0. The problem becomes intellectual and semantic because the lesion may remain localized for incredibly long periods before assuming its full cancerous potential, or may even retrogress and disappear. Cancers are not supposed to do that, and few do, but cancer must start somewhere and at some time in a cell or small group of cells and go on from there to acquire its fully malignant potential.
B. CONVENTIONAL TEST TECHNIQUES

1. Conceptual Aspects

A major difficulty in evolving reliable techniques for testing carcinogenicity is the lack of basic knowledge concerning the mechanisms of chemical carcinogenesis. Logically, the induction of cancer consists of a heritable and relatively stable loss of control of cell division (Miller, 1970). Theoretically, this loss of control may occur directly by (a) interaction of the reactive form of the carcinogen with DNA or (b) alteration of RNA or specific proteins during transcription. In the former case, alteration of the genetic information occurs and, in the latter, changes in RNA or proteins produce relatively stable alterations in genome expression. In addition, indirect disruption of cell division might occur through activation of latent viruses in the cells or selection of specific cells with a propensity to neoplastic growth (Miller, 1970). At the present time, experimental proof of any one of the above hypotheses in chemical carcinogenesis is lacking.

While there is increasing evidence that viruses are associated with tumor initiation in some laboratory animals, the contention that viruses may cause cancer in man is not established. A wide variety of chemicals are known to be carcinogenic, yet no common structural similarities are evident. The reactive form of some carcinogenic substances is known to be similar in that they are electron-deficient (Miller, 1970). But the subcellular molecular targets that may satisfy the electrophilic requirements of these chemicals during or prior to induction of neoplastic processes are unknown.

To date, no broad-spectrum screening tests for chemical carcinogens have been developed other than techniques which depend upon tumorigenesis. Miller and Miller (1971) concluded that despite the relative insensitivity and other deficiencies of conventional lifetime testing, the lifetime or two-year carcinogenicity test in small rodents remains the most logical and practical technique for establishing chemical carcinogenicity.

The difficulties inherent in assessing human toxicity by use of experimental animals are well recognized. Animals that metabolize chemicals by similar routes provide acceptable substitutes for man. No in vitro or modified in vivo procedures can
duplicate the features of in vivo testing in animals. Nevertheless, screening techniques that provide some measure of predictability should be investigated because they provide opportunities for establishing priorities in conventional lifetime studies and may offer alternative approaches as the mechanisms of chemical carcinogenesis become better understood.

2. Statistical Limitations

The need for critical statistical design in planning prolonged studies such as conventional lifetime testing is universally accepted. These requirements are recognized in carcinogenicity testing but are equally applicable to other endpoints of toxicity such as increased rate or incidence of mutation, induction of teratogenic effects, or specific metabolic alterations. Statistical limitations are a primary concern in studies requiring large numbers of animals where the induction of adverse effects, such as tumors, is anticipated in one or more generations as they age.

As noted in the recent report of the FDA Advisory Committee on Protocols for Safety Evaluation (1971), conventional lifetime tests can provide a positive answer to the question of carcinogenicity, but any negative conclusion must be qualified on the basis of the experimental design. Based upon considerations of statistical probability alone, no number of experimental animals, no matter how large, can provide an absolute probability that no carcinogenic potential exists.

The FDA Advisory Committee on Protocols for Safety Evaluation (1971) concluded that the only practicable basis for estimation of a safe dosage would be by extrapolation downward from observations at higher dosage levels. However, they recognized that this type of extrapolation may be unreliable for mathematical as well as biological reasons. They concluded,

"Clearly extrapolation from the observable range to a safe dose has many of the perplexities and imponderables of extrapolation from animal to man, and it would be imprudent to place excessive reliance on mathematical sleight of hand, particularly when the dose-response curves used are largely empirical descriptions, lacking any theoretical physical or chemical basis."
Many, but not all, scientists would agree that statistical and practical limitations prevent the establishment of absolute "safedosage levels" of carcinogens. The deliberation of the LSRO ad hoc review group suggested that the NCTR research program should include a continued effort to seek and to evaluate techniques of carcinogen testing which may possibly circumvent or reduce some of the statistical limitations inherent in lifetime studies of large numbers of animals.

3. Practical Aspects

In addition to statistical constraints, conventional lifetime carcinogenicity tests require extensive commitment of manpower, facilities, and financial support. In terms of costs versus benefits, the expenditures necessary for adequate testing of large numbers of chemical substances for tumorigenic potential without preliminary screening or purposeful selection may be beyond the benefits derived. Thus it seems logical that NCTR should consider the study and possible development of techniques for rapid screening of potentially carcinogenic chemicals.
C. POTENTIAL TECHNIQUES

Most investigators agree that in vivo lifetime testing of suspected carcinogens in susceptible animals is the most logical approach to carcinogenicity testing. The concept and techniques embodied in this approach were summarized by Weisburger and Weisburger in 1967. In the intervening 5 years, more precise analytical methodologies have been developed, but the conceptual approach of lifetime in vivo testing has remained the same.

As noted by Weisburger and Weisburger (1967), these techniques do reveal carcinogenicity of strong agents but require careful experimental design and critical management if the agents being tested are weak carcinogens. Many of the environmental contaminants appear to fall into this latter group. Because the NCTR mission includes development of test methodologies, this section of the report focuses upon some possible opportunities for early identification of cancer induction. The topics are presented as examples of possible techniques that may obviate or supplement the necessity of lifetime studies on large numbers of animals. The suggested areas for future emphasis are by no means all inclusive, but serve to reemphasize the long recognized need for development of techniques of carcinogenicity testing that do not rely solely on tumorigenesis.

1. Examination of Chemical Structures

In recent years, there has been an increasing use of three-dimensional "space filling" models of large proteins to study the precise geometry of the molecule. These models are useful in understanding the interrelationships of the various parts of the molecule and how they enter into the chemical reactions of the biological processes.

Models of large molecules provide detailed information about the reactive characteristics of the outer surface as well as the inner portions of the molecule. In these macromolecules both the intramolecular forces and intermolecular reactions are biologically significant. The classical example is the enzyme-substrate steric fitting. In this case the matching of the surface structures of both molecules appears to be a major controlling factor in the enzyme-substrate interaction. Moreover, the deep clefts that are characteristicly identified with probably active sites of biologically active molecules, e.g., lysozyme, ribonuclease, and chymotrypsin, may represent important features of the molecular fit. It seems logical to suggest that
classes of chemical carcinogens or cocarcinogens might be identifiable by these techniques of physical chemistry.

It seems evident that carcinogens induce neoplasia by reacting with cellular components inside the cell; thus, the active substance passes actively or passively through the cell membrane. In addition, it is well known that many carcinogenic substances are electrophilic reactants (Miller, 1970). DeVault et al. (1967) have suggested that electron transfer through biological structures may occur by "tunneling." Similar suggestions for electron transport in biological reactions have been made for nerve membranes. Charge transport through a biological membrane may occur by an electronic wave motion in an energy band; by a "hopping" process whereby an electron acquires sufficient energy to surmount and go over the top of an energy barrier; by quantum mechanical "tunneling" of an electron through the barrier; by charge transport involving hydrogen bonds; or by an ionic transport mechanism. On the other hand, the proton involved in the hydrogen bond may serve as an electron carrier by virtue of the electron cloud that surrounds the proton (Gutmann, 1968).

However, biological structures do not exhibit the degree of long-range order required to yield an electron energy band system. In addition, the "hopping" process is unlikely because, while it is temperature dependent, the Christov Characteristic Temperature calculated for a typical biological organic system results in an unrealistically high temperature (Christov, 1963, 1965). Therefore, charge transport at room or body temperatures strongly favors "tunneling." It is suggested that, while the actual charge transfer is by electrons, the rate determining process is most likely the sterically favorable alignment of a donor and an acceptor site. Thus, the cellular "active patch" idea is supported as the place for the favorable alignment of the electron donor and the electron acceptor.

It may be possible to identify potential carcinogens by their affinity for certain definitive areas on cell membranes of susceptible cells in vitro or in vivo. Through such basic studies, biochemical and biophysical characteristics of the cell membrane may be elucidated more fully. Knowledge of membrane characteristics may provide clues as to methods for identifying potential toxic substances at the cellular level.
2. Biochemical Tests

Of all the markers that the cancerous body may elaborate to indicate the nature of its problem, the peculiar proteins associated with plasma cell myelomas are the most readily identifiable and most reliable indicators of the presence of that particular neoplasm in man and in mice. Nevertheless, a heated argument rages on the nature of these proteins. Are they abnormal in themselves or do they represent abnormal increments of proteins formed in the healthy subject? The issue has not been resolved. This observation and its utility in clinical and experimental medicine, however, encourages search for other markers that may be associated with other specific anatomical types of cancer.

Enzymes may be useful indicators of early carcinogenesis. For example, Lum and Gambino (1972) have utilized serum y-glutamyl transpeptidase activity as an indicator of certain diseases of the liver, pancreas, and bone. In 12 patients with primary carcinoma of the lung, ovary, liver, and skin that had metastasized to the liver, they observed increased GGT activity when values were compared to those of the other transaminases studied. In patients with other liver disease and in patients with carcinoma in which the liver was not involved, normal GGT activities were present. The GGT activity was greatest in those patients with primary carcinoma of the pancreas and adenocarcinoma of the bile duct. Lum and Gambino (1972) noted that their observations confirmed the previous suggestion that GGT assay may be a useful indicator of hepatic metastases.

Dimitrov et al. (1972) have recently reported better than 90% accuracy in the detection of solid tumors by measuring the level of adenosine triphosphatase (ATPase). The test has been utilized to detect carcinoma of the breast as well as cancer of the lung and gastrointestinal tract. The investigators suggested that the increased ATPase activity in lymphocytes from cancer patients might reflect changes in the functional capacity of lymphocytes as a result of their contact with antigens from cancer cells. Preliminary studies suggest that the ATPase being stimulated is a mitochondrial enzyme rather than the membrane-bound ATPase which is present in the cell.

The enzyme systems metabolizing polycyclic hydrocarbons have been identified in many tissues. In microsomes and
the endoplasmic reticulum of liver cells, these hydroxylases function in detoxication and convert polycyclic hydrocarbons to weakly carcinogenic or noncarcinogenic compounds. Gelboin et al. (1972) have reported that one of these microsomal hydroxylases, aryl hydrocarbon hydroxylase, is present and inducible in liver cells grown in culture. The enzymic system catalyzes binding of the polycyclic hydrocarbons to both proteins and nucleic acids. Numerous carcinogenic and noncarcinogenic hydrocarbons are inducers and substrates for the enzymic system. In the model system, Gelboin et al. (1972) observed that some polycyclic hydrocarbons were converted to metabolites that were toxic and carcinogenic. While the identity of these metabolites remains to be determined, these studies point out the role of aryl hydrocarbon hydroxylase as the primary cellular receptor for polycyclic hydrocarbons.

In a related study, Nebert and Gielen (1972) concluded that aryl hydrocarbon hydroxylase induction is a simple autosomal dominant trait. They also observed differences in mouse strain susceptibility of chemical carcinogenesis that were related to either increased enzyme induction or products formed by the induced enzyme.

These studies (Gelboin et al., 1972; Nebert and Gielen, 1972) suggest that critical investigation of specific enzyme systems and the genetic basis of specific biochemical traits may provide both additional approaches to elucidation of mechanisms of chemical carcinogenesis and techniques for early identification of cellular responses to carcinogens.

Several investigators have shown that $\alpha_1$-fetoprotein, a normal component of the plasma globulins of the human fetus, is present in maximum concentrations between the 12th and 16th weeks of gestation and disappears shortly after birth. It is not normally present in children, normal adults, nonpregnant females, and females receiving oral contraceptive therapy (Breen and Schenker, 1971). However, the $\alpha_1$-fetoprotein is present in the plasma of the large proportion of patients with primary hepatic carcinoma (Smith, 1970). The specific abnormal protein can be identified readily by agarose gel immunodiffusion using the monospecific antisera from rabbits (see p. 38). Breen and Schenker (1971) indicated that the presence of $\alpha_1$-fetal protein is generally regarded as specific for hepatocellular carcinoma; however, the absolute specificity of this test has been questioned (Kroes et al., 1972). The protein may be present in certain childhood disorders and is rarely evident in
the case of other liver disease. Most evidence suggests that the test is specific for the altered metabolic state associated with hepatocellular carcinoma; however, the correlation is highly variable (Kroes et al., 1972; Mawas et al., 1970; Alpert, 1969; Keiser et al., 1972).

In addition to the α₁-fetoprotein associated with liver carcinoma, the Gold antigen is associated with carcinoma of the colon. The Gold antigen is another fetal antigen present in the normal embryo or fetus that disappears shortly after birth (Gold and Freedman, 1965).

While these antigens have been identified in man and to some extent in experimental animals, it seems logical that additional aberrant proteins may be present in cancers. In susceptible animal strains, a number of aberrant proteins may be present in blood serum or other body tissues which may indicate altered metabolism on biotransformation induced by the presence of neoplastic tissues. These types of early indicators may be useful in identifying "biochemical lesions" associated with developing tumors. The primary neoplasm must have developed sufficiently before these specific antigens are present in the blood in measurable amounts. However, it seems logical to suggest that analyses of circulating antigens may require less time or manpower than necropsy and histology of animals. Where possible, the NCTR program should include comparative studies of the relative efficiency of several methods of detecting the same endpoint of toxicity.

3. **Interactions with Genetic Material**

Because cancer is characterized by altered rates of cell division, there has been a long association between studies in carcinogenicity and investigations of mutagenicity. Active forms of numerous chemical carcinogens can react with nucleic acids. These interactions may occur in one of two general ways. The first of these is embodied in the somatic mutation concept in which replication of a chemically altered DNA leads to deletions, alterations, or rearrangements of the DNA nucleotide sequence that would be perpetuated in the neoplastic tissue.

Farber and associates (1972) have developed a method of identifying the effects of toxic substances on genetic material in situ in somatic cells. The technique assesses alteration of DNA structure following administration of the test material. The procedure is rapid, reproducible, and provides a qualitative measure of change in DNA structure by density gradient separation.
Test animals receive tritiated thymidine to provide uniform labeling of somatic cell DNA. Subsequently, the potentially toxic material is administered intraperitoneally. After incubation, the liver is removed and a whole cell suspension is rapidly prepared by the squash technique. This cell suspension is lysed in situ and is placed in the ultracentrifuge for alkaline or neutral sucrose-gradient separation and subsequent $^3$H position determination.

Unaltered DNA will appear as one distinct band similar to that observed in preparations from cells unexposed to the test substance. Altered DNA will appear in different bands. Where severe DNA fragmentation has occurred, the lighter weight fragments will appear closer to the upper segment of the sucrose gradient. If necrosis has occurred, cellular DNA and its degradation products will be present in the supernatant.

This technique provides a relatively simple method of monitoring the efficiency of DNA repair mechanisms. By repeated testing over time, the separation of DNA bands can be compared to that in previous tests. If band patterns are returning to the normal pattern, DNA repair is occurring. If no change in distribution of DNA fragment bands is evident, the DNA is permanently damaged and repair mechanisms are inoperable, at least for the time period under test.

At the present time, this test system is being used to evaluate the effects of carcinogenic substances on DNA in hepatic cells. Results suggest that some hepatic carcinogens alter DNA irreversibly.

A distinct advantage of this technique is its applicability to various types of test situations. The route of administration, test animal, or type of somatic cell is not critical provided each is standardized. Cells from any tissue or organ suspected of being affected by a toxic substance could be analyzed. In addition, the technique differs from other procedures that measure disappearance of the $^3$H label alone. The shift in position of $^3$H rather than the loss of $^3$H is correlated with changes in density gradient separation of DNA.

While functional change in DNA is not evaluated by this procedure, the technique does provide an accurate measure of reversible or irreversible physical alteration of DNA in cells exposed to toxic substances.
The second mechanism of interaction with nucleic acids involves fixations in DNA of alterations which may have occurred in RNA. Reverse transcription mediated by the enzyme, reverse transcriptase, controls RNA directed synthesis of DNA. The enzyme has been found in most oncogenic RNA viruses except certain mutants which have lost the capacity to infect and transform the normal cell. The study of this phenomenon suggests that reverse transcriptase activity is essential to the initiation of oncogenic transformation by RNA viruses (Gallo, 1972). While it is not difficult to determine reverse transcription in a virus, demonstration of activity of this enzyme in human neoplastic tissue is difficult because of problems of separating and identifying this specific enzyme from cellular DNA polymerases. DNA polymerases as a group are somewhat nonspecific and, according to Gallo (1972), there is no conclusive evidence at the present time that a true reverse transcriptase is present in normal cells, particularly mature cells including neoplastic cells.

However, studies on reverse transcription are extremely important with respect to the origin of neoplastic tissues. Gallo (1972) has noted that one of the most important uses of a viral DNA polymerase would be in the preparation of a radio labeled DNA which would be a partial copy of the 70-S RNA genome of a RNA tumor virus. The labeled DNA would be an extremely sensitive molecular probe for detecting complementary sequences of RNA in cells. If found, they would be indicative of a viral RNA genome in the host cell. Similarly, the detection of reverse transcriptase in cellular inclusions of neoplastic tissue would suggest that these particles were, in actuality, RNA viruses and potentially oncogenic. Recent in vitro studies with radiolabeled DNA products of reverse transcriptase in hybridization studies with human cellular RNA have led to the demonstration of viral RNA genome sequences in neoplastic tissues that are not detectable in extracts from normal cells (Abrell et al., 1972; Yang et al., 1972; Gurgo et al., 1972). These observations represent one of the most active areas of molecular oncology at the present time.

4. Tissue Culture Studies

In their review of test methodologies, the FDA Advisory Committee on Protocols for Safety Evaluation (1971) suggested that tissue culture was the most promising technique for screening carcinogenic chemicals. For example, tissue culture techniques have been used for evaluating the ability of oncogenic viruses to induce transformation in cells in vitro. These techniques could be used
to investigate the possible role of viruses as intermediates in the pathogenesis of neoplasms that may be induced by chemical and physical agents. The potential of cell culture and organ culture model systems for carcinogenicity testing deserves further investigation.

Presently available cell lines that could be used to test carcinogens may be of limited value in testing procarcinogens requiring activation, but cultured cell lines might be developed that could grow in the presence of other cells which activate the procarcinogen. Such systems are admittedly complex and are based on the premise that the ultimate carcinogen can be transported across cell membranes in its active form. Theoretically, the effects of potentially toxic substances could be compared in both tissue culture or a modified host-mediated assay where cultured cells are used in lieu of microorganisms. NCTR should consider research on developing techniques of monitoring transformation phenomena in cell lines known to contain or to be free of viral RNA.

5. **Exposure of Gravid Females**

Transplacental exposure to carcinogens is an interesting and important facet of environmental toxicity which began with Larsen's (1947) discovery that lung tumors could be produced in animals exposed to urethan transplacentally. The list of compounds is growing (see recent review of Swendburg et al., 1972) and the potential human hazard is emphasized by the occurrence of vaginal cancers in adolescents whose mothers had received stilbestrol during pregnancy (see review by Miller, 1971). This area needs considerably more development, including age of exposure in relation to sites and histologic types of induced tumors, mechanism of transplacental transport and metabolism, and relation to teratogenesis.
D. EPIDEMIOLOGICAL MONITORING

It is recognized that epidemiological monitoring for patterns of chemical carcinogenicity is outside the scope of the NCTR mission. However, numerous experts have suggested that monitoring of the human population for mutation, congenital defects, and diseases including cancer can provide a basis for definition of subjects or aspects that should receive more critical study or scrutiny (Crow, 1971; Neel, 1970; Higginson, 1969; Miller, 1966; Hueper and Conway, 1964). Recent emphasis on environmental quality may lead to greater understanding of human exposure to various environmental contaminants.

These investigations are not a substitute for initial toxicological evaluation of new chemicals as they are introduced into the environment, but may provide clues about substances or exposure patterns that suggest the need for further toxicological evaluation. Future NCTR research programs should take into consideration the results of epidemiological studies conducted by other agencies. Where investigations draw attention to specific substances or exposure patterns that seem related to types of cancer, or other manifestations of adverse effects, NCTR could provide facilities for long-term studies of substances implicated in the development of these adverse health effects.
X. CORRELATION OF TEST SYSTEMS

Three areas in the research program of the NCTR receiving primary emphasis are the mutagenic, carcinogenic and teratogenic effects of chemical compounds. It is recognized that chemicals may produce more than one of these three effects. For example, many and perhaps all carcinogens are potential mutagens and many but probably not all mutagens are potential carcinogens (Miller and Miller, 1971). Thus, the correlation between carcinogenic activity and mutagenic activity is far from perfect. The presence of even a high correlation between the two effects is not proof that the two effects are due to the same basic cause (Miller and Miller, 1971; Weinstein et al., 1971).

Teratology and mutagenicity are separated empirically. The relationships between genetic alteration and production of gross structural abnormalities are not firmly established. If teratology is limited by definition to development of congenital malformations or gross structural abnormalities, a number of chemical compounds are known to be both mutagenic and teratogenic (Kalter, 1971); however, these represent only a few of the chemicals tested.

Several observations based on epidemiological monitoring of human populations have indicated an association between certain abnormalities present at birth and an unusually high incidence of neoplasia (Boland, 1972; Miller, 1966, 1969). For example, there is an excessive occurrence of leukemia but not other cancers (with the possible exception of certain central nervous system tumors) in children with Down's syndrome (Miller, 1966). Wilms' tumor has been found to be associated with an excessive incidence of absence of the iris (aniridia) found in 1:50,000 to 1:100,000 in the general population, but in 1 of 73 Wilms' tumor cases (Boland, 1972).

Although correlation of teratogenic and/or mutagenic and/or carcinogenic effects of a compound may be significant, the correlation itself is not indicative of a common or single cause for the effect. Currently, the basic mechanisms of these processes are so poorly understood that additional explanation of these correlations is not possible. It is desirable that testing procedures be useful not only in assessing one of these areas but
also that they be of use for evaluation of the two other effects. However, such techniques are not currently available. As methods for study of carcinogenicity, mutagenicity and teratogenicity, continue to be developed and refined, the relationships among these processes must be considered.

Systematic study of the usefulness of a test procedure for concurrent or subsequent evaluation of other endpoints should be a primary long range goal of NCTR. For example, mutagenicity testing has been suggested as a screening method for carcinogenicity although this is not a completely accepted concept. NCTR can play a substantial role in the development of the information base necessary to establish or refute major concepts in evaluation of long-term, low-dose exposures to potentially toxic substances.
XI. APPLICATION OF THE RESULTS OF ANIMAL TESTING TO MAN

A. INTRODUCTION

The use of animal models for research on diseases of man has become a widely used and valuable experimental approach in medical science. The testing of potentially toxic drugs or chemicals in animals has become a standard and useful procedure for assessing their toxicity in man as well as animals. It is well known that compounds which are toxic for one species are likely to be toxic for others and this is especially true for the more potent toxins. However, there are numerous exceptions and there is no absolute relationship between animal toxicity data and the corresponding hazards for man.

This does not mean that toxicity studies in animals are seriously limited in their value but it does make interpretation of animal tests difficult, requiring detailed knowledge of the factors involved in the specific mechanism of toxicity for any compound.

Rall (1969), Dixon and Homan (1972 and Schein (1972) discussed the problems of extrapolating results of toxicity data from animals to man in the search for anticancer drugs. One of the striking arguments developed in Rall's review is the cited example of the search for new drugs. Assuming that 100 new compounds are tried in man within a year, with one of these 100 there is a failure of prediction from animal trials in which either death or severe disability occurs. This would be considered by the lay press to be a catastrophe. However, the predictability score of 99% would be rational from a scientific point of view. He concludes, "Therefore, the problem of predictability lies primarily in the fact that the stakes are so high, and not that the systems are necessarily so bad."

The public health problem related to the search for nontoxic anticancer drugs and the assessment of the hazards of environmental contaminants are fundamentally different. Promising antineoplastic agents, after animal toxicity screening, would be evaluated in a relatively few patients under careful medical supervision and therefore the gross potentiality for harm is relatively low. On the other
hand, the widespread distribution of a noxious chemical in the environment may involve thousands of individuals without their knowledge. In the latter instance, it will be incumbent upon the responsible health agencies to develop an animal toxicology program that will at best be reasonably predictive in a prospective sense.

Irreversible toxicity is a more serious problem than reversible toxic effects. In addition, the simultaneous exposure to a number of agents may enhance the toxic effects and it is impossible to resolve these questions with present knowledge. In general, toxicity testing that shows a low incidence of untoward effects in experimental animals will very likely show a correspondingly low incidence for toxicity in man. However, there are a number of serious exceptions to this rule and generalizations cannot be made with absolute assurance.
B. BASIS OF SPECIES VARIATIONS

Most discussions of extrapolation of animal tests to man deal with the question of species differences and similarities (Beyer 1967). Many species differences have been identified such as size, age, diet, environmental habitat and schedule of behavior (Dearborn 1967). The state of health and the anatomic physiologic and biochemical similarities have been stressed (Brodie 1962). These gross morphologic, functional and behavioral factors are embraced within the broad concepts of this discussion but will not be considered in detail.

Species differences in toxic response are related to differences in the factors involved in the mechanisms of specific toxicity. These include; the absence or presence of specific receptor sites, variations in biotransport or concentration, variations in biotransformation of compounds and a combination of the above factors.

From the biological point of view, absorption, distribution, excretion, metabolism, and mechanism of action of the toxic substance serve as a basis for comparison between species. If the compound under consideration follows a similar pattern for all of these steps (a highly unlikely possibility), then the appraisal of the toxicity of the substance will likely be very good in terms of extrapolating the data accumulated from animals to man. If the compound is not absorbed, or if it is absorbed and not distributed to target tissues, or if it is rapidly excreted, rapidly metabolized and has an unknown mechanism of action, it will be difficult to make a correlation between species. There will be a greater or lesser degree of correlation depending upon the significance of these variables (Beyer, 1967).

Receptor sites or molecular sites of action are recognized as important in toxic reactions but few have been identified. They can provide the basis for specific toxic activity and their absence or presence can account for the differences in cellular, tissue, individual or species variability in response to a chemical. The presence of sulfhydryl groups in critical enzymes has been suggested as one example of a receptor site in the toxic action of lead and other heavy metals. The absence or presence of alternate metabolic
pathways could determine whether a receptor site is critically important to a species, thus, the primary determination of toxic injury.

Biotransport and the localization and concentration of toxins at specific sites is one of the most important mechanisms of species variation. Rates of absorption and rates of excretion control blood levels and tissue dose levels of toxins. The relative concentration and distribution of a test material or its toxic metabolite in the test animal and man must be known to evaluate that species as an appropriate model for man.

The lifespan of an animal may become involved in problems of concentration. In the case of long-term, low level assessment of a toxin that is stored in the body, a mouse may not live long enough to store a toxic level that might be achieved in man during a lifetime. On the other hand long-term studies in long lived species are costly and impractical because of the time involved and the financial limitation on numbers.

The most commonly accepted proposal for toxicology evaluation in animal species, and the subsequent comparison to man, revolves around the exploration of the metabolic pathways in the two species. If the metabolic pathways are similar, it is argued the toxicity testing in the experimental animal is most likely to have a high degree of predictability for man.

Blood or tissue levels can be useful criteria for the prediction of toxicity from experimental animals to man. The most useful measurements of these criteria require analytical procedures capable of detecting the substance in extremely low levels. Fortunately, the newer analytical techniques lend themselves to this purpose. Quantities of some compounds can be detected in nanogram amounts, or parts per billion. Therefore, it is conceivable that companion studies could be undertaken in man with these extremely low levels of exposure that may serve as guides to subsequent animal tests because they may indicate similar metabolic pathways and target organ sites. Blood levels that subsequently may be proven to be hazardous for man but not in the experimental animal model can be avoided by this approach.

We are only beginning to understand the significance of metabolic pathways in the human body, let alone explore the significance of biotransformations in a wide variety of experimental
animals for the ever growing number of chemical substances that must be tested. The problem becomes monumental in scope if reviewed in this fashion. Until the general metabolic patterns at least of classes of chemical substances are better understood, it will be impossible to utilize this most fruitful approach.

The problem of biotransformation of chemical substances fortunately can be examined on a fundamental basis in terms of in vitro studies; for example, by isolated liver microsomes. This will give some useful guidelines to be employed in the exploration of suitable animal species for a given substance because these pathways can be examined rapidly and accurately without the investment of a large number of experimental animals and investigators' time.

According to this approach, it is possible to develop strains of inbred animals that possess characteristic metabolic patterns valuable in drug metabolism studies. For example, different strains of rats exist that oxidize compounds at an accelerated rate as compared to noninbred strains. These hereditary aspects of animals make them useful for drug or chemical metabolism studies. Unfortunately, the remarkable heterogeneity of man makes him less predictive in terms of his sensitivity to a specific compound. The experienced investigator recognizes that there will always be human individual differences that will confound laboratory predictions because individual variability in man is so great.
C. PRACTICAL CONSIDERATIONS

In the evaluation of animal experimental results to detect the toxicity of chemical substances for man, it is important to recognize that safety is not an absolute value. Most scientists appreciate that there is no such thing as an absolutely negative result in any toxicological study. However, as the experiments are conducted with increasing degrees of sophistication, a greater level of confidence is obtained depending upon prior experience with the species and the proven validity of, or confidence in, the test system under standardized experimental conditions. The greater the potential for lowering the absolute level of negative findings in experimental test systems, the greater will be the level of confidence on the part of the investigator in the test system itself. In general, most workers would agree that the ability to evaluate and predict animal testing results for man will be essentially qualitative in character and that any quantitative prediction is an estimation.

This report has been concerned primarily with the tools that are used to detect adverse effects in animals. As these instruments are refined, it becomes more and more difficult for the investigator to obtain negative data and it may become more and more difficult to relate animal data to man. In other words, the ability of the test system to detect some change in the biological system becomes more precise and more accurate as the refinement of the methodology is improved. As has been pointed out by Stokinger (1971), only the development of data from experimental studies will supply the level of confidence required for any given substance or class of compounds. Stokinger (1971) emphasized that environmental health standards must be based on scientific facts with reasonable limits that can be achieved with the level of knowledge we have at the present time.
XII. SUGGESTED AREAS FOR FUTURE RESEARCH

NCTR has a unique opportunity to address the need for developing techniques with predictive value that are useful in assessment of the effects of long-term, low-dosage exposures to potentially toxic substances. In many instances, selection or recommendation of techniques for toxicological evaluation are hindered by inadequate methodological validity. Many useful techniques that measure parameters such as biochemical alterations, mutagenicity, teratogenicity or carcinogenicity appear promising, but require further refinement and evaluation. NCTR should undertake research on improvement of methodology and experimental design for toxicological evaluation, and research on the basic biological processes that underlie chemical toxicity because these areas of research are necessary to determination of adverse health effects of long-term exposures to low doses of potentially toxic substances. By developing methodology and a broad data base, NCTR can make a significant contribution to interpretation of toxicological studies on animals and man.

1. General Aspects of Pathology

Pathology at the NCTR should not be purely morphological. It should be developed in a way that embraces morphology (light and EM levels), physiological and biochemical methodologies. The cadre of in-house pathologists at the NCTR should reflect this philosophy and should include pathologists who have expertise in each of the above-mentioned areas.

The natural progression of some pathological changes in experimental animals is poorly understood. The NCTR research program should include study of the progression of morphological and chemical expressions of pathological change. There is a need to relate the morphological changes described in the gross evaluation with other endpoints of toxicity such as histological evaluation, clinical chemistry profiles, and the overall health of the animal; the pathologist is much more than a morphologist.

Development of standardized nomenclature for descriptive pathology at NCTR should, as much as possible, be compatible
with the systems used by the National Cancer Institute, the Armed Forces Institute of Pathology, and the Oak Ridge National Laboratory. The use of terms should, where possible, be consistent with other programs such as the Standardized Nomenclature of Pathology (SNOP), and reference works such as *Nomina Histologica*, *Nomina Embryologica*, and *Nomina Anatomica*. It is recommended that SNOP be adapted to the purpose of animal lesions at the NCTR.

It is recommended that studies be carried out to extend toxicity testing to the cellular and subcellular levels using both morphological and biochemical techniques.

2. **Anatomical Pathology**

Toxicological evaluation depends to a great extent upon techniques in anatomical pathology. NCTR should consider developing better techniques for morphological examination of organ systems often omitted or inadequately examined in the gross morphological dissection, and particularly the significance of changes at the cellular and subcellular levels. Electron microscopic and histochemical methods should be employed to detect early changes, to develop new methods for detecting minimal yet significant changes in the intracellular milieu. Centralized electron microscopic, histopathological and histochemical laboratories should be instituted.

NCTR has a unique opportunity to develop guidelines on management and logistics of handling necropsy and histology data from experiments involving large numbers of animals.

NCTR should place emphasis on development and evaluation of automated techniques in morphology and indirect methods of morphological study such as automated pattern recognition.

3. **Biochemical and Clinical Pathology**

Recent advances in biochemical and clinical pathology suggest that the NCTR research program should include evaluation of biochemical indices of cellular injury. Although many clinical laboratory procedures are well developed for detection of abnormalities in man which facilitate accurate diagnosis of nosological entities, much more work is required to adapt such precise methods for use in other animals, especially small laboratory species.
It is recommended that an appropriate laboratory be established in which to carry out these studies, to include tests at cellular and subcellular levels.

A word of caution: Logically one might gather together a nucleus of skilled technologists who would respond to requests from the scientific staff to devise particular new methods critically important to specified objectives. This is fitting and proper. NCTR should also search for a professional scientist who is also an imaginative gadgeteer and will identify problems that he can solve that are equally or more important to the entire group than those pragmatic needs known to all. Such an individual usually becomes a focal point both to the technologically sophisticated and the scientifically oriented personnel. Such people are rare indeed.

The capacity to study many biochemical changes provides the opportunity to collect, store, and analyze the vast quantity of data necessary to correlate biochemical events with other structural and functional alternatives associated with toxicity.

4. Techniques in Mutagenicity Testing

NCTR should focus its research program on mammalian systems of evaluating mutagenicity. Emphasis should be placed upon the dominant lethal assay, in vivo cytogenetics, the host-mediated assay, and techniques revealing polygenic effects of mutagenic substances. Development of further modifications of these procedures, e.g., the dominant lethal assay, is desirable.

There is a need to correlate results from these several tests of mutagenicity in order to establish the desirability of developing a sequence of test procedures for mutagenic evaluation of substances.

In addition to the methods noted above, a number of other techniques for mutagenicity testing show promise. NCTR should plan to review opportunities for utilization of these techniques as current research establishes the validity of these methods in evaluation of mutagenicity.

Additional study of nucleic acid repair mechanisms may lead to alternative methods of detecting the effects of mutagens.
In the course of developing the mutagenicity research program, NCTR should study critically those methods that show promise of detecting synergistic mutagenic actions.

Because NCTR will be evaluating the mutagenicity of a large number of chemicals, it should consider the possibility of correlating mutagenicity with chemical structures.

5. Techniques in Testing Teratogenicity

Teratological studies can play a significant role in evaluating the toxicity of long-term, low-dose exposures to potentially toxic substances. However, there is a need for additional data on the biological bases of teratogenic responses in several animal model systems. NCTR should include research on the fundamental aspects of teratology in concert with development of methodology.

Teratogenicity testing should include more complete evaluation of functional disorders as well as prolonged observations of offspring. The NCTR program offers an unusual opportunity to collect these data. In addition, consideration should be given to future investigation of the collection and interpretation of data on similarities and differences in teratogenic effects among a number of animal strains and species; the teratogenic effects highly localized in specific organs in certain animal species for classes of chemical compounds; and the relationships between administered dose and fetal tissue concentrations of many substances to correlate the true teratogenicity of a substance and its metabolic derivatives.

The NCTR teratology program should include emphasis on the feasibility of collecting data for reference standards and techniques for teratogenicity testing; and the correlation of intermediary metabolic findings on known teratogens and those related compounds found to be nonteratogenic. This approach may assist in defining the specific nature of the toxic substance in vivo.
6. Techniques in Testing Carcinogenicity

Many attempts have been made, and indeed are continuing, to identify methods for ascribing carcinogenic potential to substances short of testing in long term, essentially life-span experiments in laboratory animals. These have been largely unsuccessful. However, the desirability of arriving at some indirect method for identifying carcinogens can not be gainsaid, and doubtless the National Cancer Institute will continue to support such work. The NCTR should be alert to monitor work toward this desirable goal wherever it may be conducted and under whatever auspices. It might be appropriate for NCTR to enter the lists, providing sufficiently novel and innovative approaches can be generated among its staff, but this should not be an overriding consideration in formulating a toxicology program where neoplastic disease is but a single endpoint.

It would seem more fitting that NCTR concentrate on defining the natural history of those neoplasms arising in the various stocks housed at the Pine Bluff facility - as surely they will. The histological criteria of malignancy applicable to most clinical cancers may apply to some neoplasms in experimental animals, but not to all. A number of lesions in rats and mice do not fulfill the criteria of malignancy so useful in human pathology. Nevertheless these are neoplasms which grow progressively and may ultimately metastasize, though the metastases themselves appear histologically identical. The majority of such neoplasms will grow progressively on transplantation to genetically appropriate hosts, and indeed are biologically malignant. The emphasis should be placed on biological attributes with definition of morphological expressions of those attributes that may be useful in interpreting the changes seen under the microscope.

Of the many compounds already tested for carcinogenic potency, some are known to be parents of metabolically derived compounds that are indeed the actual carcinogens. This facet of cancer research deserves substantial emphasis at the NCTR. Should a series of congeners be metabolized to a common compound known to be a proximate carcinogen, then that information may be extremely useful in assigning carcinogenic potency to those congeners. At present this is a working hypothesis supported by the studies of Clayson (1962), and of Miller (1970), which could be
properly put to the test at the NCTR. However, successful resolution of that issue would apply only for those species in which the end products of metabolism of the congener series were identical or very nearly so.

Carcinogenic potency is conditional. It may be conditioned by the species in which it is tested or by a host of factors which tend to inhibit or enhance the response. Most certainly carcinogenesis is similar neither to the physical nor chemical properties that an element or compound exhibits in immutable array.

7. General Aspects of Toxicological Evaluation

NCTR should consider the development of techniques utilizing general parameters of toxicity that can be incorporated into statistically sound experimental designs. These studies may be useful in development of new approaches to the difficult problem of defining factors which interact with test compounds in large-scale, long-term, low-dosage studies.

The NCTR research program should place emphasis on establishing the incidence and severity of diseases and disorders normally present in the several animal models prior to their use in definitive toxicological studies because these manifestations may interfere with detection and quantification of the subtle changes induced by long-term, low-dosage studies.

Utilization of nutrients, especially in the neonate, can be extremely sensitive to extrinsic factors. A toxic compound may indirectly cause adverse effects by interference with the nutrition of the animal. Future research should include emphasis on the nutritional requirements of each animal model, especially the infrequently or newly introduced species and specific pathogen-free animals.

NCTR should consider inclusion of reproductive and behavioral testing in future research programs because these are sensitive indicators of toxicity. Where possible, these studies should be incorporated into investigations of other endpoints of toxicity.

NCTR should make provision for publication of experimental protocols on proposed standardized methods of toxicological evaluation for use and review by the scientific community.
8. Correlation of Test Systems

Systematic study of the usefulness of a test procedure for concurrent or subsequent evaluation of other endpoints of toxicity should receive emphasis because the NCTR mission includes correlation of test systems. For example, mutagenicity testing has been suggested as a screening method for carcinogenicity although this is not a completely accepted concept. NCTR can play a substantial role in the development of the information base necessary to establish or refute major concepts in evaluation of long-term, low-dose exposures to potentially toxic substances. This systematic study should include investigations on the comparative efficacy of several methods of detecting the same or related endpoints of toxicity.

Because biochemical and clinical tests appear to be more readily automated, the NCTR research program should include correlation of results from these methods with the generally accepted morphological criteria of pathology.

NCTR should consider providing facilities for long-term studies of substances implicated in adverse health effects by epidemiological studies of other agencies.

The NCTR research program should give consideration to providing mechanisms whereby data from NCTR studies can be integrated with results of other investigations. Provisions should include correlating, disseminating, and storing these data in a manner suitable for rapid access by the world-wide community of toxicology.
XIII. BIBLIOGRAPHY

1. Aaronson, S. A.; and Todaro, G. J.
   Infectious SV40 and SV40 DNA: Rapid Fluorescent Focus
   Assay (34738).

2. Abelev, G. I.
   Antigenic Structure of Chemically-Induced Hepatomas.

3. Abrell, J. M.; Smith, R. G.; Robert, M. S.; and Gallo, R. C.
   DNA Polymerases from RNA Tumor Viruses and Human Cells.

4. Adams, A. E.
   Effects of Thyroxine on Chick Embryos.

5. Adams, L. R.; and Kamentsky, L. A.
   Machine Characterization of Human Leucocytes by Acridine
   Orange Fluorescence.

6. Adams, L. R.; and Melamed, M. R.
   A Pulse Cytofluorimeter.

7. AEI Scientific Apparatus, Ltd.
   First Symposium on Biological Applications of Combined
   Transmission Electron Microscopy and X-ray Probe Micro-

8. Alibert, A.; Rigal, M.; Bastide, P.; Turchini, J.; and
   Dastuque, G.
   Examens de Quelques Activités Enzymatiques dans le Foie
   et le Rein de Souris avant et après Administration de
   Tetrachlorure de Carbone ou de Nitrate d'Uranyle.

9. Allen, R. D.; David, G. B.; and Nomarski, G.
   The Zeiss-Nomarski Differential Interference Equipment
   for Transmitted-Light Microscopy.
10. Alpert, M. E.
α-1-Feto-protein in Human Hepatoma.

11. Ames, B. N.
The Detection of Chemical Mutagens with Enteric Bacteria.
In: Chemical Mutagens; Principles and Methods for Their

12. Anderson, A. C., Editor
The Beagle As an Experimental Dog.

13. Ashley, C. A.; and Feder, N. Glycol Methacrylate in
Histopathology: A Study of Central Necrosis of the Liver
Using a Water-Miscible Plastic as Embedding Medium.

Renal Tests in Toxicity Studies on Rats.

15. Baldwin, R. W.; Glaves, D.; Pimm, M. V.; and Vose, B. M.
Tumour Specific and Embryonic Antigen Expression of
Chemically Induced Rat Tumours.

16. Barka, T.; and Anderson, P. J.
Histochemistry: Theory, Practice and Bibliography.

17. Bateman, A. J.; and Epstein, S. S.
Dominant Lethal Mutations in Mammals.
In: Chemical Mutagens; Principles and Methods for Their

18. Ben-Bassat, H.; Inbar, M.; and Sachs, L.
Changes in Structural Organization of the Surface Membrane
in Malignant Cell Transformation.

19. Benjamin, T. L.; and Burger, M. M.
Absence of a Cell Membrane Alteration Function in
Non-transforming Mutants of Polyoma Virus.
A Study of Automated Clinical Laboratory Systems.
PB 204 923.

Limitations of Animal Data for Predicting Safety for Man.
In: Use of Human Subjects in Safety Evaluation of Food
Chemicals. NAS-NRC Publication 1491, pp 43-49 (1967).

22. Bolande, R. P.
Relationship Between Teratogenesis and Oncogenesis. Paper
presented at a Symposium on Pathobiology of Development.
American Association of Pathologists and Bacteriologists,
Annual Meeting, Cincinnati, Ohio, March 11-14, 1972.

23. Breen, K. J.; Schenker, S.
Liver Function Tests.

24. Brent, R. L.
Drug Testing in Animals for Teratogenic Effects:
Thalidomide in the Pregnant Rat.

25. Brent, R. L.
The Direct and Indirect Effects of Irradiation upon
Mammalian Zygote, Embryo and Fetus.
In: Methods for Teratological Studies in Experimental
Animals and Man. H. Nishimura and J. R. Miller, Eds.,

26. Brent, R. L.
Protecting the Public from Teratogenic and Mutagenic
Hazards.

27. Brodie, B. B.
Difficulties in Extrapolating Data on Metabolism of Drugs
from Animal to Man.

28. Brutlag, D.; and Kornberg, A.
Enzymatic Synthesis of Deoxyribonucleic Acid. XXXVI. A
Proofreading Function for the 3'→5' Exonuclease Activity
in Deoxyribonucleic Acid Polymerases.
29. Cahen, R. L.
Evaluation of the Teratogenicity of Drugs.

30. Cahen, R. L.
Experimental and Clinical Chemoteratogenesis.

31. Cahen, R.; and Fave, A.
Y-a-t-il une Relation entre l'Effet Teratogène et la
Passage Transplacentaire d'un Medicament?

32. Cappell, D. F.; and Anderson, J. R.
Muir's Textbook of Pathology, 9th Ed.
Edward Arnold Publisher Ltd., Bungay, Suffolk. 967 p (1971).

33. Caro, L. C.; and Van Tubingen, R. P.
High Resolution Autoradiography, I. Methods.

34. Carpenter, S. J.; and Ferm, V. H.
Uptake and Storage of Thorotrast by the Rodent Yolk Sac
Placenta: an Electron Microscopic Study.

35. Carr, C. J.
An Appraisal of Animal Drug Screening Techniques Used in
Psychopharmacology.
J. New Drugs **3**: 135-146 (1963).

36. Cattanach, B. M.
Specific Locus Mutation in Mice.
In: Chemical Mutagens; Principles and Methods for Their

37. Christov, St. G.
Verallgemeinerte Näherunsausdrücke für die Durchlässigkeit
von Potentialschwellen.

38. Christov, St. G.
Zur Berechnung der Mittleren Durchlässigkeit von
Potentialschwellen.
39. Clayson, D. B.
Chemical Carcinogenesis.

40. Cleaver, J. E.
Defective Repair Replication of DNA in Xeroderma Pigmentosum.

41. Clegg, D. J.
Teratology.

42. Cohen, M. M.; and Hirschhorn, K.
Cytogenic Studies in Animals.
In: Chemical Mutagens; Principles and Methods for Their

43. Conzelman, G. M., Jr.; and Moulton, J. E.
Dose-Response Relationships of the Bladder Tumorigen
2-Naphthylamine: A Study in Beagle Dogs.

44. Coons, A. M.
Fluorescent Antibody Methods.
In: General Cytochemical Methods, Vol. 1, J. F. Danielli, Ed.,

45. Cornfield, J.; Halperin, M.; and Moore, F.
Some Statistical Aspects of Safety Testing of Salk Polio-

46. Cornish, H. H.; Barth, M. L.; and Dodson, V. N.
Isozyme Profiles and Protein Patterns in Specific
Organ Damage.

47. Crenshaw, J. W.; and Yake, D. J.
Heritable Effects on Fitness of Ionizing Radiation in
Inbred Mice.

48. Crow, J. F.
Human Populations Monitoring.
In: Chemical Mutagens; Principles and Methods for Their


58. Dinman, B. D.; Fox, C. F.; Frajola, W. J.; and Rabor, A. 
   Serum Enzyme and B12 Changes in CCl4 Hepatotoxicity. 

59. Dixon, R. L.; and Homan, E. R. 
   Prediction from Laboratory Animals to Man – Quantitative 
   Relationships. 
   Fifth International Congress on Pharmacology, San Francisco, 

60. Done, A. K. 
   Developmental Pharmacology. 

61. Drake, J. W. 
   The Molecular Basis of Mutation. 

62. Drake, J. W. 
   Mutagen Screening with Virulent Bacteriophages. 
   In: Chemical Mutagens; Principles and Methods for Their 

   Formalin Fixed Cell Preparations as Standards for Use in 
   the Automated Trypan Blue Cytotoxic Assay. 
   Transplantation 14: 127-130 (1972).

64. Dubach, U. C.; and Padlina, G. 
   Aktivität der Alkalischen Phosphatase im Urin. 

65. Dugle, J. B.; and Free, A. H. 
   A Simple Blood Test for the Assessment of Renal Function 
   in Experimental Animals. 

66. Dulbecco, R. 
   Reactivation of Ultra-Violet-Inactivated Bacteriophage 
   by Visible Light. 

67. Eanes, E. D.; and Glenner, G. G. 
   X-ray Diffraction Studies on Amyloid Filaments. 
68. Epstein, S. S.; Arnold, E.; Steinberg, K.; Mackintosh, D.; Shafner, H.; and Bishop, Y.
Mutagenic and Antifertility Effects of TEPA and METEPA in Mice.

69. Epstein, S. S.; Arnold, E.; Andrea, J.; Bass, W.; and Bishop, Y.
Detection of Chemical Mutagens by the Dominant Lethal Assay in the Mouse.

70. Epstein, S. S.; and Röhrborn, G.
Recommended Procedures for Testing Genetic Hazards from Chemicals, Based on the Induction of Dominant Lethal Mutations in Mammals.

71. Epstein, S. S.; and Shafner, H.
Chemical Mutagens in the Human Environment.

72. Ericsson, J. L. E.; and Trump, B. F.
Observation on the Application to Electron Microscopy of the Lead Phosphate Technique for the Demonstration of Acid Phosphatase.

73. Farber, E.
Personal Communication (1972).

74. Ferm, V. H.
The Rapid Detection of Teratogenic Activity.

75. Ferm, V. H.
The Synerteratogenic Effect of Lead and Cadmium.

76. Ferm, V. H.
The Teratogenic Effects of Metals on Mammalian Embryos.

77. Ferm, V. H.; and Ferm, D. W.
The Specificity of the Teratogenic Effect of Lead in the Golden Hamster.
78. Fisher, R. M.
Electron Microscopy - 1 Million Volts and Beyond.

79. Fitzhugh, O. G.
Reproduction Tests.
In: Modern Trends in Toxicology, I. E. Boyland and
(1968).

80. Food and Drug Administration Advisory Committee on
Protocols for Safety Evaluation.
Panel on Reproduction Report on Reproduction Studies in
the Safety Evaluation of Food Additives and Pesticide Residues.

81. Food and Drug Administration Advisory Committee on Protocols
for Safety Evaluation.
Panel on Carcinogenesis Report in Cancer Testing in the
Safety Evaluation of Food Additives and Pesticides.

82. Foulds, L.
Neoplastic Development. Vol. 1

83. Franz, J.; and Degenhardt, K. H.
A Model in Comparative Teratogenesis. II. Response to
5-Fluoro-2-Deoxyctydine at Successive Stages in Organogenesis.

84. Freese, E.
Molecular Mechanisms of Mutations.
In: Chemical Mutagens; Principles and Methods for Their

85. Gabridge, M. G.; and Legator, M. S.
A Host-Mediated Microbial Assay for the Detection of
Mutagenic Compounds (33666).

86. Gallo, R. C.
Cancer Prophylaxis and Remission Maintenance: Concepts
Derived from Studies on Viral Oncogenesis.
Fifth International Congress on Pharmacology, San Francisco,
87. Garant, P. R.
The Demonstration of Complex Gap Junctions between the Cells of the Enamel Organ with Lanthanum Nitrate.

88. Gelboin, H. V.; Kinoshita, N.; and Wiebel, F. J.
Microsomal Hydroxylases: Induction and Role in Polycyclic Hydrocarbon Carcinogenesis and Toxicity.

89. George, L. A.; and Bustad, L. K.
Comparative Effects of Beta Irradiation of Swine, Sheep and Rabbit Skin.

90. Ginn, F. L.; Shelburne, J.; and Trump, B. F.
Disorders of Cell Volume Regulation. I. Effects of Inhibition of Plasma Membrane Adenosine Triphosphatase with Ouabain.
Amer. J. Pathol. 53: 1041-1071 (1968).

Creation of "Amyloid" Fibrils from Bence Jones Proteins in Vitro.

Murine Amyloid Fibril Protein: Isolation, Purification and Characterization.

93. Glenner, G. G.; Terry, W.; Harada, M.; Isersky, C.; and Page, D.
Amyloid Fibril Proteins: Proof of Homology with Immunoglobulin Light Chains by Sequence Analyses.

94. Gold, P.; and Freedman, S. O.
Specific Carcinoembryonic Antigens of the Human Digestive System.
95. Goodale, R. H.; and Widmann, F. K.
Clinical Interpretation of Laboratory Tests, 6th Ed.

96. Graham, R. C., Jr.; and Karnovsky, M. J.
The Early Stages of Absorption of Injected Horseradish
Peroxidase in the Proximal Tubules of Mouse Kidney;
Ultrastructural Cytochemistry by a New Technique.

97. Green, D. E.
In Vivo Detection of Drugs.

98. Green, D. E.
Automated Detection of Abused Drugs by Direct Mass
Fragmentography.

99. Greenawalt, J. W.; Rossi, C. S.; and Lehninger, A. L.
Effect of Acute Accumulation of Calcium and Phosphate
Ions on the Structure of Rat Liver Mitochondria.

100. Grice, H. C.
The Changing Role of Pathology in Modern Safety Evaluation.

101. Gurgo, C.; Ray, R.; and Green, M.
Rifamycin Derivatives Strongly Inhibiting RNA Leads to
DNA Polymerase (Reverse Transcriptase) of Murine
Sarcoma Viruses.

102. Gurkin, M.; and Kallet, E. A.
An Instrument for Quantitative Microspectrofluorometry.

103. Gutmann, F.
Electron Tunneling in Biological Structures.
104. Hackenbrock, C. R.
Ultrastructural Bases for Metabolically Linked Mechanical
Activity in Mitochondria. I. Reversible Ultrastructural
Changes with Change in Metabolic Steady State in Isolated
Liver Mitochondria.

105. Hackenbrock, C. R.
Ultrastructural Bases for Metabolically Linked Mechanical
Activity in Mitochondria. II. Electron Transport-Linked
Ultrastructural Transformations in Mitochondria.

106. Hanawalt, P. C.
Repair of Genetic Material in Living Cells.

107. Hashimoto, K.
Intercellular Spaces of the Human Epidermis as Demonstrated
with Lanthanum.

108. Hay, E. D.; and Revel, J. P.
The Fine Structure of the DNP Component of the Nucleus;
An Electron Microscopic Study Utilizing Autoradiography
to Localize DNA Synthesis.

109. Heming, A. E.; and Brown, J. H. U.
Chemical Mutagenesis.

110. Herriott, R. M.
Effects on DNA: Transforming Principle.
In: Chemical Mutagens; Principles and Methods for Their

111. Higginson, J.
Present Trends in Cancer Epidemiology.

112. Hinchliffe, J.R.; and Ede, D. A.
Limb Development in the Polydactyous Talpid Mutant of the
Fowl.
113. Hollaender, A., Editor
Chemical Mutagens; Principles and Methods for Their
Detection, Vol. 1 and 2.

114. Hollaender, A.
Conclusion.
In: Chemical Mutagens; Principles and Methods for Their

Preservation of Integrity of Rat Tissues for Cytochemical
Staining Purposes.

116. Hueper, W. C.; and Conway, W. D.
Clinical Carcinogens and Cancers.

117. Hulett, H. R.; Coukell, A.; and Bodner, W.
Tissue-Typing Instrumentation Using the Fluorochromatic
Cytotoxicity Assay.

118. Irwin, S.
Prediction of Drug Effects from Animals to Man.
In: Ciba Foundation Symposium: Animal Behavior and Drug
Action. A. V. S. de Reuck and J. Knight, Eds., Little, Brown

119. Janigan, D. T.
The Effect of Aldehyde Fixation on Acid Phosphatase Activity
in Tissue Blocks.

120. Judah, J. D.; Ahmed, K.; and McLean, A. E. M.
Possible Role of Iron Shifts in Liver Injury.
In: Ciba Foundation Symposium: Cellular Injury. A. V. S.
de Reuck and J. Knight, Eds., Little, Brown and Co.,

121. Kalter, H.
The Inheritance of Susceptibility to the Teratogenic Action
of Cortisone in Mice.
122. Kalter, H.
Factors Influencing the Frequency of Cortison-Induced Cleft Palate in Mice.

123. Kalter, H.
Correlation Between Teratogenic and Mutagenic Effects of Chemicals in Mammals.

124. Kalter, H.

125. Kalter, H.; and Warkany, J.
Experimental Production of Congenital Malformations in Mammals by Metabolic Procedure.

126. Kassirer, J. P.

127. Keiser, H. R.; Vogel, C. L.; and Sadikali, F.
Protocollagen Proline Hydroxylase in Sera of Ugandans with Hepatocellular Carcinoma.

128. Kelner, A.
Effect of Visible Light on the Recovery of Streptomyces Griseus Conida from Ultraviolet Irradiation Injury.

129. Knox, W. E.
The Protoplasmic Patterns of Tissues and Tumors.
130. Koehler, J. K.  
The Technique and Application of Freeze-Etching in  
Ultrastructure Research.  

Morphological, Neurochemical, and Behavior Correlates of  
Lead Intoxication and Undernourishment in Developing Rats.  
Abstract of paper presented at FASEB 56th Annual Meeting,  
April 9-14, 1972.  

132. Kroes, R.; Williams, G. M.; and Weisburger, J. M.  
Early Appearance of Serum $\alpha$-Fetoprotein Hepatocarcinogenesis  
as a Function of Age of Rats and Extent of Treatment with  
3'-Methyl-4-dimethylaminoazobenzene.  
Cancer Res. **32**: 1526-1532 (1972).

133. Landauer, W.  
The Hatchability of Chicken Eggs as Influenced by Environment  
and Heredity.  

134. Larsen, C. D.  
Pulmonary Tumor Induction by Transplacental Exposure to  
Urethane.  

135. Lavappa, K. S.; and Yerganian, G.  
Latent Meiotic Anomalies Related to an Ancestral  
Exposure to a Mutagenic Agent.  

136. Leduc, E. H.; Marinozzi, V.; and Barnhard, W.  
The Use of Water-Soluble Glycol Methacrylate in Ultra-  
structural Cytochemistry.  

137. Legator, M. S.  
Chemical Mutagenesis Comes of Age.  

138. Legator, M.S.; and Malling, H. V.  
The Host-Mediated Assay, a Practical Procedure for  
evaluating Potential Mutagenic Agents in Mammals.  
In: Chemical Mutagens: Principles and Methods for Their  
139. Legrand, J.; Kreigel, A.; and Jost, A.
Deficience Thyroidienne et Maturation du Cervelet
Chez de Rat Blanc.

140. Leighton, J.; Merkow, L.; and Locker, M.
Alteration in Size of the Heart of Late Chick Embryos
after Incubation at Varied Temperatures.

141. Levin, S.; and Richter, W. R.
A Systematic Method for the Detection of Induced Hyper-
plasia and Early Neoplasia in the Urinary Bladder.
Mimeographed report of the Department of Pathology and
A. J. Carlson Animal Research Facility, University of

142. Lillie, R. D.
Histopathologic Techniques and Practical Histochemistry,
3rd Ed.

143. Lindsey, C. C.; and Moodie, G. E. E.
The Effect of Incubation Temperature on Vertebral Count
in the Chicken.

144. Luft, J. H.
Ruthenium Red and Violet. I. Chemistry, Purification,
Methods of Use for Electron Microscopy and Mechanism of
Action.

145. Luft, J. H.
Ruthenium Red and Violet. II. Fine Structural Localization
in Animal Tissues.

146. Lum, G.; and Gambino, S. R.
Serum Gamma-Glutamyl Transpeptidase Activity as an
Indicator of Disease of Liver, Pancreas, or Bone.
147. Luna, L.G., Editor
Manual of Histologic Staining Methods of the Armed Forces
Institute of Pathology, 3rd Ed.

148. Lyon, M. F.; and Morris, T.
Mutation Rates at a New Set of Specific Loci in the Mouse.

149. Magee, P. N.
Tests for Carcinogenic Potential.
In: Methods in Toxicology. G. E. Paget, Ed., F. A. Davis

150. Malling, H. V.
Chemical Mutagens as a Possible Genetic Hazard in
Human Populations.

151. Mantel, N.; and Bryan, W. R.

152. Mawas, C.; Buffe, D.; and Burtin, P.
Influence of Age on alpha-Fetoprotein Incidence.

153. Mayer, P.
Notiz über Hämostein und Hämalaun.

154. McLean, J. D.; and Singer, S. J.
A General Method for the Specific Staining of Intracellular
Antigens with Ferritin-Antibody Conjugates.

155. Meier-Ruge, W.
Experimental Investigation of the Morphogenesis of
Chloroquine Retinopathy.

156. Meissner, W. A.; and Warren, S.
Neoplasms.

Acridine Orange Metachromasia for Characterization of Leukocytes in Leukemia, Lymphoma and Other Neoplasms
Cancer 29: 1361-1368 (1972b).

159. Miller, E. C.; and Miller, J. A.
The Mutagenicity of Chemical Carcinogens: Correlations, Problems, and Interpretations.

160. Miller, J. A.
Cancer Res. 30: 559-576 (1970).

161. Miller, R. W.
Relation Between Cancer and Congenital Defects in Man.

162. Miller, R. W.

163. Miller, R. W.
Transplacental Chemical Carcinogenesis in Mice.

164. Moller, J. H.; Amplatz, K.; and Edwards, J. E.
Congenital Heart Disease. Universities Associated for Research and Education in Pathology, Inc., Monograph.
The Upjohn Co., Kalamazoo, Michigan, 83 p (1971).

165. Montesano, R.; and Saffiotti, U.
Carcinogenic Response of the Respiratory Tract of Syrian Golden Hamsters to Different Doses of Diethylnitrosamine.
Cancer Res. 28: 2197-2210 (1968).
166. Moriarity, G. C.; and Halmi, N. S.
Electron Microscopic Study of the Adrenocorticotropic-Producing Cell with the Use of Unlabeled Antibody and the Soluble Peroxidase-Antiperoxidase Complex.

167. Moss, M. L.; Horton, C. A.; and White, J. C.
Clinical Biochemistry.

168. Nairn, R. C.; Richmond, H. G.; McEntegart, M. G.; and Fothergill, J. E.
Immunological Differences between Normal and Malignant Cells.


Nutrient Requirements of Laboratory Animals: Cat, Guinea Pig, Hamster, Monkey, Mouse and Rat, 2nd Rev. Ed.

171. National Institute of General Medical Sciences.

172. Nebert, D. W.; and Gielen, J. E.
Genetic Regulation of Aryl Hydrocarbon Hydroxylase Induction in the Mouse.

173. Neel, J. V.
Evaluation of the Effects of Chemical Mutagens on Man: The Long Road Ahead.

174. Neel, J. V.
The Detection of Increased Mutation Rates in Human Populations.


181. Pickett, J. P.; Greene, W. B.; and Sommer, J. R. Improved Film Strip Technique for the Laboratory. Arch. Pathol. 77: 429-433 (1964).


185. Raab, P.  
Diagnostic Value of Urinary Enzyme Determinations.  

186. Rall, D. P.  
Difficulties in Extrapolating the Results of Toxicity Studies in Laboratory Animals to Man.  

187. Rall, D. P.  
Problems in Chemical Carcinogenesis and Mutagenesis.  
Fifth International Congress on Pharmacology,  

188. Robbins, S. L., and Angell, M.  
Basic Pathology.  

189. Röhrborn, G.  
Mutagenicity Tests in Mice. I. The Dominant Lethal Method and the Control Problem.  

190. Röth, L. J.; and Stumpf, W. E., Editors  
Autoradiography of Diffusable Substances.  

191. Russell, W. L.  
X-ray-Induced Mutations in Mice.  

192. Rutenburg, A. M.; Goldberg, J. A.; and Pineda, E. P.  
Serum y-Glutamyl Transpeptidase Activity in Hepato-biliary Pancreatic Disease.  

193. Rygaard, J.; and Olsen, W.  
Interference Filters for Improved Immunofluorescence Microscopy.  

194. Salpeter, M. M.; and Bachmann, L. J.  
Autoradiography with the Electron Microscope; A Procedure for Improving Resolution, Sensitivity and Contrast.  
195. Salpeter, M. M.; Bachmann, L. J.; and Salpeter, E. E. 
Resolution in Electron Microscope Radioautography. 

196. Saunders, L. Z. 
Ophthalmic Pathology in Rats and Mice. 
In: Pathology of Laboratory Rats and Mice. E. Cotchin and 

197. Saxen, L. 
Tissue Interactions and Teratogenesis. Paper presented 
at a Symposium on Pathobiology of Development. American 
Association of Pathologists and Bacteriologists, Annual 
Meeting, Cincinnati, Ohio, March 11-14, 1972.

198. Scarpelli, D. G.; and Trump, B. F. 
Cell Injury. Universities Associated for Research and 
Education in Pathology, Inc., Monograph. 
The Upjohn Co., Kalamazoo, Michigan, 67 p (1971).

199. Schein, P.S. 
Qualitative Aspects of Drug Toxicity in Prediction from 
Laboratory Animals to Man. 
Fifth International Congress on Pharmacology, San Fran- 

Schein, D. R.; and Rall, D. P. 
The Evaluation of Anticancer Drugs in Dogs and Monkeys 
for the Prediction of Qualitative Toxicities in Man. 

201. Schneiderman, M. A. 
A Method for Determining the Dose Compatible with Some 
"Acceptable" Level of Risk. Appendix III to Federal 
Environmental Pesticide Control Act Hearings before the 
Subcommittee on Agricultural Research and General Legisla- 
tion of the Committee on Agriculture and Forestry, U.S. 
Senate, Ninety-second Congress, First Session, on S.232, 
202. Shea, S. M.  
Lanthanum Staining of the Surface Coat of Cells. Its Enhancement by the Use of Fixatives Containing Alcian Blue or Cetylpyridinium Chloride.  

203. Slater, T. F.  

204. Smith, J. B.  
Alpha-fetoprotein: Occurrence in Certain Malignant Diseases and Review of Clinical Applications.  

205. Soares, E. R.  
Estimating the Frequency of Induced Dominant Lethals in Mice by Uterine Dissection after Weaning Progeny.  

206. Soares, E. R.; and Crenshaw, J. W.  
Quantitative Measurement of the Polygenic Effects of Ethyl Methanesulfonate (EMS) on Mice.  

207. Soares, E. R.; and Crenshaw, J. W.  
Qualitative and Quantitative Measurement of the Mutagenic Effects of Ethyl Methanesulfonate on Inbred Mice.  

208. Spivey-Fox, M. R.; and Harrison, B. N.  
Use of Japanese Quail for the Study of Zinc Deficiency.  

209. Spyker, J. M.; Sparber, S. B.; and Goldberg, A. M.  
Subtle Consequences of Methylmercury Exposure: Behavioral Deviations in Offspring of Treated Mothers.  

210. Srb, A. M.; Owen, R. D.; and Edgar, R. S.  
General Genetics, 2nd Ed.  

211. Stein, P. G.; Lipkin, L. E.; and Shapiro, H. M.  
Spectre II: General-Purpose Microscope Input for a Computer.  
212. Sterling, T. D.
Difficulty of Evaluating the Toxicity and Teratogenicity of 2,4,5-T from Existing Animal Experiments.
Science **174**: 1358-1359 (1971).

213. Stokingter, H. E.
Sanity in Research and Evaluation of Environmental Health; How to Achieve a Realistic Evaluation (in Seven Commandments).

214. Sutton, H. E.
Workshop on Monitoring of Human Mutagenesis.
Teratology **4**: 103-108 (1971).

215. Swendburg, J. A.; Koestner, A.; Wechsler, W; and Denlinger, R. H.
Quantitative Aspects of Transplacental Tumor Induction.
Cancer Res. **32**: 2656-2660 (1972).

216. Testerman, M. K.; and Bond, R.
In press (1972).

217. Thomassen, R. W.; Howbert, J. P.; Winn, D. F., Jr.; and Thompson, S. W.
The Occurrence and Characterization of Emboli Associated with the Use of a Silicone Antifoaming Agent.

218. Thompson, S. W.; and Hunt, R. D.
Spontaneous Tumors in the Sprague-Dawley Rat: Incidence Rates of Some Types of Neoplasms as Determined by Serial Sections Versus Single Section Technics.

219. Tosteson, D. C.
Regulation of Cell Volume by Sodium and Potassium Transport.

220. Traut, H.
On the Calculation of Human Mutation Rates from Changes in Sex Ratio.
221. Tuchmann-Duplessis, H.
Teratogenic Drug Screening. Present Procedures and Requirements.
Teratology 5: 271-286 (1972)

222. Uchida, I. A.; and Lin, C. C.
Identification of Triploid Genome by Fluorescence Microscopy.
Science 176: 304-305 (1972)

223. Vogel, F.; and Rohrborn, G.
Concluding Remarks.

224. Vogt, M. T.; and Farber, E.
On the Molecular Pathology of Ischemic Renal Cell Death.

225. Vrensen, G. F. J. M.
Some New Aspects of Efficiency of Electron Microscopic Autoradiography with Tritium.

226. Walker, N. E.
Distribution of Chemicals Injected into Fertile Eggs and Its Effect upon Apparent Toxicity.

227. Weinstein, L.B.; Grunberger, D.; Fujimura, S.; and Fink, L. M.
Chemical Carcinogens and RNA.
Cancer Res. 31: 651-655 (1971).

228. Weisburger, J. H.; and Weisburger, E. K.
Tests for Chemical Carcinogens.

229. Wied, G. L.; and Bahr, G. F., Editors
Automated Cell Identification and Cell Sorting.

230. Williamson, A. P.; Blattner, R. J.; and Lutz, H. L.
Abnormalities in Chick Embryos Following Thalidomide and Other Insoluble Compounds in the Amniotic Cavity.
231. Wilson, J. G.
Differentiation and the Reaction of Rat Embryos to Radiation.

232. Wilson, J. G.
Embryological Considerations in Teratology.
In: Teratology: Principles and Techniques. J. G. Wilson
and J. Warkany, Eds., University of Chicago Press,
Chicago, pp 251-261 (1965).

233. Wilson, J. G.
Principles of Teratology. Paper presented at a Symposium
on Pathobiology of Development. American Association of
Pathologists and Bacteriologists, Annual Meeting, Cincinnati,
Ohio, March 11-14, 1972.

234. Wilson, J. W.; and Pickett, J. P.
Serial Sections of Lung: The Use of a 70-Millimeter
Film-strip Technique for Large Tissue Sections.

235. Wolman, S. R.; Phillips, T. F.; and Becker, F. F.
Fluorescent Banding Patterns of Rat Chromosomes in Normal
Cells and Primary Hepatocellular Carcinomas.

236. Woo, J; and Cater, D. B.
Foetal-Type Antigen on the Surface of Hepatoma Cells--
Evidence from Electrophoresis.

237. World Health Organization.
Procedures for Investigating Intentional and Unintentional
Food Additives.

238. World Health Organization.
Evaluation and Testing of Drugs for Mutagenicity:
Principles and Problems.

239. Yang, S. S.; Herrera, F. M.; Smith, R. G.; Reitz, M. S.;
Lancini, G.; Ting, R. C.; and Gallo, R. C.
Rifamycin Antibiotics: Inhibitors of Rauscher Murine
Leukemia Virus Reverse Transcriptase and of Purified DNA
Polymerases from Human Normal and Leukemic Lymphoblasts.
240. Zigmond, S. H.; and Hirsch, J. G.
Effects of Cytochalasin B on Polymorphonuclear Leucocyte
Locomotion, Phagocytosis and Glycolysis.

ADDITIONAL REFERENCES

12a. Andervont, H. B. and Dunn, T. B.
Transplantation of Spontaneous and Induced Hepatomas in
Inbred Mice.

77a. Firminger, H. I.
Histopathology of Carcinogenesis and Tumors of the Liver
in Rats.

187a. Reuber, M. D.
Histopathology of Transplantable Hepatic Carcinomas Induced
by Chemical Carcinogens in Rats.
Gann (Monograph) 1: 43-54 (1966).

187b. Reuber, M. D.
Morphologic and Biologic Correlation of Hyperplastic and
Neoplastic Lesions Occurring "Spontaneously" in C3HxY Hybrid
Mice.
B. OTHER CONSULTANTS

Berwin A. Cole, Ph.D.
Deputy Associate Commissioner for Science
Food and Drug Administration
Health, Education and Welfare
5600 Fishers Lane (14-57)
Rockville, Maryland 20852

Stephen Douglas, M.S.
National Center for Toxicological Research
Jefferson, Arkansas 72079

Emmanuel Farber, M.D., Ph.D.
Fels Research Institute
Temple University Medical School
Philadelphia, Pennsylvania 19140

Vergil H. Ferm, M.D., Ph.D.
Department of Pathology
Dartmouth Medical School
Hanover, New Hampshire 03755

Thomas J. Haley, Ph.D.
National Center for Toxicological Research
Jefferson, Arkansas 72079

Mr. Lee Luna
CH, Histopathology Labs
Armed Forces Institute of Pathology
Washington, D.C. 20305

G. Burroughs Mider, M.D.
Executive Officer*
Universities Associated for Research and Education in Pathology, Inc.
Bethesda, Maryland 20014

Mr. Robert Pendergrass
Department of Pathology
University of Maryland
Baltimore, Maryland 21201

David P. Rall, M.D., Ph.D.
Director
National Institute of Environmental Health Sciences
P.O. Box 12233
Research Triangle Park
North Carolina 27709

Melvin D. Ruber, M.D.
Department of Pathology
University of Maryland
Baltimore, Maryland 21201

Umberto Saffiotti, M.D.
Associate Scientific Director for Carcinogenesis, DCCP
National Cancer Institute
National Institutes of Health
Bethesda, Maryland 20014

Mr. David T. Smith
National Center for Toxicological Research
Jefferson, Arkansas 72079

* As of August 1972
Katharine Snell, M.D.
National Cancer Institute
Laboratory of Pathology
Building 10, Room 2N110
National Institutes of Health
Bethesda, Maryland 20014

Arthur C. Upton, M.D.
Professor of Pathology and Dean
School of Basic Health Sciences
State University of New York
Stony Brook, New York 11790

Harold L. Stewart, M.D.
Consultant
Registry of Experimental Cancers
National Cancer Institute
Eye Research Foundation Building
Room 202
8710 Old Georgetown Road
Bethesda, Maryland 20014

Robert W. Wissler, M.D.
Professor and Chairman
of Pathology
University of Chicago
Chicago, Illinois 60637

John B. Storer, M.D.
Scientific Director for Pathology
and Immunology
Biology Division
Oak Ridge National Laboratory
P.O. Box Y
Oak Ridge, Tennessee 37830

David M. Young, D.V.M., Ph.D.
Comparative Pathologist
Laboratory of Toxicology
National Cancer Institute
National Institutes of Health
Bethesda, Maryland 20014

F. William Sunderman, Jr., M.D.
Professor and Head
Department of Laboratory Medicine
University of Connecticut
School of Medicine
Drawer B
Newington, Connecticut 06111