Methods of Animal Experimentation

WILLIAM I. GAY

Volume II

Methods of Animal Experimentation

EDITED BY

WILLIAM I. GAY

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List of Contributors

Numbers in parentheses indicate the pages on which the authors' contributions begin.

Robert J. Byrne,* *Department of Veterinary Science, University of Maryland, College Park, Maryland (481)*

Cesar A. Caceres, *Instrumentation Field Station, Heart Disease Control Program, Division of Chronic Diseases, U.S. Public Health Service, Department of Health, Education and Welfare, Washington, D.C. (527)*

Juan B. Calatayud, *Department of Medicine, George Washington University, School of Medicine, Washington, D.C. (527)*

Roy Yorke Calne, *Surgical Unit, Westminster Hospital, London, England (251)*

James E. Corbin, *Ralston Purina Company, St. Louis, Missouri (451)*

Patrick A. Gorman, *Department of Medicine, George Washington University, School of Medicine, Washington, D.C. (527)*

Charles E. Hall, *Medical School, University of Texas, Galveston, Texas (223)*

Raymond J. Hock, *White Mountain Research Station, University of California, Bishop, California (273)*

Neal S. Nelson, *Section on Nuclear Medicine, Department of Pharmacology, University of Chicago, Chicago, Illinois (1, 59)*

John H. Rust, *Section of Nuclear Medicine, Department of Pharmacology, University of Chicago, Chicago, Illinois (1, 59)*

Kanematsu Sugiura, *Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, New York, New York (171)*

Paola S. Timiras, *Department of Physiology, University of California, Berkeley, California (333)*

Charles C. Wunder, *Department of Physiology, State University of Iowa, Iowa City, Iowa (371)*

* Present Address: Laboratory Aids Branch, Division of Research Services, National Institutes of Health, Bethesda, Maryland.

Preface

Although much has been written about methods for the care and production of laboratory animals, there has been a lack of compiled information on the use of animals in various fields of research. In the belief that such compilation would benefit investigators conducting research dependent upon the use of animals, this book was written. "Methods of Animal Experimentation" provides information on the most common methods for using animals as tools in the search for new biological knowledge. The authors of the individual contributions and the editor believe that the techniques described will facilitate the most efficient use of research animals and provide guidelines for their utmost comfort and welfare.

The descriptions of both fundamental and well-developed techniques of animal experimentation in various research fields should be useful to graduate students and experienced scientists who must consider variations in research approaches. The book is a source of information for the scientist administrator who is frequently confronted with different proposed approaches to biological research projects utilizing animals.

The text is arranged according to specific research methods rather than to organ system or disease category. This approach gives the reader a broad view of the techniques involved in specific fields and describes the range of usefulness of these techniques. This approach also made it possible to select contributors expert in experimental methods and equally well qualified in the care of laboratory animals.

The common diseases of laboratory animals are not discussed in the text unless they are related closely to the experimental methods described. Although not intended to be a general guide on conventional methods of the production, care, and feeding of laboratory animals, the book thoroughly reviews special animal care associated with each experimental technique. The book also discusses hazards involved in using the various experimental techniques and suggests necessary safety precautions. The reader is referred to W. Lane-Petter's "Animals in Research" (Academic Press, New York, 1963) for basic information on laboratory animal care and maintenance.

The first five chapters of Volume I describe basic information, methods, and principles involved in managing animals for experimental procedures. The remaining chapters in Volume I and those in Volume II deal with special techniques which have been demonstrated to be distinct, useful methods for using laboratory animals as a basic biomedical research tool.

The editor is extremely grateful for the splendid cooperation and support given by the contributors of this volume. He is also especially indebted to Dr. Robert J. Schnitzer for his expert counsel, guidance, and inspiration.

August, 1965 WILLIAM I. GAY

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Introduction

Volumes on methodology associated with the basic science disciplines have become commonplace. It is surprising that the techniques for using animals in biomedical research have not been previously compiled. This text will provide a cross section of information dealing with the techniques of a wide variety of experimental procedures.

It is alarming to discover the lack of knowledge of this subject by many graduate students in the biological sciences. The recent trend in many disciplines toward studying isolated phenomena is resulting in a lack of appreciation for the considerable fundamental knowledge which may be gained by making use of experiments utilizing information from the "whole animal." Many students in the biological sciences receive all of their research training using tissue cultures or isolated microorganisms. No doubt these students will reach a point in their investigations where it will be essential to test concepts in experimental animals. Hopefully this text will stimulate incorporation of courses in methods of animal experimentation into the curricula of these students.

It has become increasingly obvious that ideas for new avenues of research are dependent upon a general knowledge of the experimental methods that have been used in both related and unrelated research areas. Many of the so-called "original" investigations have involved adapting methods used in other types of studies to the individual's problem. This text will doubtless stimulate new approaches by making investigators aware of the methods used in other fields.

This text covers a variety of topics by individuals actively using the techniques they describe. The descriptions of the techniques in any specific category is not intended to be exhaustive but each has an ample bibliography and will serve to orient the reader to the pertinent literature.

THOMAS B. CLARKSON

Bowman Gray School of Medicine,
Wake Forest College,
Winston-Salem, North Carolina
January, 1965

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CHAPTER 1

Radiation Hygiene

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I. The Nature and Assessment of Ionizing Radiation Effects

The management of animals for radiobiological studies has become an increasingly important problem for those interested in the care of laboratory animals. It is well that one have some familiarity with the radiation injury before he attempts to make decisions regarding proper and adequate care. It will be noted that there are many unique and radically different situations that may arise. Probably the best capsule advice is to proceed with caution when managing irradiated animals.

A. The Biological Target

1. *Biological Unit*

The unit of most interest in understanding the biological effects of ionizing radiation is the cell. To be even more specific, it seems to be a sensitive substance and is sometimes called "sensitive volume" of a cell. Most students of radiobiology believe it to be related to the deoxyribonucleic acid (DNA) (Alexander and Bacq, 1961). To have an effect an ionizing radiation must in some manner interact with that sensitive volume. It may be a direct or indirect interaction. A photon may either pass through the sensitive volume, ionize, and establish an injury, or create an

undefined active substance that is transported to the sensitive volume. It is quite evident that some effects may be repaired, partially repaired, or reversed. In the extreme case the effect of the interaction of the photon or active substance with the sensitive volume may be fatal to the cell immediately, though the fatal outcome is in most cases delayed until the cell attempts to divide. Some cells, i.e., those that are partially repaired plus those that are only moderately injured, are able to divide, but the daughter cells bear the injury so long as they and the animal of which they are a part survive. Finally, there will be a substantial number of cells that show no injury. If they are in adequate numbers and their environment is not unfavorable they will repopulate the organ of which they are a part. It is in this manner that healing and recovery of an irradiated animal take place, or on the other hand may fail to take place. In general, the greater the exposure the greater the cell injury and loss. The greater the cell loss the less likelihood there is that the animal will survive. This explains why shielding a part of the body of an animal from ionizing radiation increases its chance of survival under many circumstances (Cronkite and Bond, 1960a).

Radiation given at a slow rate is less damaging than that given at a rapid rate. From this it can be deduced that the ionizing events affecting the cell sensitive volume must take place within a relatively short space of time for the maximum injury. This is especially true for somatic cells but for genetic cells there is some question whether this be considered an acceptable statement. But even in genetic cells it appears to be true in some special instances (Russell *et al.*, 1958). It is the concern for the irreparable damage to genetic resources that has dominated the formulation of radiation protection recommendations in recent years.

2. *Biological Dose Effect*

When considering the response of an organism to ionizing radiation, the investigator must be aware not only of the nature of the sensitive target or cell, but also the problem of quantal response which is observed in living organisms.

The quantal response, i.e., all or none, life or death, is an individual response to radiation exposure. When all quantal responses within a population are considered at various levels of exposure, they produce a graded effect which can be expressed as a sigmoid curve if response is plotted against the various exposures.

Many biological responses of an organism to ionizing radiation tend to follow the typical sigmoid curve so commonly seen in bioassay estimates. The reasons for this response are complex and in many cases unknown.

A consideration of the problem will point out some of the known and unknown facts and assumptions.

In considering the effects of ionizing radiation, it must be remembered that some portion of a population of cells or cell structures, such as an organ, can survive at almost any dose level. The response of the complete organism depends on the proportion of surviving cells in the cell population at risk. The actual amount of direct injury must be very small; i.e., in the case of a cell receiving 10 kilorads of radiation, if the cell is estimated to contain 3×10^{22} molecules/gm, only 1 or 2 molecules per million will be ionized or "injured." The extremely small magnitude of direct interaction with the biological material indicates that there must be some biological amplification of the injury. The exact mode of action is unknown, but there is a biological amplification which may be through the interruption of essential DNA, enzyme systems, etc.

a. *Time Sequence of Radiation Effects.* The time sequence for a radiation injury to be manifest is variable. Duration of the sequence is governed by the length of time required for the expression of the end point chosen to be observed. Physically, the time required for the direct action of radiation is very rapid following irradiation; for example, (1) by 10^{-16} second the photon has passed through the target and caused an ionized path to form, (2) by 10^{-12} second free radicals have started to form and disperse through the medium, (3) by 10^{-3} second free radicals have either recombined or reacted with sensitive molecules, and (4) in less than one second all radiochemical reactions have taken place.

On the other hand, the time sequence for expression of the biological injury is much longer. In this case:

(1) In less than 1 hour biological changes are observed in the most radiosensitive cells. Most of the observed changes are in the meiotic and mitotic processes of the cell nuclei, i.e., paling of chromosomes, stickiness and bridging of chromosomes, rupture of chromosomes, and other aberrations that occur in the "hit" area.

(2) By 5 days most histological changes have occurred, i.e., decreases in the numbers of sensitive cells remaining. However, this injury may not be expressed for some days, depending on the rate of turnover and replacement of the cell population.

(3) By 1 month most of the acute lethal effects are observed.

(4) In 1 or more years most of the carcinogenic or aging effects are observed.

(5) After one or more generations genetic effects may be observed.

b. *Biological Response Curve.* Depending on the selected end point, the biological response curve may be of the sigmoid type; the curve of response of individual cells, however, is generally of the exponential type.

Lea (1955) proposed a "target theory" where there are target molecules in the cell sensitive to ionizing radiation and that is applicable to the conditions where a single ionizing event or "hit" would cause the effect. At low doses the number of hits is proportional to the dose of radiation. But, at increasingly larger doses of radiation there is an overkill, i.e., a single target volume is "hit" by ionizing events many times even though only one hit is required to "kill" the target.

Therefore the response is no longer proportional to the dose but is exponential.

The curve of response of a homogeneous population of cells is

$$N = N_o \, e^{-kd}$$

where N = number of survivors, N_o = original number in population, k = sensitivity of the population, and d = dose of radiation. This is also used to give the probable number of survivors in a given population.

If it is assumed that only one ionizing event is required to inactivate a cell, then the probability of survival in the population is:

$$P = \frac{N}{N_o} = \frac{(VD)^n \, e^{-VD}}{n!}$$

where P = the probability of survival, N = the number of survivors, N_o = the original number of cells in the population, V = the sensitive volume, D = the dose in "hits"/cm^3, VD = the expected number of "hits"/sensitive volume, and in this example, if $VD = 1$, then one hit ($n = 1$) inactivates the cell. n = the number of ionizing events ("hits") actually occurring in volume V. Ideally, at $VD{=}n$ the cell is inactivated.

The case where more than one "hit" is required to inactivate the target or more than one "target" is present in each cell can be reduced to a "single hit" case by calculating the probability of have $n_c{-}1$ hits. The "multihit" case for $n_c{-}1$ hits may be expressed as

$$P = \left[e^{-VD} \sum_{k=0}^{n_c-1} \frac{(VD)^k}{k!} \right]^n$$

where P = the probability of having $n_c{-}1$ hits, V = the sensitive volume, D = the dose in "hits"/cm^3, VD = the expected number of hits $(n_c{-}1)$/sensitive volume, n = the number of ionizing events ("hits") actually occurring in volume V, k = the number of sites or "targets" to be hit, and n_c = the number of hits required to inactivate the "target" ($n_c = nk$). Ideally, at $VD = nk$ the cell is inactivated.

Lea also introduced the concept of the identity:

$$\text{Mean lethal dose (MLD)} = \text{Inactivation dose} = D_{37}$$

D_{37} is the dose corresponding to an average of one hit per target and which leaves 36.8% of the population surviving. This may be expressed:

$$P = \frac{dN}{N_o} = \frac{dD}{D_0}$$

where N_o = the original number of organisms, N = the number of organisms surviving dose D, dD = the increment in dose, $D_0 = D_{37}$, P = the probability of survival after change in dose to $D + dD$, and dN = an increment in survivors with dD. This expression integrates to:

$$P = \frac{N}{N_o} = e^{-D/D_0}$$

where P = the probability of survival, N_o = the original number of organisms, N = the number of survivors, D = the administered dose, and $D_0 = D_{37}$. In the case of a sigmoid or cumulative-effect type of curve, $\text{MLD} = \text{LD}_{50}$.

The sigmoid curve seen when an entire multicellular organism is considered is probably due to the fact that a heterogeneous population is being considered. Each of the different cell populations making up the organism has a different radiosensitivity. Thus, the whole organism must be considered as a problem of multihit kinetics.

This multihit curve can be obtained by a summation of the probability of survival for each of the cell populations involved:

$$P(s) = \sum_{i=1} P(c)_i$$

where $P(s)$ = probability of survival of organism, and $P(c)_i$ = probability of survival of the ith cell population.

Data are generally presented as the dose of radiation that will produce the desired response in 50% of the experimental animals. This form of presentation is easily used since the steepest slope of the sigmoid curve of biological response is generally through the 50% level. Greatest accuracy in the determination of the curve is generally between the 20% and 80% levels. And even if probit analysis is used the tails of the curve may be indistinct and variable.

Data may also be presented as the dose that will produce the desired response in 63% of the population or in 100% of the population, but these are not considered as accurate an end point for the experiment.

A time parameter, or limit, is also included in the data, especially with mortality data, because every large organism will die at some time. The time parameter may be combined with or replaced by some expression of the size of the population at risk.