THE MOUSE IN BIOMEDICAL RESEARCH

VOLUME III

NORMATIVE BIOLOGY, IMMUNOLOGY, AND HUSBANDRY

EDITORS:

Henry L. Foster, J. David Small, James G. Fox

American College Of Laboratory Animal Medicine Series

The Mouse in Biomedical Research

Volume III

Normative Biology, Immunology, and Husbandry

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The Mouse in Biomedical Research

Volume III
Normative Biology, Immunology, and Husbandry

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Foreword

This volume on normative biology, immunology, and husbandry of laboratory mice is the third of the four-volume treatise on "The Mouse in Biomedical Research." It is, however, the most basic in that the information presented forms the groundwork for the use of the mouse in research. The first six chapters deal with management and husbandry and are of obvious importance to those who are concerned with the production and maintenance of colonies of mice. The last twelve chapters deal with normal biology. They are not only of importance to those who use mice in the laboratory, but to those who produce and maintain mice because the information presented forms the biological basis for mouse husbandry.

One of the reasons that the mouse came into early popularity as a research animal was that it was relatively easy to raise in large numbers and maintain in the laboratory. This popularity in turn provided the impetus to develop more refined and carefully controlled husbandry methods. The mouse, true to form, responded well to the more sophisticated methods of production and care. For example, the mouse presented no special problems with respect to cesarean derivation and barrier rearing. The large-scale application of these techniques has transformed the quality not only of mice but also other animals available for laboratory use today. One shudders to think what would have happened to the field of cesarean derivation and barrier rearing had the mouse, as the premier research animal, presented the obstacles to germfree derivation that the hamster has.

The chapters on gnotobiotics, gastrointestinal microflora, animal health surveillance and health delivery systems, and environmental monitoring taken in concert with contributions on mouse diseases in other volumes of this treatise give us an unparalleled system for disease control. The papers on management and design of breeding and research facilities, applied breeding methods, and nutrition provide valuable information for production and maintenance of mouse colonies and elimination of unwanted variables. Though written for the mouse, the principles discussed can be applied to other laboratory animals. That the mouse is most frequently the prototype for improved quality and greater definition of other laboratory animals is well illustrated.

The basic biology of the mouse is given in the chapters on anatomy, embryology, reproductive physiology, physiology, endocrinology, hematology, clinical biochemistry, and gastrointestinal microflora. This information is important to the care and management of production and research colonies of mice because it provides the scientific basis for husbandry practices. These chapters also include the biological background relevant to specific research uses of the mouse discussed in other volumes of this treatise. The breadth and depth of these chapters on normal biology are indicative of the wide range of uses of the mouse in research. The existence of the large number of stocks and strains of mice adds to the diversity and complexity of this species.

The chapters on immunoglobulins and immunoglobulin genes, lymphocyte immunogenetics, and immune response disorders provide a framework for the use of the mouse in the burgeoning field of immunology. They are also testimony to the importance of the mouse in immunogenetics and related research.

The chapter on surgical techniques provides valuable information on surgical preparations that contribute to the usefulness of the mouse in a number of fields.

The impressive amount of information on normative biology, husbandry, and management in this volume is an affirmation of the primacy of the mouse as a research animal. The availability of so much information is one of the reasons the

mouse is used so extensively, and this extensive use is continuously adding to our understanding of the biology of the mouse.

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Preface

The American College of Laboratory Animal Medicine (ACLAM) was formed in 1957 in response to the need for specialists in laboratory animal medicine. The College has promoted high standards for laboratory animal medicine by providing a structured framework to achieve certification for professional competency and by stressing the need for scientific inquiry and exchange via progressive continuing education programs. The multivolume treatise, "The Mouse in Biomedical Research," is a part of the College's effort to fulfill those goals. It is one of a series of comprehensive texts on laboratory animals developed by ACLAM over the past decade: "The Biology of the Laboratory Rabbit" was published in 1974, "The Biology of the Guinea Pig" in 1976, and a two-volume work "Biology of the Laboratory Rat" in 1979 and 1980. Also, in 1979 the College published a two-volume text on "Spontaneous Animal Models of Human Disease."

The annual use of approximately 50 million mice worldwide attests to the importance of the mouse in experimental research. In no other species of animal has such a wealth of experimental data been utilized for scientific pursuits. Knowledge of the mouse that has been accumulated is, for the most part, scattered throughout a multitude of journals, monographs, and symposia. It has been fifteen years since the publication of the second edition of "The Biology of the Laboratory Mouse" edited by E. L. Green and the scientific staff of the Jackson Laboratories. It is not the intent of this work simply to update and duplicate this earlier effort, but to build upon its framework. We are indeed fortunate to have Dr. Green and many of his colleagues at The Jackson Laboratory as contributors to this treatise. It is the intended purpose of this text to assemble established scientific data emphasizing recent information on the biology and use of the laboratory mouse. Separation of the material into multiple volumes was essential because of the number of subject areas covered.

The contents of Volume I are presented in fourteen chapters and provide information on taxonomy, nomenclature, breeding systems, and a historical perspective on the development and origins of the laboratory and wild mouse. Six chapters deal specifically with the ever-increasing diversity of inbred strains of mice, including coverage of methods of developing and the genetic monitoring and testing of these strains. The emphasis of this volume on genetics is also manifested by chapters discussing the *H-2* complex, cytogenetics, radiation genetics, and pharmacogenetics.

Because of the impact of spontaneous diseases on interpretation of and potential for complicating experimental research, it is of paramount importance for investigators to recognize these diseases and their effect on the mouse. Volume II, for the first time, compiles in one format a narrative detailing infectious diseases of the mouse; the chapters cover bacterial, mycotic, viral, protozoal, rickettseal, and parasitic diseases. Also, nonneoplastic and metabolic diseases are covered as well as the topic of zoonoses.

Volume III provides comprehensive coverage of selected material related to normative biology and management and care of the laboratory mouse. Developmental, anatomical, nutritional, physiological, and biochemical paramters of the mouse are compiled in several chapters and will be of great interest and an important resource for normal biological profiles. A review of the histologic features was not included because of space constraints and the availability of this information in previous texts. Environmental monitoring and disease surveillance as well as management and design of animal facilities will be particularly useful for those individuals responsible for the management of mouse colonies. The chapters on gnotobiotics and gastrointestinal flora represent the state of the art in gnotobiology. The three chapters on selected aspects of immunology in the mouse serve to highlight the explosive

progress being made in immunologic techniques and instrumentation and the underlying importance of genetic differentiation.

The fourth volume includes selected applications of the mouse in research. Several chapters discuss the use of the mouse in infectious disease research, while others range from eye research to the use of the mouse in experimental embryology. The chapters devoted to the use of the mouse in oncological research follow a body system format. Research topics in other disciplines have not been included, but hopefully will be included in future editions.

This treatise was conceived with the intent to offer information suitable to a wide cross section of the scientific community. It is hoped that it will serve as a standard reference source. Students embarking on scientific careers will benefit from the broad coverage of material presented in compendia format. Certainly, specialists in laboratory animal science will benefit from these volumes; technicians in both animal care and research will find topics on surgical techniques, management, and environmental monitoring of particular value.

The editors wish to extend special appreciation to the contributors to these volumes. Authors were selected because of knowledge and expertise in their respective fields. Each individual contributed his or her time, expertise, and considerable effort to compile this resource treatise. In addition, the contributors and editors of this book, as with all volumes of the ACLAM series texts, have donated publication royalties to the American College of Laboratory Animal Medicine for the purpose of continuing education in laboratory animal science. This book could not have been completed without the full support and resources of the editors' parent institutions which allowed time and freedom to assemble this text. A special thanks is also extended to the numerous reviewers of the edited work whose suggestions helped the authors and editors present the material in a meaningful and concise manner. We acknowledge and thank Rosanne Brown and Sara Spanos for their secretarial assistance. Also, the assistance provided to us by the staff of Academic Press was greatly appreciated.

Finally, we especially acknowledge with deep appreciation the editorial assistance of Patricia Bergenheim, whose dedication and tireless commitment to this project were of immeasurable benefit to the editors in the completion of this text.

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Chapter 1

Gnotobiotics

P. C. Trexler

I. INTRODUCTION

Ordinarily animals are symbiotic complexes, composed of a host animal and a myriad of intimately associated microorganisms (microbiota). Some of these microbes are beneficial to the host, others may produce disease, while the great majority probably have little effect on the host although, because of their actions on various substrates, they do produce alterations in the internal and external milieu. The net effect of this microbiota upon the performance of the host will vary with changes in the composition of the microbiota and with changes in the resistance of the host. Uniformity in the characteristics and

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performance of an animal depends to a great extent upon the control of the associated microbiota. The exercise of such control forms one aspect of the sciencie of gnotobiotics and it is this aspect that is considered here.

A. Definition and Historical Development

The first rearing of germfree animals (guinea pigs) was reported by Nuttall and Thierfelder (1895–1896). They were obtained by caesarian section, placed in a sterile bell jar isolator, and fed sterile milk. The rearing of germfree mice was not reported until 1959 by Pleasants. In the interval between these two reports, publications appeared describing the successful rearing in a sterile environment of chicken, goat, monkey, rabbit, and rat, as well as many of the lower forms of animals including fish. The term "germfree" was widely used to designate these animals, but because the word "germ" has several meanings, germfree was often used for those animals that were merely pathogen-free; furthermore, many of these animals were deliberately associated with pure cultures of microorganisms and could not then correctly be called germfree.

In order to use terms to which precise definitions could be given, and thus avoid ambiguity, Baker and Ferguson (1942) proposed "axenic" (from the Greek *a,* without, and *xenos,* strangers) to describe "individuals of the species free from any demonstrable life apart from that produced by their own protoplasm." Reyniers *et al.* (1949) proposed the term "gnotobiotics" to designate the field of investigation concerned with rearing animals and plants which were free of all microbes or associated only with known species. The basic term "gnotobiota" is derived from the Greek root *gnotos,* meaning well known, and *biota,* meaning the total biological complement of a region (Ward and Trexler, 1958). A series of terms to designate animals with one or more associates (mono-, di-, tri-, and synxenic) was proposed by Dougherty (1953). Raibaud *et al.* (1966) proposed a series of terms using both gnoto- and -xenic. The term gnotoxenic refers to gnotobiotes which may be mono-, di-, tri-, or polygnotoxenic. The term agnotoxenic refers to animals which are either holoxenic, having an unaltered microbiota (conventional) or are heteroxenic (i.e., specific pathogen-free, SPF). In the first monograph on the subject, Luckey (1963) proposed the term "gnotobiology" under which to group studies made with gnotobiotes and, this term has often been used interchangeably with gnotobiotics, the term which involves the entire field of investigation, including husbandry.

Reproducing colonies of gnotobiotic mice have been maintained continuously since 1954 (Gordon and Pesti, 1971). Most of these colonies have been derived from animals hand reared at Lobund (Pleasants, 1959) either directly or by foster suckling. Syukuda *et al.* (1973) hand-reared baby mice in 1967, and these served as nucleus stock for many of the Japanese colonies. The hand-rearing procedure is very tedious and has seldom been used; foster suckling on gnotobiotic stock is far simpler.

B. Uses

1. Research

Studies involving gnotobiotic mice have been included in the reviews of Gordon and Pesti (1971) and of Coates (1975) and in the reports of international meetings by Heneghan (1973) and Fliedner *et al.* (1979). The absence of microorganisms ordinarily associated with a mouse results in a great many changes in that mouse, particularly in those tissues which come in direct contact with microbes, e.g., the alimentary and respiratory tracts and the various defense systems. A greatly distended cecum is the most prominent characteristic of a mouse with a deficient gut flora. In the absence of bacteria, intestinal contents are aerobic rather than strictly anaerobic, the feces have a considerable quantity of active enzymes, and the bile acid cycle is modified. The interpretation of observations made on animals either lacking in or having a grossly altered microbiota, must be made with considerable care because of the many ways in which such animals can differ from the usual laboratory animals; nevertheless, such studies contribute much to the understanding of physiological and metabolic phenomena. In the absence of microorganisms these phenomena can be studied uncomplicated by microbial activity; it is also possible to study the effect of one or more species of microorganisms.

For many purposes the above anomalies are of little significance; mice with no readily cultivatable associates provide a source of living sterile tissues and products. Such animals also serve as living test tubes for the cultivation of a variety of microbes and floras. Patte *et al.* (1979) use axenic mice in the course of microbiological examination of meconium. Gibbons *et al.* (1964) established in the mouse a human oral flora consisting of thirteen species, and many authors have reported the use of axenic mice for the passage of various fractions of gut flora. Gnotobiotic mice have been used to study the antagonisms present between species composing the gut flora, e.g., *Shigella* are not pathogenic in conventional mice but can infect some gnotobiotes depending upon the presence or absence of antagonists.

Gnotobiotic mice are particularly useful when studying their defense system because in the absence of living microbes the development of both cellular and humoral elements is retarded, and precise stimulants can then be applied. Diets, essentially antigen-free, have been devised (Wostmann, 1975), and these further reduce antibody production, although great care must

be taken to evaluate the nutritional and physiological effects of these low molecular weight diets. It is also true that in the absence of microbes, pathological processes can continue far beyond the stage which usually is terminated by an infection. Studies of the effects of radiation have benefited particularly by controlling the bacteria in the gut of animals. Gnotobiotic animals have been very useful in the study of organ and tissue transplants, since many stable chimeras can be produced in the absence of potential pathogens.

2. Nucleus Stocks Free of Infectious Diseases

The gnotobiotic technique is particularly useful for the removal of pathogens from breeding stock. The barrier system, used to protect a breeding colony from specific pathogens, is a compromise between attempted economical operation and real microbiological security: by comparison the isolator provides an absolute barrier. An isolator is used to protect small numbers of animals from pathogens in the environment or to protect the environment from pathogens carried by the animals.

Though it is possible to obtain "clean" breeding stock and introduce it directly into a barrier room, it is usually more economical to maintain such stocks in isolators where they can be examined microbiologically before being moved into the barrier rooms. If a pathogen-free stock is to be derived from existing infected colonies, the foster mothers should be kept bacteria-free to facilitate the testing of the young for the presence of bacteria which would indicate a fault in technique. The litter must be tested for the presence of all the pathogens from which the stock is to be freed. The number of foster mothers and litter which can be maintained in a single isolator depends upon the probability of infection by the pathogens concerned. If there is no danger of the pathogens being transmitted *in utero,* there is no theoretical limit to the number of litters that can be reared within a single isolator.

3. Gnotobiotes as Laboratory Animals

An ideal laboratory animal is often considered to have a normal flora but to be free of potential pathogens, which is a contradiction. Unfortunately, the procedures for obtaining pathogen-free stock exert a considerable effect upon the flora. Frequently a "bacterial cocktail" is given in an attempt to rectify this flora deficiency. Both gnotobiotes and the best specific pathogen-free (SPF) animal colonies are the same initially; the gnotobiotic colonies are maintained under strict microbiological control, while the SPF colonies are permitted to become contaminated by any organism other than the specified pathogens. The microbiological differences between various SPF colonies can be important, and are particularly so when animals are used in life span studies. The two classes of animals also differ in the probability of "breakdown," i.e., the introduction of pathogens which interfere with their usefulness. It is general accepted that SPF colonies will have to be rederived periodically and the problems associated with infections in user's laboratories, particularly on long-term studies, are well known. Gnotobiotic animals have rarely been accidentally contaminated with a pathogen; contamination is notably absent in a well-run gnotobiotic laboratory. Even when contamination occurs it is usually due to common benign species.

At present there are two limitations to the wider use of gnotobiotic animals: (1) a reluctance to extrapolate the observations to man and ordinary animals (this is due to the lack of a "normal" flora) and (2) their expense. At the present time there is sufficient knowledge of murine microbiota to produce a gnotobiote with characteristics and performance falling within the accepted range of normal variability (Pesti, 1979); the problem area lies with the accidental contaminants which characterize a SPF breeding room. The only feature in common between these contaminants and the flora of man and ordinary animals is the fact that they are uncontrolled; laboratory science would be far better served by determining the microbial species essential for a given study, making certain they are present and that all irrelevant species are absent.

The actual cost of using gnotobiotic animals for many of the applications for which SPF animals are now used depends not only upon the initial cost of the animal and its maintenance, but also upon a comparison between the numbers required and the reliability of the observations made. There has been little effort devoted to the reduction of the costs of gnotobiote production and use because present markets are sporadic and ask for only small numbers of animals.

II. STERILE ENVIRONMENT

Gnotobiotic animals, whether axenic or associated with microbiota, must be placed within a sterile environment and maintained without introducing contaminants. A colony of axenic mice serves as an excellent test for the adequacy with which a sterile environment is maintained because of the reliability with which contaminates can be detected in the absence of a microflora. The axenic colony serves to demonstrate the effectiveness of both apparatus and procedure.

Although sterile workspace can be maintained in an open room when meticulous care is employed to avoid contact contamination and unidirectional (laminar) airflows are present to reduce airborne contamination, it is far easier to use physical barriers to prevent contamination. Isolators consist of a single continuous physical barrier arranged to prevent contamination of a workspace without interfering with the use of the space. A great many different isolators have been used, some of which are available commercially, while others are "homemade."

A. Types of Isolators and Their Sterilization

Rigid-walled isolators, particularly those made of stainless steel, are very sturdy and although their initial cost is high they may be economical when used continuously over long periods. Metal isolators are usually sterilized with steam under pressure, either as a pressure vessel or within a larger autoclave (Sacquet, 1968). Isolators made with rigid walls are more difficult to use than those having flexible walls because of the limitation imposed on the movements of the operator and the greater air pressure differential that has to be maintained across the walls to compensate for air displacement caused by the operator. For these reasons most new installations now use flexible film isolators and these will be the only ones considered here.

Flexible-walled isolators have been made from a great variety of plastic materials, e.g., polyvinychloride (PVC), polyethylene, polypropylene, nylons, vinylidene chloride, polyurethane; PVC is by far the most commonly used material. While some of the plastic films can be autoclaved, it has been found more practical to use chemical sterilizing agents. Peracetic acid solutions (Trexler and Reynolds, 1957) are widely used for this purpose because they are sporicidal in both liquid and vapor phases and are volatile leaving no toxic residues.

1. Glove Isolators

Glove Isolators (Fig. 1) ordinarily have a rigid base (plywood or chipboard) upon which the envelope rests and to which is attached the entry port, ventilation equipment, possibly cage rack supports, and a frame to position the top of the envelope. The size of the envelope used on isolators for mice ranges from 1 m high, 1 m wide, and 2 m long to about 0.4 m^3. In the smaller isolators, mouse cages are placed on the floor; racks may be used in the larger ones. Racks or shelves can be assembled within the isolator as self-standing units, or they can be made of metal tubing which passes through the envelope walls to be supported externally.

Fig. 1. Glove isolator.

Entry ports consist of a short length of plastic or sheet metal tube attached to the isolator base and passing through the plastic film wall. The tube is circular in section so that a tight joint can be made with the envelope. The port is closed by means of PVC caps held in place by rubber bands or adhesive tape. The position of the entry port is dictated by the operations carried on within the isolator and the arrangement within the isolator room. Materials can be moved readily into and out of ports that are directly in front of the operator where the envelope width is no greater than 700 mm. The front wall seems to be the most convenient port location for isolators on racks against a wall or those more than 700 mm wide, since animals and materials can be passed through the port without moving the isolator. The gloves, however, must be positioned to provide access to the port. The newly introduced transfer isolator (Trexler, 1980) considerably reduces the restrictions on the position of ports, since a port need not be capped from within its main isolator and materials can be passed through the port from the transfer isolator. Entry ports on the side walls are harder to use, and there appears to be no advantage to this except in surgical isolators.

Rubber gloves, either shoulder or wrist length, are used for handling materials within an isolator. Shoulder-length gloves can be very durable, lasting for years. They are, however, expensive, difficult to replace while the isolator is in operation, cannot be used for fine work, and restrict movement because of the limited size of the arm opening. The attachment of wrist-length gloves to either rubber or plastic sleeves makes it possible to change gloves without contaminating the isolator, (Fig. 2); this is useful if the glove is damaged or if it is neces-

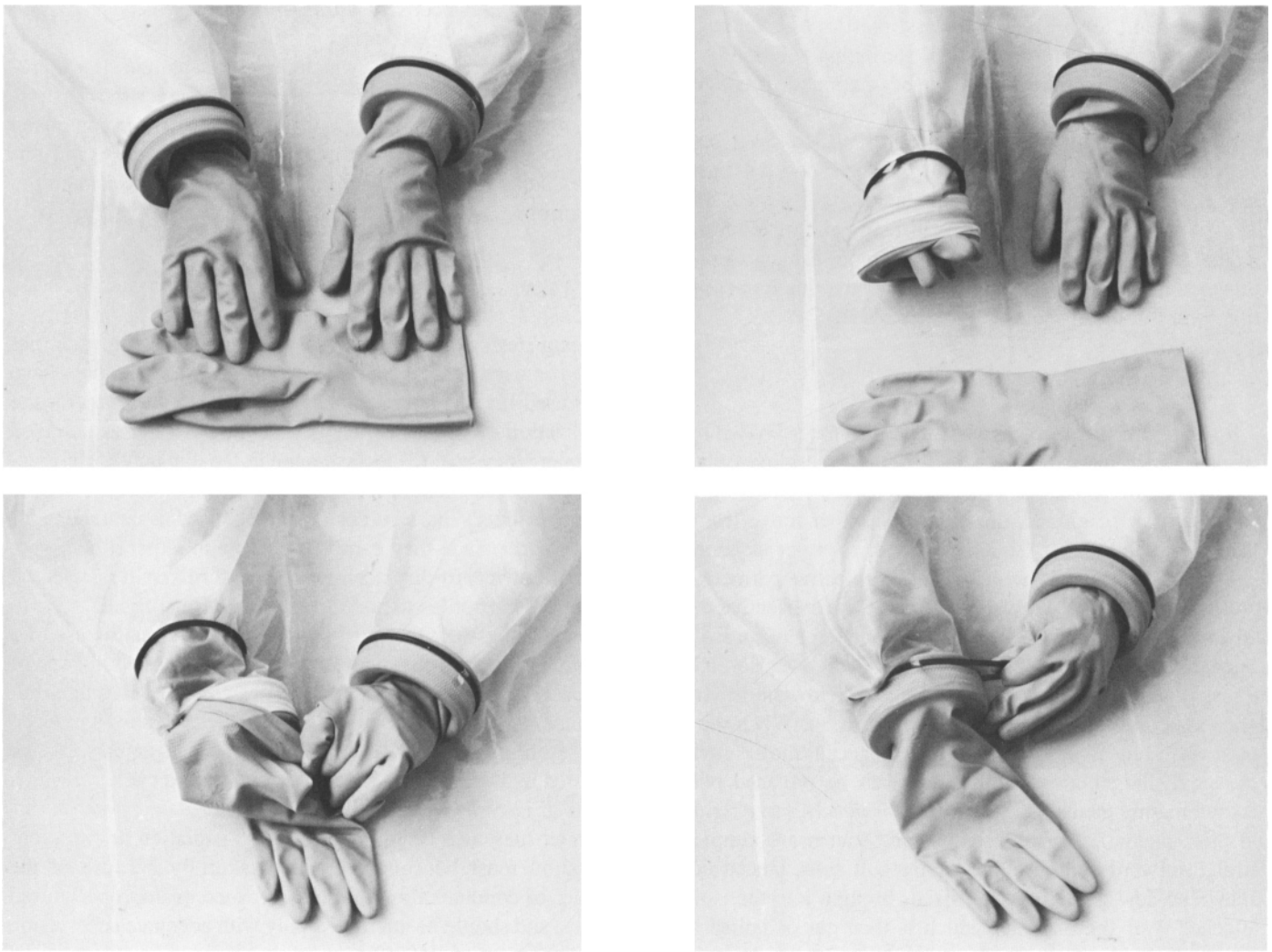

Fig. 2. Glove changing while isolator is in use.

sary occasionally to perform delicate manipulations. Sleeves made of soft flexible plastic film can be very durable if made with lap welds. Plastic sleeves can be welded to the envelope using any size or shape of opening to provide maximum movement; if damaged they can be repaired with adhesive tape.

An isolator is leak tested before sterilization by inflating it and allowing it to stand, usually overnight, or it can be inflated with Freon and tested using an electronic halogen leak detector. Usually an isolator is loaded as completely as possible before being sterilized to reduce the number of sterile entries required later. A 2% aqueous solution of peracetic acid with wetting agent is commonly used to sterilize isolators. All surfaces are wetted with this solution because the liquid kills spores 10 to 20 times as rapidly as the vapor, and the liquid will penetrate small amounts of organic matter (e.g., blood films) while the vapor will not. Peracetic acid is rapidly destroyed by organic matter and the common metals, other than stainless steel; for this reason, glass, plastic, or stainless steel atomizers are used to disperse the sterilizing solution.

Once the sterilizing solution has been applied, it is allowed to act for at least 1 hr before venting to remove the remaining peracetic acid. Personnel should be protected from contact with the sterilant because it is an irritant and it has been reported to be potentially cocarcinogenic (Bock *et al.*, 1975). The odor of peracetic acid can be virtually eliminated from the laboratory by diluting it in a chemical hood, using an atomizer powered manually or by Freon, and by removing it in conjunction with a transfer isolator (Trexler, 1980).

2. Half-Suit Isolators

It is obvious that the size of a glove isolator is limited by the working area that can be reached through the gloves, and although the working area can be increased by attaching more than one pair of gloves, the user must either move from one pair to another or assistants are needed. A larger isolator area can be used efficiently by means of a protective garment. The upper part of a protective garment is the most effective design when attached at the waist to the wall or floor of an isolator. Such a half-suit is a continuation of the isolator barrier; it has a transparent facepiece which provides excellent visibility, and it allows the user to move easily from the waist. When the skirt of a half-suit is lengthened, the user can move over increasingly larger areas. A half-suit can be replaced without contaminating the isolator provided it is attached to a rigid ring in the isolator floor; replacement procedures are simple and straightforward for an isolator having half-suits, placed side by side. The new garment is sterilized, brought into the isolator, attached over the old one, which is then cut or pulled away from the outside. The use of two half-suits in an isolator is also convenient for any procedure requiring assistance and provides a spare, if one is damaged. Half-suits attached to walls are not ordinarily replaced.

Great care should be taken to ensure that personnel have an adequate supply of fresh air for breathing and cooling. Everyone working in a protective garment having an enclosed headgear should be aware of the dangers resulting from a lack of an adequate air supply.

B. Supplies: Preparation and Sterilization

Continuous maintenance of gnotobiotic animal colonies requires impeccable sterile processing. The probability that any living microbes present will grow and multiply sufficiently to be detected eventually is increased by (1) the absence of preservatives, (2) the activity within the isolator which can expose the microbe to a great variety of microenvironments, and (3) the extended time periods, which permit adaptation. It is therefore essential that all sterile processing be under the supervision of adequately trained personnel. Sterilization is a two-step operation: (1) the treatment necessary to inactivate living microbes and (2) the packaging necessary to prevent contamination prior to supplying the isolator.

1. Water

Steam sterilization is the usual method of treating water before it is used in an isolator. The following procedures have been used for this purpose: (1) filling bottles designed for the sterilization of fluids, (2) canned or tinned water as supplied for emergency use, (3) incorporated in the diet before sterilization, using a thickener such agar, and (4) piped directly from a water sterilizer. Glass bottles are widely used as containers for sterile water since they can be treated with either a high-vacuum or downward-displacement steam sterilizer. It is essential to subject the bottles to pressurized steam for sufficient time to assure sterilization; the most satisfactory check is the use of a recording thermometer with the sensing element within one of the batch of water bottles. Vented caps could be used to eliminate the danger of an explosion when the pressure is reduced after sterilizing and before cooling. The danger can also be avoided by the use of an autoclave with a fluid cycle, in which the load is cooled before the door is opened.

Water may also be sterilized using γ-radiation or filtration. Filtration must be conducted very carefully because of the danger of contamination through damaged or improperly used filters, and should be attempted only with adequate supervision (Ducluzeau *et al.*, 1979).

2. Bedding

Bedding and all other resistant materials should be subjected to sterile treatment sufficient to assure sterilization beyond all doubt; any possible contamination is then limited to packaging and sterile transfers. Pressurized steam, γ irradiation, and ethylene oxide have been used routinely for sterilizing bedding.

Steam sterilization of bedding requires the removal of air to assure complete penetration by saturated steam for an adequate time/pressure period, after which it has to be dried; originally all this was done in a special autoclave attached to an isolator. The bedding was placed in the autoclave in porous bags, air was removed by high vacuum and after the maintenance of a saturated steam pressure for a period related to the pressure used, the bedding was dried *in situ* by a flow of sterile air. At present bedding and other materials are sterilized usually in a high vacuum autoclave; they are placed in special sterile drums to minimize the risk of contamination between autoclaving and passing into an isolator. A sterile drum (Fig. 3) consists of a cylinder, made of aluminum or stainless steel, which is open at one end and has a glass wool sterilizing filter situated circumferentially at the midline; a smaller filter may be used, placed at an opening in the closed end. After loading is complete, the open end of the drum is closed by a heat-resistant plastic film which is taped into place; this end is attached later to an entry port by means of a plastic sleeve.

The requirements for sterilizing materials in these drums are far more exacting than the usual autoclaving procedure because filters, tight enough to hold bacteria, restrict the passage of both air and steam. The drum must be packed loosely to allow circulation of air and steam. The autoclave should have an absolute vacuum gauge to indicate the actual pressure remaining within the autoclave chamber, and the leak rate should be determined. Much of the air leaking into the chamber during a vacuum cycle will be forced into the drum by the buildup of steam pressure. If the air within the chamber is heated by the autoclave jacket, compression by the incoming steam can raise the temperature within the drum above that of the steam, but since the steam within the drum is not saturated, heat-resistant spores will survive. Since air can leak into the chamber through a faulty gland or door gasket, it is essential that leak rates be determined. A pulsed cycle, i.e., a succession of three prevacuums, reduces but does not annul this requirement.

Sterile drums can be used in a downward displacement autoclave provided the drum has a valved drain and this drain is connected to atmosphere through a valved opening in the door, the wall, or through a pipeline from the chamber. The two valves are kept open as the steam enters the chambers; the outer valve is closed only when the steam emerging has no air in it. After the sterilizing cycle is complete, the autoclave door is opened and the valve on the drum is closed before the line attached to the other valve is disconnected. This method does away with the need for a high-vacuum autoclave, but the load cannot be cooled and dried quickly.

Bedding for sterilization by γ radiation (5 Mrad) is placed in plastic bags of a size suitable to pass through an entry port. The bedding is double bagged or it can be put in a protective cardboard box before sterilization. Either procedure preserves a sterile surface on the bag when later passed into an isolator. The bags (0.12–0.25 mm polyethylene) are vacuum packed and visually inspected prior to use for the presence of a vacuum as a check for leaks.

3. Diet

Pelleted food is sterilized by γ radiation in Great Britain (Ley *et al.*, 1969) and by autoclaving in the United States (Williams *et al.*, 1968). Fortified diets capable of being either irradiated or steam sterilized are available commercially, and some companies offer presterilized animal feeds. Diets sterilized by either method with and without vitamin supplements have been used for breeding and maintenance of animals. It is necessary to be on the alert for the appearance of signs of diet difficiency because a chosen diet may prove to be inadequate, adequacy being a function of the mouse strain, the gnotobiotic condition, the process of sterilization, and the shelf life of particular batch of feed before and after sterilization.

The packaging and sterilizing of diet by γ radiation is much the same as that for sterilizing bedding. If a diet has a sufficiently low bacterial count, it can be sterilized by 2.5 Mrads. Usually 4–5 Mrads are used and is considered to be the routine dosage, because virus is known to be more resistant to radiation than bacteria.

The process of sterilizing food by steam is much the same as sterilizing bedding except that care must be taken to minimize loss of vitamin and reduction in nutritive value. The initial moisture content of the diet and the time and temperature used are important (Zimmerman and Wostmann, 1963). A high temperature and vacuum autoclave are preferred for steam sterilizing food because of its short treatment time and rapid cooling. Steam sterilization alters the physical properties of pelleted diet more than irradiation; pellets are usually coated with a talcum or silicate powder to prevent sticking.

4. Heat-Labile Materials

Heat-labile solutions are usually sterilized by filtration on a sterile workbench and placed in ampules or filtered directly into an isolator. Other heat-sensitive materials can be sterilized by several other methods, e.g., (1) ethylene oxide, (2) treated