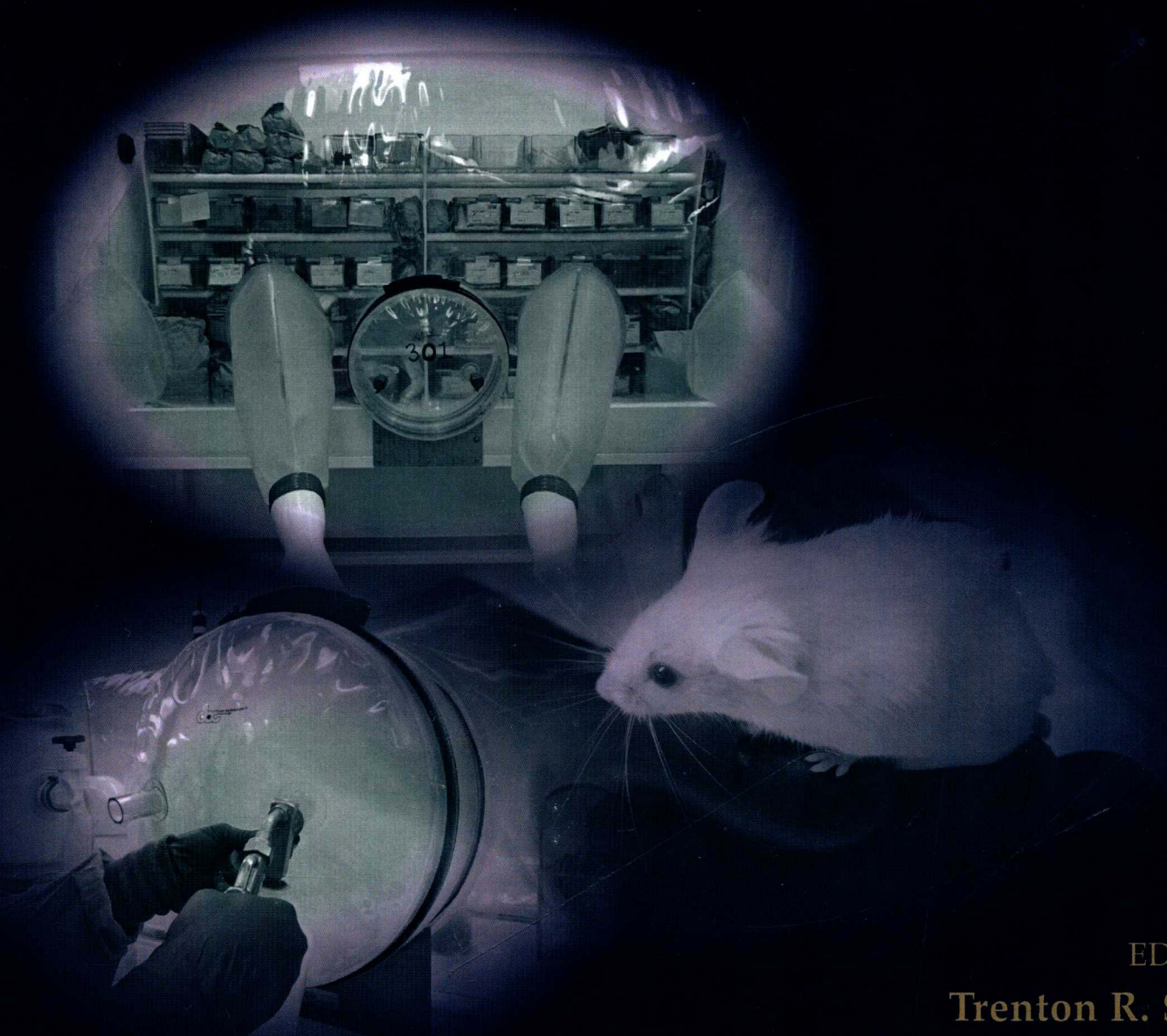
Gnotobiotics



EDITED BY
Trenton R. Schoeb
Kathryn A. Eaton

American College of Laboratory
Animal Medicine Series

Gnotobiotics

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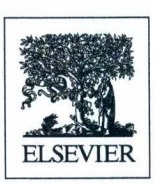

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Gnotobiotics

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Foreword

The American College of Laboratory Animal Medicine (ACLAM) was founded in 1957 to encourage education, training, and research in laboratory animal medicine and to recognize veterinary medical specialists in the field by certification and other means. Continuing education has been an important activity in ACLAM from its inception. A series of volumes on the biology and diseases of laboratory animals and related topics have been published by ACLAM over the past four decades. The first in this series, *The Biology of the Laboratory Rabbit*, was published in 1974, with a second edition in 1994; *The Biology of the Guinea Pig* in 1976; and a two-volume work, *Biology of the Laboratory Rat*, in 1979 and 1980, with a second edition in 2006. The publication *Laboratory Hamsters* appeared in 1987. In 1979, the College published a two-volume text on *Spontaneous Animal Models of Human Disease*. In 1981–83, four volumes of *The Mouse in Biomedical Research* were published, followed by four updated volumes in 2007. A two-volume treatise on *Nonhuman Primates in Biomedical Research* was published in 1995 (Vol. 1) and 1998 (Vol. 2), a second two-volume edition in 2012, and a text *Anesthesia and Analgesia in Laboratory Animals* in 1997, followed by a second edition in 2008. *Flynn's Parasites of Laboratory Animals* came out in 2007. *Planning and Design of Research Animal Facilities* was published in 2009; *Biology and Medicine of Rabbits and Rodents, 5th Edition* in 2010; *The Laboratory Rabbit, Guinea Pig, Hamster and Other Rodents* in 2012; *Laboratory Animal Welfare* in 2013; and, most recently, the third edition of *Biology and Diseases of the Ferret* in 2014. The third edition of a teaching text published in 2014, *Laboratory Animal Medicine* (first edition published in 1984 and the second edition in 2002), reflects the College's continuing effort to foster education. The newest in the series, *Swine in the Laboratory: Surgery, Anesthesia, Imaging, and Experimental Techniques*, third edition, was published in 2015. Finally, the second edition of *Clinical Chemistry of Laboratory Animals* will be published in 2017. Continuing the tradition of publishing texts, the College has commissioned the sixth edition of *The Biology and Medicine of Rabbits and Rodents*, *The Biology and Diseases of Captive Marmosets*, and the third edition of *The Laboratory Rat*.

The majority of advances in biology and medicine have depended, in one way or another, on the study of animals. During the past generation, the health, genetic integrity, and environmental surroundings of the animals have been recognized as important factors to be taken into account in planning animal studies. The ultimate responsibility for ensuring the validity of scientific results, together with humane and scientifically appropriate animal care, resides with two categories of scientists: veterinarians responsible for the acquisition, care, nutrition, anesthesia, and other aspects of humane animal use; and scientific investigators, their staff and students, who use animals as subjects of study. This book, *Gnotobiotics*, therefore is intended for these individuals and others in the fields of biology and medicine who utilize gnotobiotic animals in biomedical research. Although a chapter in the first and second editions of *The Mouse in Biomedical Research* was devoted to gnotobiotics, this new text will update and expand our knowledge base on the subject. The editors and contributors are particularly hopeful that the text will prove useful in introducing students and scientists embarking on the use of various species of gnotobiotic animals to important concepts on host–microbiome interactions.

The contents of this text on gnotobiotics, edited by Trenton R. Schoeb and Kathryn A. Eaton, are presented in eight chapters that provide information central to the successful use of gnotobiotic animals, including rodents, swine, and fish. Chapter 1 provides a historical overview of the development and use of gnotobiotic science and technology. With the increasing awareness of the importance of the microbiome in health and disease of various species of animals, the use of gnotobiotic technology has increased dramatically in the past decade and is expected to continue to grow for the foreseeable future (Fig. 1). Given the complexities of establishing and operating a gnotobiotic facility, in Chapter 2 the authors describe, in considerable detail, the "nuts and bolts" required to successfully maintain a gnotobiotic facility. Chapter 3 details requirements for germfree manipulations and handling of rodents, including step-by-step instructions and numerous illustrations. Proper acknowledgments are given to the laboratory of Jeffrey Gordon, MD, a pioneer in the sophisticated use of gnotobiotic mice in studying the interface of the microbiome and the host, the recent development of a gnotobiotic rodent facility at Baylor University, the established gnotobiotic units at the University of Alabama and University of Michigan, and the longstanding expertise of Taconic Farms in providing germfree mice for the research community.

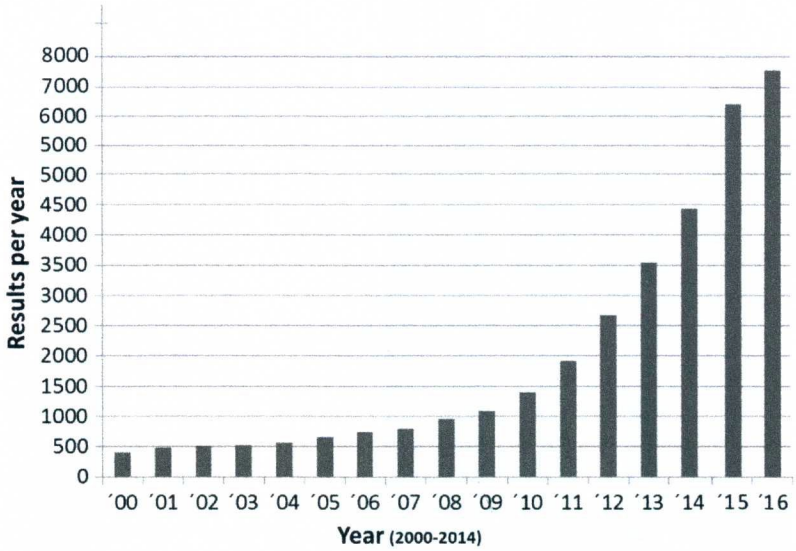

FIGURE 1 PubMed search using terms microbiome or microbiota, total hits per year. *Modified from Bleich, A., Fox, J.G., 2015. The mammalian microbiome and its importance in laboratory animal medicine. ILAR 56 (2), 153–158.*

A critical element for successfully operating a gnotobiotic facility is developing and implementing a sound business plan. These elements are discussed in Chapter 4 under the headings "Operational management considerations in establishing a gnotobiotics program," "Developing a business model," "Identifying expenses," "Establishing fees," "Subsidization," and "Financial reviews." As stated by the authors, "The effort necessary in developing a business plan unique to your program, though challenging and requiring a significant time investment, will provide many benefits."

The use of gnotobiotic pigs has developed and prospered in a small number of academic units during the past 50 years. The reported initial use of germfree pigs was described in 1960 by Dr. Jaroslav Sterzl and colleagues from the Department of Immunology in the Czech Academy's Institute of Microbiology. Highlighted in Chapter 5 are numerous examples of the use of the gnotobiotic pig in the study of the pathogenesis of various infectious agents, including bacterial, fungal, and viral pathogens. Importantly, the ontogeny of the porcine immune system has, in large part, elucidated using the gnotobiotic pig. Given the physiologic and immunologic similarities of the pig and humans, it is anticipated that use of the gnotobiotic pig will continue to be an important large animal model for biomedical research.

As a departure from the study of mammalian hosts in gnotobiotic settings, Chapter 6 introduces the reader to the use of gnotobiotic fish to study host–microbe interactions. The development and use of gnotobiotic fish have advantages not available when using mammalian species. Individual female fish can release 80 to several thousand eggs at once, and these embryos are protected with a resistant chorion to selected chemicals, thus enabling surface sterilization and subsequent development of the eggs under gnotobiotic conditions. Although germfree fish of a variety of species have been developed over the past 50 years, it was not until the development of the germfree zebrafish that detailed studies on host–microbe interactions were undertaken. This model has proven to be particularly attractive, and the authors review the required maintenance of gnotobiotic fish used to ensure continued research involving this fascinating model.

Chapter 7 concentrates on the use of gnotobiotic mice to study metabolic syndrome, a term used to define obesity and its interrelated metabolic abnormalities, which is an increasingly important disease in humans. The authors take advantage of the growing number of studies describing approaches used to investigate these metabolic abnormalities and how the microbiome influences these perturbations.

Chapter 8 provides a detailed overview of the use of gnotobiotic mouse and rat models to study the pathogenesis of inflammatory bowel disease (IBD), which, as the author indicates, affects 1.5 million humans in the United States and over 2 million individuals in Europe. He rightly points out that the use of gnotobiotic rodents clearly demonstrated that spontaneous intestinal inflammation in immunomodulated rodents was the result of intestinal microbiota. These studies provided a critical framework for the increasing utilization of gnotobiotic rodents to study various immunological and genetic components important in the development of IBD. This chapter is replete with important studies highlighting the utility of gnotobiotic rats and mice, immunocompromised and/or other targeted genetically modified rodents, plus a select number of inbred strains of mice exposed to various microorganisms, in understanding the intricacy of this important disease.

In summary, this text provides the biomedical research community an essential text on the successful operation of a gnotobiotic research facility and provides compelling examples of the use of gnotobiotic animals in understanding the complexities of how the microbiome plays an indispensable role in shaping of host's physiological and immunological responses required to maintain homeostasis, or when coping with a perturbed state due to infections or metabolic disturbances.

James G. Fox

Contents

Chapter 1

Historical Overview

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Chapter Outline

The history of gnotobiotics, or portions or summaries of it, has been presented in several previous sources (Carter and Foster, 2005; Coates and Gustafsson, 1984; Foster, 1980a; Gustafsson, 1959a; Heine, 1998; Kirk, 2012; Lindsey and Baker, 2005; Luckey, 1963c; Mickelsen, 1962; Pleasants, 1965, 1974; Pollard, 1964; Rahija, 2006; Reyniers, 1959b; Trexler, 1983, 1985; Trexler and Orcutt, 1999; Wostmann, 1996). In the interest of a convenient source of background and perspective, an overview is provided here. For those interested in more detail, Thomas Luckey's 1963 book, *Germfree Life and Gnotobiology* (Luckey, 1963c), which we have relied on considerably, provides the most comprehensive account of history up to the early 1960s. It is available in electronic form from Elsevier (http://store.elsevier.com), Amazon.com (www.amazon.com), and Books-A-Million (www.booksamillion.com), and original copies can occasionally be obtained via the used book market using services and vendors such as Google Books (books.google.com), Amazon.com, and AbeBooks.com (www.abebooks.com). [A nutritional biochemist, Luckey published a short autobiography (Luckey, 2007) a few years before his death in 2014 at age 94. Aficionados will find it interesting; he was evidently somewhat eccentric.] A number of other original works contain not only additional detailed information but also many interesting illustrations not included in those cited above. Unfortunately, much of the material is difficult to access, if not impossible for practical purposes, because of age or language. Some sources, although published in English, were of limited distribution and can be difficult or impossible to locate, although some of the books and symposium proceedings cited herein can occasionally be obtained from used book vendors. Services such as WorldCat (www.worldcat.org) are helpful in locating materials accessible only in library archives, such as those of the University of Notre Dame. On the other hand, some publications in scientific periodicals, though older, are nevertheless freely available online; these are indicated below where cited.

In keeping with the purpose of this book, this overview is focused on developments of greatest relevance to today's biomedical researchers and laboratory animal professionals. However, a variety of species other than those emphasized in this book have been derived germfree (Luckey, 1963d; Pleasants, 1974). The guinea pig and chicken have been widely used, especially by early investigators, because of their ease of introduction into a germfree environment and lack of need for intensive neonatal care (Luckey, 1963d; Pleasants, 1974). Other mammals include the rabbit, gerbil (Wostmann, 1996), *Millardia meltada* (an Indian wild rodent) (Saito and Nomura, 1984), dog, cat (Fletcher et al., 1991), rhesus and cynomolgus macaques, African green monkey, baboon (Kalter et al., 1973), chimpanzee (Eichberg et al., 1979), black-mantled tamarin (Eichberg et al., 1979), goat, lamb, calf, and foal. Birds include Japanese quail and turkey; amphibians include frogs and toads; and fish include Atlantic halibut (Verner-Jeffreys et al., 2003), platyfish, sea bass, Nile tilapia (Situmorang et al., 2014), and salmon (Trust, 1974); see also Chapter 6. A wide variety of invertebrates also have been studied under germfree conditions (Luckey, 1963d), including *Drosophila melanogaster*, which, in addition to mammals and fish, is currently used to study host–microbiota relationships (Lee and Brey, 2013; Ma et al., 2015; Newell et al., 2014).

Most reviews of the history of gnotobiotics begin with Louis Pasteur's comments, reproduced on page 61 of Luckey (1963d), to the French Academy of Science in 1885 regarding his "preconceived idea that [germfree animal life] would be impossible," to which Marceli Nencki, a Swiss chemist, responded that animals without bacteria should be healthier because they would not be exposed to toxic by-products of bacterial digestion (Nencki, 1886, cited in Luckey, 1963d). However, a broader perspective recognizes critical developments in early microbiology by Pasteur and others, notably Ignaz Semmelweiss (antiseptics and antiseptic practices), Joseph Lister (antisepsis and pure cultures in liquid media), Paul Ehrlich (bacterial staining), and Robert Koch (solid culture media) (Joklik et al., 1992; Luckey, 1963a, 1963d; Perkins, 1969). Also crucial was the introduction of pressure steam sterilization by Charles Chamberland, whose first sterilizer

Gnotobiotics. http://dx.doi.org/10.1016/B978-0-12-804561-9.00001-3

resembled a pressure cooker (Luckey, 1963a; Perkins, 1969). Such developments were necessary for successful attempts at germfree derivation to be possible.

The first attempt actually to test the competing hypotheses of Pasteur and Nencki was by George Nuttal and Hans Thierfelder at the University of Berlin (Nuttal and Thierfelder, 1895, cited in Luckey, 1963d), who used an apparatus based on a bell jar to house a cesarean-derived guinea pig, which was fed sterile milk (Luckey, 1963d). The animal seemed healthy until it was sacrificed after 8 days and found to be germfree. In subsequent experiments, in which the animals were fed a vegetable diet, some guinea pigs were contaminated, but others remained germfree when sacrificed at 10 days of age, although they grew poorly (Nuttal and Thierfelder, 1896, cited in Luckey, 1963d). They also tried to derive germfree chicks, but were unsuccessful (Nuttal and Thierfelder, 1897, cited in Luckey, 1963d). Although these investigators did not conduct further germfree experiments, they introduced procedures that remain basic to gnotobiotics over 100 years later, including aseptic cesarean surgery and transfer of neonates to a sterile enclosure, sterilization of air and food, collection of excreta, use of rubber gloves for manipulations inside the enclosure, use of a germicidal trap, and hand-feeding of sterile milk to neonates, and they observed the enlarged cecum that is characteristic of germfree rodents (Luckey, 1963d). Nonetheless, the limited success with guinea pigs was not sufficient to settle the question of whether life without bacteria was possible for more than brief periods. Olga Metchnikoff thought the results of Nuttal's and Thierfelder's experiments supported the possibility, as the guinea pigs did gain weight, but Max Schottelius disagreed, believing that the weight gains were due to the accumulation of contents of the digestive tract (Luckey, 1963d).

Although Nuttal and Thierfelder were not successful in deriving germfree chicks, their finding that chick embryos were sterile suggested a simpler way to obtain germfree animals, inasmuch as the external surface of eggs should be chemically sterilizable, and, moreover, chicks are capable of independent life when hatched and do not require hand-feeding of sterilized milk (Luckey, 1963d). In 1899, Schottelius, at the University of Freiburg in Breisgau, Germany, reported that he had obtained sterile chicks (Schottelius, 1899, cited in Luckey, 1963d). Although they seemed healthy for the first few days, they soon became weak and then moribund and were sacrificed, and he concluded that bacteria were necessary for chicks to utilize their diet. Schottelius continued his work with chicks for a number of years, but, because of the undeveloped knowledge of nutrition of the time, he never was able to maintain them long term (Luckey, 1963d). He did, however, serve as mentor of two other pioneers of gnotobiotics, Michel Cohendy and Ernst G. F. Küster. Cohendy went to Schottelius's laboratory to learn germfree technology in 1907 at the behest of Élie (Ilya) Metchnikoff, then director of the Pasteur Institute in Paris. Cohendy began conducting experiments with germfree chicks after his return to the Pasteur Institute in 1908 and was able to develop diets that supported growth (Luckey, 1963d). He and Eugene Wollman reared germfree guinea pigs and used them to study vitamin C deficiency (Cohendy and Wollman, 1914, cited in Luckey, 1963d). Küster joined Schottelius in 1910, conducted his initial experiment with a germfree goat in 1911, and by 1913 had kept a germfree goat alive for 35 days (Küster, 1913a, cited in Luckey, 1963a). His isolator, which featured an airlock (Küster, 1913b, cited in Luckey, 1963a) (Fig. 1.1), is considered the first to embody all of the major features of modern isolators (Heine, 1998; Trexler, 1985).

Although their accomplishments were important, early workers faced major obstacles to further progress that were insurmountable at the time, including lack of knowledge of nutritional requirements, particularly regarding vitamins and their susceptibility to degradation by steam sterilization; difficulties of hand-rearing small mammals; and equipment that had to be custom-built in each laboratory, was usually too small for more than one or a few animals, and was cumbersome to use. That began to change in the late 1920s and early 1930s. In particular, the sciences of nutrition and biochemistry were advancing rapidly, and between 1912 (thiamine and vitamin C) and 1941 (folic acid) the B vitamins and vitamins A, C, D, E, and K all had been discovered (Eggersdorfer et al., 2012). Advancements in gnotobiotics during the 1930s through the 1950s occurred primarily at the University of Lund in Sweden; Notre Dame University in Indiana, United States; and Nagoya University in Japan.

In 1928, Gösta Glimstedt began working with germfree guinea pigs at the University of Lund and continued these studies into the 1930s (Luckey, 1963a). [His photograph appears on page 5 of Miyakawa and Luckey (1968).] During this period, Bengt Gustafsson (Fig. 1.2), a major figure in development of germfree rats, began his career in Glimstedt's laboratory and began working to produce germfree rats in 1943 (Luckey, 1963a). His first apparatus consisted of two chambers connected by a germicidal dip tank. Pups were obtained by hysterotomy (Cesarean section) in one chamber and passed through the germicide in a container into the second chamber, which was supplied with heat-sterilized air. The apparatus, containing the fortified cow's milk used to rear the pups, was sterilized by autoclaving. Pups were fed every 4 h until weaning at 21 days of age. Gustafsson was able to rear some pups to 28 days of age without contamination, but he was not able to keep them any longer because of the small size of the chamber. He next constructed a rectangular stainless steel isolator that could hold 10 rats and then a larger one that could hold up to 30 (Figs. 1.3–1.5) (Gustafsson, 1959b, available online). The top was a sheet of plate glass sealed with a rubber gasket (Fig. 1.5). Gloves were fitted to the sides and a germicidal trap to the end. The isolator was sterilized in a large autoclave before use. For derivations, a

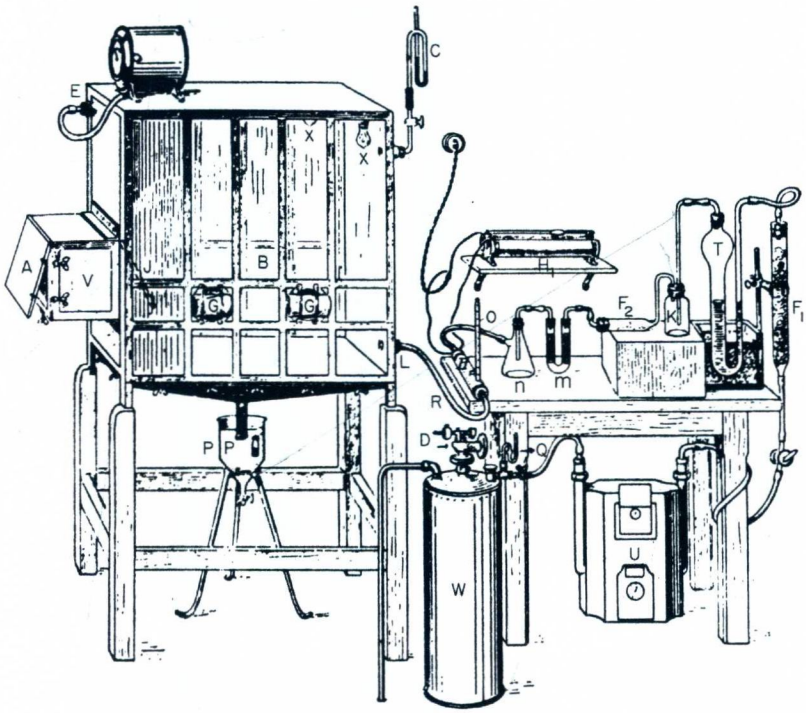

FIGURE 1.1 Ernst Küster's isolator and associated equipment for rearing germfree goats. A, outer door of "ante chamber" (airlock); B, chamber with glass windows; C, manometer to monitor pressure inside the chamber; D, air supply safety valve; E, air outlet; F, cotton air filters; G, rubber gloves attached with gaskets; H, electric heater for incoming air; J, inner door of airlock; K, trap for sulfuric acid from T; L, entry point of incoming air; m, drying tube; n, dust trap to protect the electric heater; O, thermometer; P, paraffin oil trap sealing the funnel that collected excrement from the bottom of the chamber; Q, air supply manometer; R, air supply line; T, sulfuric acid drying tower; U, flow meter; V, airlock; W, air storage tank; X, electric lights. The air pump and prefilter that supplied air to the air storage tank were under the floor. *From Fig. 3.8, p. 108, of Luckey, T.D., 1963c. Germfree Life and Gnotobiology. Academic Press, New York. Used with permission.*

FIGURE 1.2 Bengt Erik Gustafsson, University of Lund and Karolinska Institutet, developer of isolators and methods for rearing germfree rats. *From Fig. 1.42, p. 35, of Lindsey, J.R., Baker, H.J., 2005. Historical foundations. In: Suckow, M.A., Weisbroth, S.H., Franklin, C.L. (Eds.), The Laboratory Rat, second ed. Elsevier Academic Press, San Diego, CA, pp. 1–52. Used with permission.*

separate chamber was used, and pups were transferred through the germicide in a container. Gustafsson also solved the problems of developing an adequate sterilizable milk substitute and the technique of hand-feeding the pups via intubation and gavage. He raised germfree pups to weaning in 1946, although they did not grow well, but by 1947 he was able to raise them to maturity (Gustafsson, 1947, 1948). Gustafsson established the Germfree Research Laboratory at the

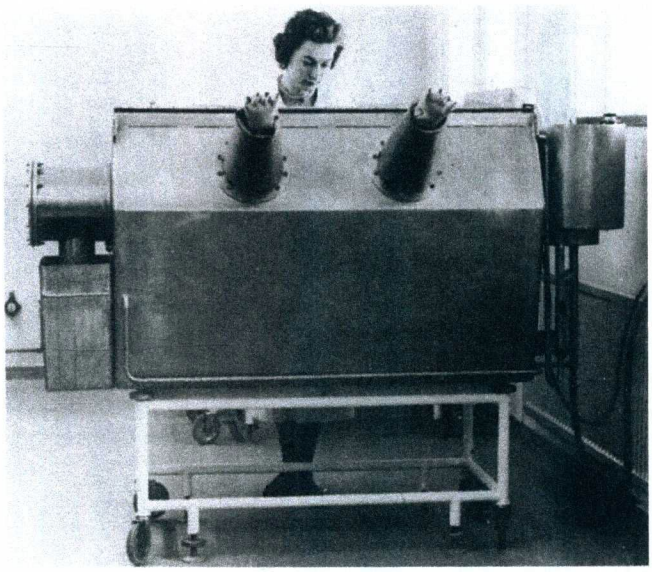

FIGURE 1.3 Gustafsson's lightweight stainless steel isolator "type 3," which had a capacity of 20–30 rats. *From Fig. 1.45, p. 37, of Lindsey, J.R., Baker, H.J., 2005. Historical foundations. In: Suckow, M.A., Weisbroth, S.H., Franklin, C.L. (Eds.), The Laboratory Rat, second ed. Elsevier Academic Press, San Diego, CA, pp. 1–52. Used with permission.*

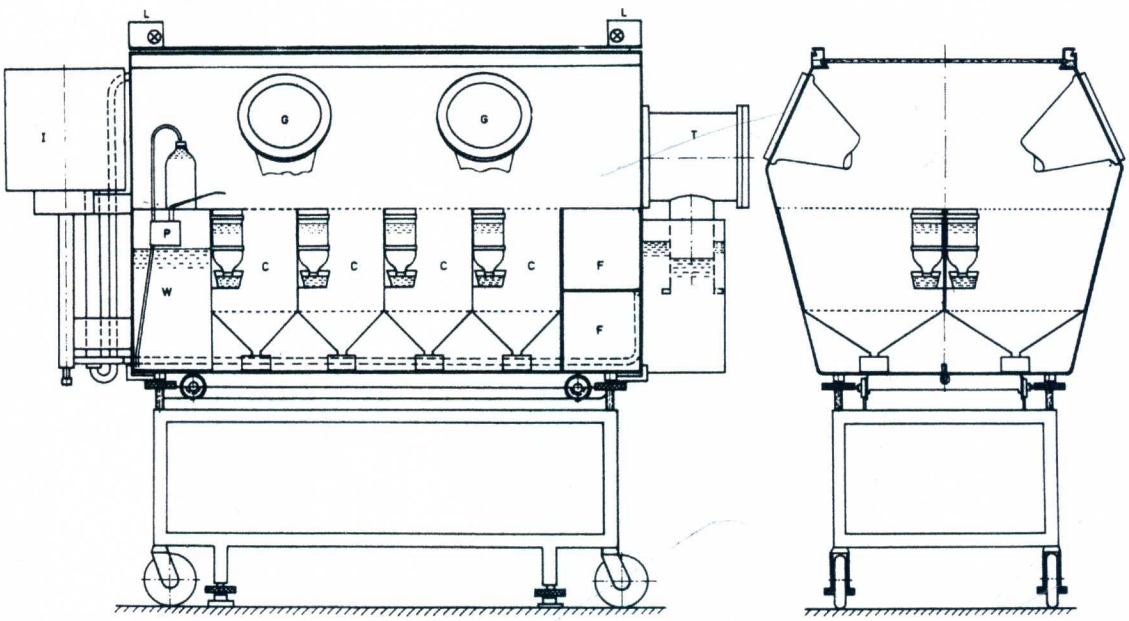

FIGURE 1.4 Diagram of the isolator shown in Fig. 1.3. L, lights (top); I, air sterilizer; W, water tank; P, water pump; C, cages; G, glove ports; F, food containers; T, food autoclave and transfer unit. *From Fig. 1.46, p. 37, of Lindsey, J.R., Baker, H.J., 2005. Historical foundations. In: Suckow, M.A., Weisbroth, S.H., Franklin, C.L. (Eds.), The Laboratory Rat, second ed. Elsevier Academic Press, San Diego, CA, pp. 1–52. Used with permission.*

Karolinska Institutet in Stockholm, Sweden, in 1961. PubMed lists 75 papers he authored or coauthored related to his work with gnotobiotic rats by the time of his death in 1986 at the age of 79. Some of his original isolators were still in use in 2002 (Lindsey and Baker, 2005).

James Arthur (Art) Reyniers (Fig. 1.6) began his work at the University of Notre Dame in 1928 (Pleasants, 1965, available online). The account of Trexler (1985), summarized by Lindsey and Baker (2005), provides an interesting personal perspective. According to Trexler, Reyniers "had no formal training in research" when he came to the University of Notre Dame to teach bacteriology, having "left the University of Chicago shortly after enrolling as a graduate student." At the time, investigation of bacterial inheritance and variation was his primary interest; germfree animals were

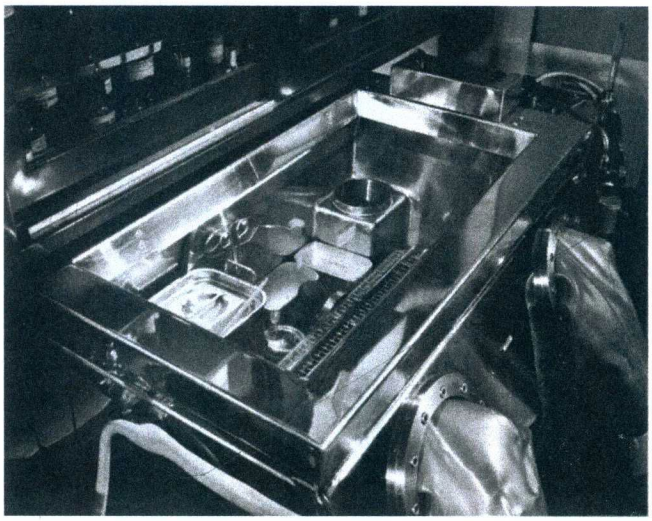

FIGURE 1.5 Gustafsson stainless steel isolator showing a view of the interior through the plate-glass top. *From Fig. 7, p. 223, of Newton, W.L., 1965. Methods in germfree animal research. In: Gay, W.I. (Ed.), Methods of Animal Experimentation, Vol. I. Academic Press, New York, pp. 215–271. Used with permission.*

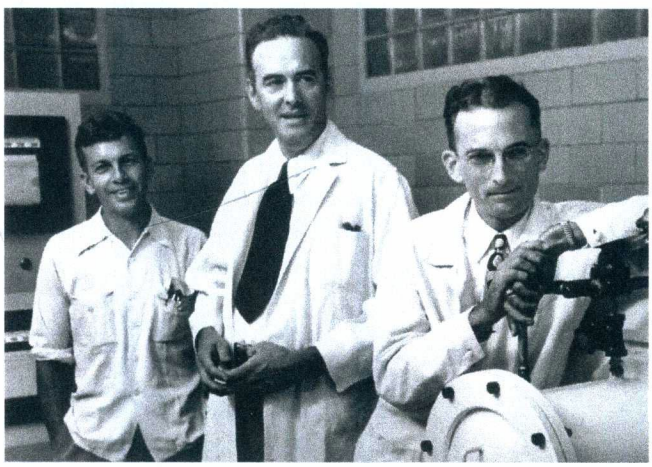

FIGURE 1.6 The leadership team of the LOBUND Institute in 1949. Left to right: Philip "Trex" Trexler, Associate Director; James Arthur "Art" Reyniers, Director; and Robert "Bob" Ervin, Associate Director. *From Fig. 1.49, p. 40, of Lindsey, J.R., Baker, H.J., 2005. Historical foundations. In: Suckow, M.A., Weisbroth, S.H., Franklin, C.L. (Eds.), The Laboratory Rat, second ed. Elsevier Academic Press, San Diego, CA, pp. 1–52. Used with permission.*

needed "to make certain that no contamination occurred during the study of bacteria associated with a host." However, Trexler considered him "undoubtedly a genius as a promoter." He was very successful in obtaining funding, raising over $3 million for germfree research, according to Robert Ervin (Fig. 1.6) (Trexler, 2002). [Trexler does not give a date for the figure, but the entire annual budget appropriation for the National Institutes of Health did not exceed $3 million until 1946 (NIH, 2016). According to the US Bureau of Labor Statistics, $3 million at that time would be equivalent to over $37 million in 2016.] Reyniers also was highly successful in attracting other scientists to the University of Notre Dame, such as Robert Ervin (Assistant Director, Administration), Helmut Gordon (physiology), Thomas Luckey (biochemistry), Arthur Phillips (biological engineering), Julian Pleasants (microbiology), John Reback (virology), Bernard Teah (production), Philip Trexler (Assistant Director, Research), Morris Wagner (bacteriology), and others (Lindsey and Baker, 2005). Reyniers thus led the development of a program that became the leading center of germfree research into the 1960s (Lindsey and Baker, 2005). It was later known as the LOBUND Institute, "LOBUND" being an acronym for Laboratories of Bacteriology, University of Notre Dame (Kirk, 2012; Lindsey and Baker, 2005; Pleasants, 1965). (Although "LOBUND Institute" was the title given by the university, variations such as "Lobund Institute," "Lobund Laboratory," or simply "LOBUND" or "Lobund" have been used inconsistently in publications, including those by