

63-5-2

CATALOGED BY ASTIA  
AS AD NO. 402065

**FINAL REPORT**

**September 1, 1960 through September 30, 1962**

**The Design Development and Study of Germfree Isolators  
for Use with Patients and Research**

**Principal Investigator: P. C. Trexler**

**LOBUND Laboratories**

**University of Notre Dame**

**Grant # MEDDH - 61 - 16**

**402 065**



**FINAL TECHNICAL REPORT**

**1 September 1960 - 30 September 1962**

**Principal Investigator: P. C. Trexler, Professor of Biology  
University of Notre Dame  
Notre Dame, Ind.**

**THE DESIGN, DEVELOPMENT AND STUDY OF GERMFREE ISOLATORS  
FOR USE WITH PATIENTS AND IN RESEARCH**

**Grant No. MEDDH-61-16**

**QUALIFIED REQUESTORS MAY OBTAIN COPIES OF THIS REPORT FROM ASTIA**

## ABSTRACT

Preparing Institution: University of Notre Dame

Title of Report: The Design, Development and Study of Germfree Isolators  
for Use with Patients and in Research

Principal Investigator: P. C. Trexler, Professor of Biology

Pages        illustrations    5, 25 March, 1963

Grant No.: MEDDH-61-16

Supported by: U. S. Army Medical Research and Development Command  
Department of the Army  
Washington 25, D. C.

Practical methods for using flexible film isolators to produce and distribute germfree mice and rats were developed to the extent that commercial laboratory animal breeders now engage in this activity. The methods of using these animals in the laboratory have been simplified to eliminate unusual requirements of equipment and space for animal studies. A preliminary study to evaluate pertussis vaccine indicated that these animals may be suitable for routine biological testing.

A pure culture of *Cl. difficile* will eliminate the principle anomaly of the germfree animal (distended cecum) to provide the first step in the development of a general purpose laboratory mouse with a completely defined microflora. Methods have been developed for using the larger animals (swine and sheep) in the laboratory with either a total absence of microbes or with pure cultures. The same principles of isolator construction have been used to build isolators for providing a sterile area for human surgery. Isolators have been constructed for either protecting a patient from the microbic environment of the hospital ward or preventing the spread of pathogens from a sick patient.

Note: Copies of this report are filed with the Armed Services Technical Information Agency, Arlington Hall Station, Arlington 12, Virginia, and may be obtained from that agency by qualified investigators working under government contract.

## TABLE OF CONTENTS

Abstract	ii
Significance of this Research	1
Aims	3
Introduction	3
A. Apparatus and Methods	6
1. Glove isolators	6
2. Introduction of supplies	8
3. Removal of specimens	10
4. The transfer of large specimens	11
5. The use of neutralized peracetic acid	13
B. Laboratory Animal Production and Distribution	16
1. Production methods	16
2. The transportation of animals	18
3. Studies with gnotobiotic animals	19
a) The distended cecum in the axenic animal	19
b) A comparison of the protection by pertussis vaccine to the intracerebral challenge of bordetella pertussis in SPF and axenic mice	22
c) Collaborative study with the Indiana University Medical Center, Indianapolis, Ind.	22
d) The removal of gnotobiotic mice from a sterile environment	24
4. Isolators for use with large animals and man	26
a) Large animal isolators	26
b) Patient isolators	27
References	29
Publications Resulting from this Study	31

## SIGNIFICANCE OF THIS RESEARCH

Studies supported by this Grant together with Contract DA-49-007-MD-878 and RG-6728 and 5752 from the National Institutes of Health have made it possible to greatly reduce the cost of producing and using gnotobiotic animals. The former methods required the use of expensive stainless steel apparatus and specialized plumbing installations. The present system can be used in any laboratory equipped for sterile operations. The new system is based upon the use of lightweight flexible film plastic isolators that can be easily moved and stored on shelves or racks. This "stacking" provides five or six times as much usable "germfree" space per floor area as with the former stainless steel isolators.

With assistance from the Institute for Laboratory Animal Resources a series of Workshop Programs were held to provide the training of institutional and commercial laboratory animal breeders to use the germfree isolator technic. As a result of this program gnotobiotic animals are produced at Fort Detrick and five commercial breeders now offer them for sale. This is a considerable contribution to the national research effort because now any laboratory can use gnotobiotics without the need to setup their own production facilities or to obtain animals from some research institute that has such facilities. Because of the pooling of requirements as to age and sex distribution and the utilization of animal production "know-how" the animals can be supplied by commercial breeders at less cost than in most research facilities.

A series of studies have been initiated which now are being continued by two commercial breeders to extend the isolator technique to the production of quantities of defined microflora laboratory animals. Theoretically these animals should be far superior to the present SPF animals because the microbiological component of the animal will be controlled. It was not possible to continue this development at LOBUND because more animals were produced in the smallest significant pilot operation than could be used by the LOBUND staff and those with whom I was permitted to collaborate.

Isolator technics have been extended to the production of large animals such as swine and sheep. It seems likely that these animals will be useful in the future for the production of sterile biological products such as blood, serum or cells for tissue culture. Studies with the smaller laboratory animals have shown that antisera produced in gnotobiotic animals are more specific than sera produced in comparable animals with a conventional or uncontrolled microflora. The development of methods for the maintenance of large animals is continuing at Ohio State University and at SPF Laboratories Inc., Liberty, Ind. The methods used can be adapted to routine operations by those interested in the production of biologicals.

These technological developments make feasible the more extensive use of the gnotobiotic. Microbiologically the animal without microbic contamination is a unique culture medium well suited to the study of the multifactorial etiology of disease. Pure culture studies can be extended from the in vitro to the in vivo environment which would appear to be of virological significance. There is a growing body of evidence that the microflora of vertebrates is of considerable significance in health as well as disease. The gnotobiotic is an ideal model for exploratory studies in this area. The control of the microbic component of the laboratory animal provides a more uniform and reproducible organism for biological testing and experimentation.

In collaboration with a group at WRAIR headed by Dr. Levenson, a series of isolators were developed for use in the operating room and for the treatment and care of patients on the wards. The isolator is a mechanical system for preventing microbic contamination and is a more precise method than the aseptic and sterile technic as now practiced. It seems likely with adequate development and mass production flexible film isolators should be relatively inexpensive. The use of isolators could alter considerably the aseptic and sanitary regimen of the hospital.

Surgical isolators could be developed to provide a presterilized working area in a small disposable package. These could be used to by-pass much of the housekeeping that is now a prerequisite to the setup of an operating room. This development would be of assistance in establishing operating facilities in a hurry as required at mobile field stations or at a disaster site, or wherever it is difficult to maintain aseptic conditions.

Isolators may also be used to confine a patient either to prevent the spread of pathogenic microorganisms or to protect a patient from pathogens in the hospital environment. The isolator is a mechanical device to simplify the task of maintaining asepsis or isolation and eventually will reduce the requirement for trained personnel for this operation. This type of apparatus appears to be potentially very useful in the treatment of wounded soldiers particularly those with extensive wounds complicated by renal failure or radiation injury which renders them particularly susceptible to infection. It also seems likely that isolators will be effective in the treatment of burn patients where either the entire patient or just the burned area may be isolated. Since the humidity can be easily controlled within the isolator the physician in charge could elect to reduce the humidity to promote eschar formation and a dry wound or increase the humidity to reduce the energy required by the evaporation of moisture from the wound surface. The amount of water lost through evaporation could be measured in the air effluent from the isolator to aid in the precise calculation of the fluid replacement requirements. Isolators should also be of assistance in the use of wet bandages or sprays for wound treatment.

Isolators will make it possible to unequivocally determine the role of microbes carried by the patient and those from the environment as the source of the causative agents of infectious disease.

## AIMS

1. To develop apparatus and methods which will make it possible to conduct a series of investigations in a sterile environment with a minimum investment in equipment and a minimum of technical limitations.
2. To apply these procedures to the production of laboratory animals in a manner that may make it feasible to use approximately the same pattern of production, distribution and use as with conventional animals.
3. To apply these methods to the treatment and care of patients.

## INTRODUCTION

The first report on the rearing of germfree animals at the University of Notre Dame was published in 1932 by Reyniers. Investigations of apparatus and methods, rearing of animals and their use has been conducted almost continuously since 1934 by the principle investigator at the University of Notre Dame. Apparatus for the routine rearing and use of small laboratory animals was developed early in the program. Basic units termed the Reyniers Isolator consist essentially of a modified autoclave in which a sterile environment can be maintained indefinitely, supplies introduced and removed at will. With this equipment most of the common laboratory animals were obtained and reared in a sterile environment. The first reproduction of a vertebrate in a sterile environment was reported in 1946 (Reyniers et al.) A large steam sterilized tank (Reyniers 1946) was developed in which the caretaker entered the sterile area through a germicidal trap. The caretaker wore a completely sealed plastic garment so as not to contaminate the sterile environment. This type of apparatus was replaced by the jacket isolator which provides a more reliable and less expensive operation.

Experience at the University of Notre Dame Lobund Laboratory, National Institutes of Health, Walter Reed Army Institute of Research and several laboratories in foreign countries clearly demonstrated that the Reyniers Isolator can be used to maintain a sterile environment indefinitely. In this apparatus, a variety of animals have been reared and a large number of studies made. The apparatus is expensive to procure and operate, because it is essentially heavy industrial equipment. Modifications of the apparatus for specific experiments is difficult and expensive.

In 1957, light weight inexpensive flexible film isolators were described (Trexler and Reynolds). The Lobund Laboratories were reorganized and a new laboratory (Gnotobiotics Laboratory) was established in order to continue the development of the inexpensive equipment. This work was supported by the U. S. Public Health Service Grant #D-631, and by a contract with the Army Surgeon General's Office #DA-49-007-MD-878. The flexible film isolator was developed, and a relatively inexpensive model was produced (less than \$150.00). Operating procedures were developed so that non-professional personnel could maintain animals in this equipment. This type of isolator can be used in any laboratory equipped for sterile operations without the necessity of installing special plumbing. However, the use of the modern high vacuum autoclave reduces the manipulations involved in sterilizing supplies. Such equipment is required for a large scale operation. The application of these methods to the mass production

of rat and mouse colonies was demonstrated together with the means for shipping these animals by common carrier. The entire operation approaches that pattern now used for the production, distribution and use of conventional laboratory animals.

A work shop program supported jointly by this activity and the Institute for Laboratory Animal Resources was held in 1960 and 61. Ten commercial animal breeders and one Government laboratory participated in the program. Six of these now produce germfree animals. As a result, an investigator can order certain species of axenic animals in the same manner as other supplies. This development is of primary importance because a laboratory no longer must engage in the long-term space and time consuming project of producing gnotobiotic animals in order to use them in research.

Intercurrent infection in laboratory animals has complicated the execution of many experiments and tests and equivocated the interpretation of many observations. The number of such infections has been greatly reduced by means of the specific pathogen free laboratory animal program inaugurated at the WRAIR animal colonies in 1954 by Dr. R. D. Henthorne of the U. S. Army Veterinary Corps. The procedures involve the separation of an animal from the microfauna and flora of the parent animal by means of hysterotomy or hysterectomy and the rearing of the animal with varying degrees of isolation. Several attempts were made by various committees established at the Institute for Laboratory Animal Resources and the Animal Care Panel to establish standards for this procedure. No standards could be established because it was not possible to determine the microbic component of the animal even qualitatively due to the difficulty in demonstrating the absence of the large variety of pathogens that are of importance to the health of the laboratory animal, both in the environment of the rearing colony and the tremendous variety of experimental environments. Such standardization is further complicated because the animals then develop an "artificial" microflora from the pasteurized foods, bedding and other supplies, contact with the caretaker and the incompletely sterilized environment.

It seems obvious that practical microbic monitoring could be established if these animals were associated with a few pure cultures. A demonstration of contamination in these cultures would then indicate that the sterile barrier had been broken and there was a danger that a potential pathogen was admitted (Trexler 1959). The development of relatively simple and inexpensive isolator system as mentioned above makes it feasible to consider the maintenance of a sterile barrier for the production of laboratory animals. Such a consideration requires that studies be made as to which pure cultures should be introduced or associated with laboratory animals. A comparison of germfree and conventional animals as now available indicates that the microbic flora does contribute to the health and well-being of the animals.

From the theoretical viewpoint, it seems that the control of the microbic component of the laboratory animal together with its genetics and environment should make it possible to produce much more uniform animals than heretofore available. Heretofore, the microbes associated with animals presented an indeterminable variable. The widespread production of these animals will also make it possible to extend pure culture techniques to the animal for the maintenance of viral and other parasitic cultures. Complete control of microbic contamination



of laboratory animals will make it possible to devise experiments to elucidate the role microbes play in man and domestic animals in health and disease. This aspect combined with the development of more standard and uniform animals represents a fundamental advancement in the development of test media and models for use in experimental biology and medicine.

In association with an improved laboratory animal, similar methods have been devised for handling larger animals of importance to agriculture and more important to the production of vaccines and antisera. Sheep, goats and swine have been reared for varying periods of time in a sterile environment. It is noteworthy that a swine repopulation program has been underway for the past seven years. As a result, it has been possible to eliminate some diseases of economic importance from the swine population. Application of flexible film isolators to this basic procedure, has made it possible to obtain axenic or germfree swine as a by-product of this operation. One commercial laboratory alone produces 1,500 surplus male piglets a year (S.P.F. Laboratories, Inc., Liberty, Indiana). This operation provides a germfree swine as a laboratory animal without the investment required to obtain them from pregnant sows. These animals provide large quantities of sterile products such as tissue, serum, blood and a variety of hormones and enzymes elaborated by a living animal. In this regard, it may be of interest to note that five axenic monkeys were obtained at Lobund during the late thirties. One was maintained for almost a year.

Since the isolator method or closed system has been used so successfully in controlling microbic contamination in the laboratory, it seems desirable to determine if the same principles can be used for the control of microbic contamination in the hospital. In the spring of 1957, this problem was reviewed at length with the Hospital Division at the American Sterilizer Company. Several isolators were designed which were similar to those used to handle small animals, that is, regular glove isolators. Procedures used in an operating room were reviewed by their nursing staff. However, their consulting surgeons felt that the problems of infection and contamination in the operating room were of rather minor importance, and there was no point in continuing this line of development. Later on that year Dr. McElvanney, an Orthopedist at the Northwestern University Medical School and Mr. Snyder, President of Snyder Manufacturing Company, a manufacturer of isolators and protective garments, discussed the problem with me. A system was designed which combined sterile air flow with jackets and flexible film shields to provide a sterile area, but not within a closed system. This project never got beyond the talking stage.

In 1958, Dr. Levenson told me of his interest in this problem and several methods were discussed. Following the Symposium on Gnotobiotic Technology at Lobund, arrangements were made for me to witness an operation at the Walter Reed Army Medical Hospital. An isolator was designed (U. S. Patent #3,051,164) based upon the jacket isolator which had been developed for use in the production of large colonies of laboratory animals. An isolator was built in the Gnotobiotics Laboratory, and taken to Walter Reed Army Institute of Research where it was used for dog surgery. Further design and development has been carried on in collaboration with Dr. Levenson, the team at WRAIR and the Standard Safety Equipment Company of Palatine, Illinois. Four operations have been performed on human patients at the Walter Reed Army General Hospital, and we have co-operated with the Curtiss Wright Company in developing another model.

## PROBLEMS INVESTIGATED

### A. Apparatus and Methods

#### 1. Glove Isolators

The central component of the simplified glove isolator developed as indicated above is a replaceable vinyl plastic bag, rectangular in shape and attached by one edge to a supporting base. This bag contains gloves, air inlet filter and outlet trap and a simple cylindrical sleeve which served as the entry port of the sterile lock. The end of the sleeve is closed off by means of a rubber cap made of the same type of material as the rubber gloves. A series of forty isolators were set up for the rearing of animals, and maintained on a routine basis. This was done to determine their reliability and the problems involved in routine operation. We have demonstrated that a skilled technician can maintain adequate sterility using rather crude equipment. The problem is to develop apparatus and methods that can be used routinely by relatively untrained personnel.

Contamination experience during the period reported (Table 1), verified an earlier conclusion that it is imperative to operate the isolator in such a manner that it is possible to identify the source of contaminations. In this way corrective measures can be taken if the incidence warrants such action.

Two instructive incidents occurred during the period reported. A series of contaminants were experienced in which there was no apparent cause such as faulty operation of the sterile lock or glove punctures. The variety of organisms seemed to indicate air-borne contamination. Eventually, some of the filters were found to be damaged by a thin wire used to hold the filter media in place. The filters were redesigned to substitute a screen or tape for the wire to protect against rough handling. A considerable reduction in the contamination rate was obtained. However, contaminations continued to occur sporadically for six months. It was felt that this was due to the presence of latent contaminants within the isolator. This was confirmed by observations of the isolators housing the rat colony. When the filters were replaced, the rat colony had been reduced to two isolators. Cecal smears from rats in one isolator showed a peculiar micro-organism which could not be cultivated in the laboratory. After three months of study, the organism was cultivated, due more to changes in the organism than to different procedures in the laboratory. The organism was tentatively assigned to the genus *Microcycilus*. The off-spring from the original isolator had been distributed to six other isolators, making a group of seven containing the *Microcycilus*. When the air conditioning system was shut off for spring repairs, the temperature and humidity increased in all the isolators. The seven isolators that contained the *Microcycilus* then developed either a micrococcus or a mold contamination. None of the thirty some other isolators showed such contamination though they were subject to the same environmental conditions. The mold and micrococcus contaminants probably were introduced at the same time as the *Microcycilus* but were in such microenvironments that they remained dormant.

Another series of contaminants occurred in which a source was not readily discernible. Careful checking revealed that these were probably caused by the use of a new filter medium, Medium FM004. This medium was similar to PF-115 with which our original tests had been made. The newer material manufactured

TABLE 1. Primary Contaminations in Isolators. 1 July 61-30 September 62

<u>Date</u>	<u>Probable cause</u>
7/27/61	8 mil film chamber punctured-possible during set-up
8/17/61	Mould *
8/18/61	Diplococcus *
9/ 7/61	Rat escaped cage and chewed isolator chamber
9/ 8/61	Micrococcus *
9/ 8/61	Splinter from support rack punctured top of chamber
9/20/61	Chamber cut by cage lid during set-up
9/28/61	Chamber punctured by cage lid
10/ 6/61	Micrococcus *
10/ 6/61	Mould *
10/23/61	Improper assembly of isolator
11/ 3/61	Gram pos. rod *
11/17/61	Gram pos. rod *
11/17/61	Gram pos. rod *
12/ 5/61	Overage chamber in operation 413 days
12/ 5/61	Inadequate sterilization of food
12/11/61	Inadequate sterilization of food
1/16/62	Yeast *
2/12/62	Micrococcus *
3/12/62	Failure of 8 mil chamber due to excessive air pressure
3/13/62	Improper closure of inner door of sterile lock
3/30/62	Failure of old rigid inner door of sterile lock
4/ 4/62	Gram neg. rods. Door surfaces not properly cleaned
4/ 5/62	Overage plastic chamber
5/15/62	Micrococcus
5/16/62	Yeast
6/ 1/62	Punctured glove in jacket isolator
* Correlated with use of new filter medium in sterilization canisters.	

to use as an air filter, used a similar fiber, but the fiber diameters were more uniform. PF-115 contains a number of rather coarse long fibers. In examining this filter medium after use, it was found that the large fibers apparently contributed to the resiliency of the medium. This resiliency kept the filter media in contact with retaining band on the end of the filter. The heating of the filter and bands resulted in thermal expansion during sterilization. Without the necessary resiliency, there was a small amount of leakage around the air filters. This series of contaminants terminated with the reuse of the PF-115 filter media. Since this filter media was used on the sterilization canisters rather than on the air filters to the isolators no latency was noted. These two incidences have demonstrated the importance of using reliable air filters on isolator systems.

Another series of contaminations occurred which were traced to punctures in the film wall of the chamber. These were caused by pinching the film between the lid of an animal cage in the wall of the retaining box of the isolator. These cages had been used for two years with no difficulty. The damage was caused by a single individual with little mechanical dexterity though with excellent professional training in the handling of animals. Additional padding placed on the retaining box eliminated further punctures.

Considerable difficulty has been experienced with the inner door of the sterile lock on the older isolators. The sterile lock on some models is 12 inches in diameter and on others 18 inches in diameter, in contrast to the 9 inch diameter of the Reyniers Isolators. The larger diameter permits the passage of larger cages and animals. The doors are removable to avoid the loss of floor space required by hinged operation. Difficulties with gaskets and door warpage resulted in their replacement by a flexible rubber cap or a vinyl film cap held on with a rubber strap. Little difficulty was experienced with the caps. One contamination was caused by an incorrectly applied retaining strap and another by an old rubber cap that was no longer resilient.

Several chambers were made in which the expensive shoulder length rubber glove was replaced by a plastic sleeve made of the same material as the wall of the isolator. A wrist length rubber glove was attached to the sleeve by means of a supporting ring and sealing tape. In addition to economy, this arrangement increases the shoulder diameter providing more freedom of movement. The vinyl sleeves originally were made with straight dies resulting in a square section. The corners were weak and eventually leaked but no difficulty was experienced with circular section sleeves.

Three new films were investigated for use as chamber material, polypropylene, Nylon-11, and Tedlar. Four isolators were made of polypropylene. Though we maintained animals for eight months in one isolator, the film appears to be too fragile for routine use. Isolators made from Nylon-11 and Tedlar appeared to be satisfactory. All three types of film are steam sterilizable. The last two films can be stretched readily, so they can be used with the attachment ports in the same way as the vinyl film.

## 2. Introduction of Supplies

Background: Two methods of introducing supplies to flexible film isolators have been developed. (a) Direct attachment to a steam sterilizer. A small steri-

lizer such as used with a Reyniers Isolator can be attached to the flexible film isolator. To protect the plastic film from the high temperature, the isolator is attached to a stainless steel flange approximately 18 inches in diameter and bearing the sterilizer. The amount of supplies that can be sterilized at one time can be greatly increased by loading the entire Reyniers Isolator. After sterilization, a vinyl isolator is attached to the door, and the entire contents of the Reyniers Isolator transferred to the flexible film isolator. The latter isolator serves to transport the supplies to other isolators as needed. The flexible film isolator is much easier to move in the laboratory than is the Reyniers unit. (b) The supplies may be packaged, steam sterilized and the exterior of the package decontaminated in the germicidal lock of the isolator. If the quantity of supplies involved is small, a sealed package can be used provided excess air is removed before sealing to prevent excessive pressure during sterilization. Heat transfer is accomplished by conduction rather than condensation. The moisture necessary for sterilization must be contained within the package or diffuses through the plastic film. Somewhat longer length of sterilization is required because the steam is uncompletely saturated due to entrapped air. Some plastic films such as cellulose, Nylon-6 or 11 and polypropylene transmit considerable steam. However, a vented bag in which a filter such as a cotton plug prevents contamination can be used to process large amounts of supplies. Residual air is removed by a prevacuum drawn in the sterilizer. After sterilization, the vent on the plastic bag is sealed at the base of the filter and the filter can then be cut off the bag. The sealed package can then be passed through a germicidal lock. (Trexler and Reynolds, 1957).

Present Investigation: The use of a Reyniers Isolator for the sterilization of supplies requires the investment in an expensive specialized piece of apparatus and the quantity of supplies that can be processed on a sterilization cycle is limited by the reach of the operators arms in the attached rubber gloves. This can be avoided by using a standard steam sterilizer and packaging the supplies so that they can be handled in a contaminated room.

Earlier, we found that the plastic film package described above was too fragile for routine use in quantity. A metal cylinder or canister was developed to provide a sturdier package.

The supplies to be sterilized are placed in the cylinder and the open end of the cylinder is then covered by Mylar film which is taped securely around the opening to form a drum head. The interior of the container is vented by openings that are covered with glass wool filter pads to prevent contamination. With the small canisters, 12 inches diameter by eighteen inches long, these openings are arranged around the center of the cylinder. Larger cylinders which may be eighteen inches in diameter and thirty-six inches long are usually sterilized in the vertical position and the condensate is removed through a liquid trap.

The sterilization of these canisters is a critical operation as shown by the difficulties experienced by many workers. In order to obtain adequate sterilization, it is necessary to pull a high vacuum initially in the autoclave to remove the entrapped air from the interior of the canister. A vacuum of 20 mm. absolute pressure as obtained in the newer autoclaves seems desirable. The remaining air within the sterilizer chamber must then be displaced by the incoming steam to prevent accumulation in the canister. This can be accomplished provided the vacuum pump operates until the residual air is removed, the vacuum is drawn from

the lower portion of the chamber and the steam is admitted slowly enough to permit stratification. As with other sterile operations, it is necessary to run spore inactivation tests to determine the end point of sterilization. The canisters as used at Lobund have an end point of around thirteen to fifteen minutes, using resistant *B. stearothermophilus* as an indicator. A twenty-five minute sterilization period is used to provide an adequate safety factor.

The entire colony and animals on experiment (1800 total) were supplied by means of the canisters. The only contaminations traced to this method of sterilization and introduction were caused when filter media other than PF-115 was used as mentioned in the previous section. The successful routine maintenance of approximately 60 isolators for six months by this method is a demonstration that it can be used successfully.

Before this method was adequately tested in the laboratory it was recommended to commercial laboratory animal breeders as an alternative to the use of a Reyniers Isolator as a sterilizer. At first, all of the breeders and laboratories that tried the method were unable to successfully maintain animals in a sterile environment. The failures were examined carefully and were found to be caused by either the failure to pull an adequate vacuum or the failure to displace the residual air from the chamber of the sterilizer after the vacuum cycle. We also noted that after about twenty sterilizing cycles the filter becomes matted and impairs the passage of gas. It should be changed before it develops a high resistance and becomes channeled. The above difficulties can be detected by determining the sterilization end point and then at least doubling the sterilization time to provide a safety factor.

The canister method is now used by five commercial laboratory animal breeders, Ohio State University, Northwestern Medical School, Kansas State Medical School, University of Minnesota and the University of Notre Dame. This demonstrates that gnotobiotic animals can be reared and used upon a routine basis without the necessity of investing in expensive special apparatus.

### 3. Removal of Specimens

Background: Three methods have been used to remove specimens; (1) the sterile lock, (2) a liquid germicidal trap and (3) a sealable polyethylene sleeve. Specimens can be placed in the sterile lock after the supplies are introduced or following a transfer from another isolator. Such a sterile lock usually contains a residual amount of germicide, this is hazardous when microbiological specimens are removed. These specimens are usually protected by being placed in a bottle with a secure cap or rubber stopper.

The removal of specimens and waste through a germicidal bath was described by Küster in 1915. It is also used by Gustafsson (1948). However, liquid traps are not routinely used with flexible film isolators since these isolators are frequently moved and may be stacked on shelves. This movement could spill the germicide. The use of a sealable polyethylene sleeve was described as an adjunct to the original flexible film isolator (Trexler and Reynolds, 1957) though it has not been used beyond the original study.

Present Investigation: The removal of specimens through the sterile lock can be accomplished when the isolator is entered for other purposes if the animals

are maintained on an experiment that requires few specimens or if the animals are being reared. A more rapid method is required for frequent sampling. This situation arose at both the Gnotobiotics Laboratory at Lobund and at WRAIR.

The sealable polyethylene tubing method was simplified for a more routine use. A short section of a 2" plastic pipe is sealed through the wall of the isolator. A rubber stopper serves as the inner door and a three or four foot length of flat polyethylene tubing is attached to the outside of the 2" pipe. This is sterilized by collapsing the tube around a peracetate acid spray nozzle. The interior of the sleeve and the rubber stopper are treated with peracetate acid spray. As the sleeve is extended and the peracetate acid completely covers the inner surfaces of the sleeve. Nozzle is removed and a cotton plug sterilized in the oven introduced to protect the opening. After a holding period of twenty minutes, the stopper on the inside of the isolator is removed and the air pressure within the isolator removes the peracetate acid by ventilating the sleeve. Specimens to be removed are put in the distal end of the sleeve, and the sleeve sealed off by means of a flat ribbon sealer. The specimens encased in the plastic bag are then removed by cutting through the seal. This is repeated until the flexible tubing is used up. The old tubing can be removed and a fresh piece put on.

Specimens in test tubes have been removed by simply removing the rubber stopper from the one inch tygon tube through which spray is introduced into the isolator. The operation must be done reasonably fast to prevent deflation of the isolator. After the specimen is removed, the end of the rubber stopper is dipped in germicide and replaced in the tube. It is theoretically possible to contaminate the isolator because as air leaves a tube or duct back eddies may occur along the walls so that contaminating dust particles can creep upstream. About two dozen specimens were removed from the isolator without detecting contamination. It is recommended that this procedure be limited to isolators that are used for short term experiments, or that it be used near the termination of a study in an isolator. This procedure can simplify the use of isolators because frequently at the termination of an experiment a number of specimens must be removed over a period of a few hours as the animals are sacrificed.

#### 4. The Transfer of Large Specimens

Background: The size of the specimens that could be transferred from one isolator to another without contamination has been limited to the size of the sterile lock. With the Reyniers system this is 9" in diameter, with the flexible film isolator this has been increased to 18 inches in diameter. We have found the eighteen inch opening to be more satisfactory because it will pass practically all of the cages used to maintain rats and mice. Large animals such as sheep and lambs have been transferred through this opening after tranquilization. We have found it difficult to increase the diameter of the opening and still obtain a Freon tight seal on the door. Larger doors could be made using mechanical seals such as on autoclaves, but these are expensive and heavy.

Present Investigation: It seems possible that larger specimens could be transferred from one isolator to another using the sealing properties of plastic

rather than depending upon a mechanical door. The split seam transfer method (Trexler, 1959) could be used, however, it is difficult to reseal a large opening because the flat plastic film must be positioned and held during the sealing process.

Another method was devised using a clamp type sealer. A flare must be provided around the edges to be sealed. Two flares on adjacent ends of the isolators can be sealed together and the interior of the sealed portion sterilized with peracetic acid. Ethylene oxide-Freon mixture could also be used. The joining of the two isolators and the sterilization of the joint is accomplished from the exterior of the isolator. After the sterilization cycle, the germicidal gas can be flushed out and a passage way cut through the two adjacent ends of the isolators. The cut is made down the center of the "trough" sealed to the wall on the inside of the receiving isolator. After the specimen has passed through, the opening in the receiving isolator can be sealed by means of a clamped type sealer after the edges of the cut "trough" are brought together.

If the specimens can be introduced through one end of an isolator and removed through the other, this procedure can be carried on indefinitely. With this method it seems likely that large cages containing for example full-sized cattle can be moved from one isolator to another, or the animal transferred from one cage to another. The cages can be handled by means of an overhead trolley, supported by an A frame. These supports can readily be passed through the walls of the isolator

An old patient isolator (see below) was used for the developmental study. This method of transfer will make it possible to transfer a patient from one isolator to another without the risk of contamination from the environment. Considerable development will be needed to provide a procedure for handling the patient.

The method as developed will make it possible to transfer large animals (sheep, cattle, swine) from one isolator to another. A dependable long-term maintenance of gnotobiotic animals requires transfer for repair and cleaning of cages. A large animal maintained for years would be an excellent source of sterile or immune serum. Such sera will have fewer cross reaction because of the absence of a contaminating flora. Some agents can be grown in the environment or could be used to provide a long-term infection in the host and a continuous antibody stimulant.

Patent application is being made on the above device through the University of Notre Dame.



## 5. The Use of Neutralized Peracetic Acid

**Background:** Peracetic acid has been used at Lobund since about 1949 (Greenspan, Johnsen and Trexler 1955) without finding specifically resistant organisms. A 2% solution will inactivate the most resistant bacteria and mold spores in about thirty seconds in the liquid phase, and about 12½ minutes in the vapor phase.

**Present Investigation:** Peracetic acid because of its volatility has a pungent odor and is irritating to the eyes and when inhaled. Normal usage within the sterile lock apparently does not affect the animals. Peracetic acid should not be used with patients or perhaps animals on some types of experiments. Non-volatile salts are equally germicidal but less stable than the acid. When the peracetic acid is neutralized, oxygen may be evolved with loss of germicidal property.

A series of studies were made in an attempt to improve the stability of neutralized solutions. The effect of raising the pH of peracetic acid solutions by adding 9N NaOH, upon the stability of peracetic acid solutions is reported in Table 2. The addition of Calgon for stabilization is recommended by the manufacturer. Solutions within a pH range of 6.0 to 7.0 appear to be usable both because of reduction in odor and stability.

The addition of NaOH solutions must be made carefully and the pH determined to avoid the decomposition attending higher pHs. This problem can be avoided by diluting the acid in a buffer solution rather than distilled water. A 1M solution of potassium dibasic phosphate will provide a solution of pH 6.75  $\pm$  0.1 and appears somewhat more stable than solutions with Calgon.

The sporicidal activity of the acid and buffered solution were compared in suspensions of *Bacillus stearothermophilus* spores 5000 per ml. (Table 3). The comparatively dilute suspensions of spores were used because these solutions are to be used to sterilize clean surfaces. The activity of the buffered solution in the presence of calf serum is reported in Table 4.

The buffered sporicidal solution can be easily prepared by diluting the stock peracetic acid with 1M dibasic potassium phosphate. Peracetic acid is ordinarily used in 2% solution primarily because of its vapor phase activity. However in the liquid phase it is an effective sporicide in 0.25% solutions with a holding period of 60 seconds. This solution can be used for sterilizing surfaces rapidly in the laboratory and possibly in the operating room. A more thorough study appears warranted using pathogens and accepted procedures.

**TABLE 2**  
**Stability of Peracetic Acid Solutions**

pH	0 Min.	15 Min.	30 Min.	1 Hour	4 Hrs.	8 Hrs.	24 Hrs.
5.5	2%	1.8%	1.6%	1.6%	1.5%	1.2%	0.90%
6.0	2%	1.7%	1.6%	1.6% (1.8%)	1.4% (1.6%)	1.3% (1.54%)	0.81%
7.0	2%	1.1%	1.1%	0.93% (1.75%)	0.66% (1.55%)	0.66% (1.36%)	0.60%
8.0	2%	0.82%	0.75%	0.60% (1.10%)	0.49% (0.96%)	0.47% (0.85%)	0.45%

Note: Initial conc of  $\text{CH}_3\text{COOOH}$ -2% at pH 2.2.  
 % =  $\text{CH}_3\text{COOOH}$  no Calgon  
 (% =  $\text{CH}_3\text{COOOH}$  with Calgon)

**TABLE 3**  
**Sporicidal Activity of Acid and Buffered P. A. Solutions**  
**% Peracetic Acid**

	2.0%	1.5%	1.0%	0.5%	0.25%	0.1%	0.01%	0.001%	0.0001%	0.00001%
<u>I. pH = 2.2</u>										
30 Sec.						5-	4+ 1-	4+ 1-		
60 Sec.						5-	2+ 3-	5+		
300 Sec.						5-	5-	5+		
<u>II. pH = 6.75</u>										
30 Sec.						1+ 9-	9+ 1-	5+	5+	
60 Sec.	5-	5-	5-	10-	5-	2+ 13-	7+ 3-	10+	10+	4+ 1-
150 Sec.	5-	5-	5-	5-						
300 Sec.	5-	5-	5-	10-	5-	15-	5+ 5-	9+ 1-	9+ 1-	5+

Note: pH adjusted with molar  $\text{K}_2\text{HPO}_4$  Solution Sporocidal activity determined against a suspension of *Bacillus stearothermophilus* spores  $5 \times 10^3/\text{ml}$ .

TABLE 4 .  
Sporicidal Activity of Buffered P. A. Solutions in the Presence of Serum

% P. A. with buffer	2.0		1.0	0.5	0.25	0.2						0.1					
	4.7	---	---	---	---	10	7.5	6.0	5.0	4.0	---	15.0	12.0	10.0	9.0	7.5	5.0
% Calf Blood																	---
60 Seconds	2-	2-	2-	2-	2-	3+	3+	6-	5- 2+	6-	9- 1+	3+	3+	3+	3+	3+	3- 1+
150 Seconds	2-	2-	2-	2-	2-	3+	3+	5- 1+	8-	6-	9-	3+	3+	3+	3+	3+	14-
300 Seconds	2-	2-	2-	2-	2-	3+	3+	6-	8-	6-	9-	3+	3+	3+	3+	3+	14

## B. Laboratory Animal Production and Distribution

**Background:** In the past, investigators had to obtain germfree animals from some institution that possessed a colony with a surplus of animals, or raise their own. A large size colony is required to give the proper number within the age and sex range required for an experiment. In addition, the production of germfree animals requires that a surplus number be available as standby in case of an accidental contamination. For these reasons, it seems obvious that a more satisfactory operation could be obtained at least for the majority of applications if animals were reared in commercial establishments which could then supply animals to a number of using laboratories. This is the usual production and distribution pattern of laboratory animals. The development of flexible film isolators holds promise for providing an operation that is economically feasible.

### Present Investigation:

#### 1. Production Methods

The labor involved in maintaining animals in the isolators described above is considerably greater than in the conventional open animal room; due to the small number of cages that can be handled through one pair of gloves, because of the fixed armhole attachment at the chamber wall. In addition, the small storage space in the isolator requires that frequent entries of supplies and the removal of waste materials be made. The development of jacket isolators, see Figure 1, greatly increases the area that can be serviced by personnel on a single entry to the isolator. This area can be further extended by using a mechanical device for handling the animal cages. Figure 1a. A series of jacket isolators have been used for the production of gnotobiotic rat and mouse colonies and the maintenance of gnotobiotic goats and sheep during the period studied primarily to determine the feasibility of the operation.

An analysis of the contaminations experienced in jacket isolators, shows that one was caused by failure of the air supply. The jacket deflated for an hour and contamination undoubtedly entered through the air outlet system. Another contamination occurred when the film on the floor of the isolator was punctured, probably by a piece of grain. The floor arrangement of this jacket isolator was identical with that on the glove isolators. It is apparent that because the greater amount of work and heavier cages used in the jacket isolator, a solid base is more satisfactory than the flexible film. Such bases have been made from both glass fiber reinforced polyester and sheet metal. The remaining contaminations were due to failure of the jacket. The first jackets were made of flexible film vinyl, the same as the glove isolators. These were then replaced by jackets made of vinyl coated cotton cloth. These were sturdier, but it still appears necessary to check the jacket for leaks after every ten entries and then replace the jacket as needed. Replacement can be readily accomplished using the germicidal moat that surrounds the manhole and a cover to seal-off the jacket from the isolator.

At the present time, all of the food used to rear gnotobiotic animals is sterilized by the laboratories that operate the colonies. Steam is routinely used to obtain sterilization, and the various diets are fortified so that they will have adequate nutriment for the animals. It is no longer necessary to

prepare the special diets. Two companies provide open formula rations made to specifications of the users, General Biochemicals, Inc. and Nutritional Biochemicals, Inc. One company - Ralston Purina Company - provides a closed formula ration 5010C which is comparatively inexpensive. This diet has been used in these studies.

Several companies now offer animal rations that have been pasteurized but not sterilized. These rations are suitable for the rearing of specific pathogen free animals. The production of sterile rations is being considered by several companies.

The production and use of gnotobiotics would be greatly simplified if rations could be reliably sterilized and delivered to the using laboratory in a package that can be readily introduced into the isolator.

At the present time water is steam sterilized in two-liter Square-Pak Flasks (American Sterilizer Company) prior to introduction into the isolator. This operation requires considerable labor for the maintenance of either large colonies or large animals. Several years ago we built and tested a 20 and a 40 gallon water sterilizer attached to an isolator. A reliable operation required a rather complex valving arrangement to reduce the danger of contamination through a valve leak. A simple system for the continuous sterilization of water was designed using three standard heat exchangers. It was not possible to test the unit due to the early termination of this grant. However, the heat-exchanger system provides the simplest design for the sterilization and introduction of large quantities of water into the isolator.

The above methods appear to be capable of expansion to obtain greater efficiency. Further expansion was not justified because the animals produced could not be used efficiently by either the staff at LOBUND or WRAIR. Several attempts were made to determine a useful production cost figure. Both the facilities and personnel at the University are more compatible with research than animal production operations so it was not possible to arrive at costs that would reflect the commercial value of the animals. Such figures can be obtained now from the price lists of those firms that completed the Workshop Program mentioned above and are now in production.

The Workshop Program as mentioned above was inaugurated in part to determine if isolator methods could be used for the production of the so-called SPF animals. These animals are currently derived from stock that has been obtained through caesarian section and maintained with varying degrees of protection. Access to production rooms is limited to personnel that have scrubbed, donned sterile garments and masks. Mr. A. R. Schmidt of Schmidt and Company, Milwaukee, Wisc., is building new quarters and installing isolators to mass produce SPF animals at prices competitive with the other systems. This operation provides a means of testing the design of isolators for the mass production of animals without requiring that gnotobiotics be produced and used found for them. With the experience obtained in the design and development of a variety of isolators systems for maintaining gnotobiotic animals, it seems likely that apparatus can be designed for the production of gnotobiotics in mass quantity based upon experience with the SPF animals. Dr. Foster of the Charles River Breeding Laboratories is also cooperating in this program.

There seems to be obvious advantages in the development of a system for controlling the environment in which animals are raised, so that these animals can be produced in a sterile environment, or one including pure cultures, or an environment in which little control is exercised over the micro-organisms originally associated with the animals, but that subsequent contamination particularly from the caretaker is avoided.

With the present methods of producing SPF animals, contaminating micro-organisms are added to the microfauna and microflora by the caretakers, pasteurized supplies and errors in quarantine procedures. There does not appear to be a practical microbiological monitoring system for routinely checking for the presence of the great variety of pathogens that can interfere with many of the studies for which laboratory animals are used. The need for such a routine monitoring for pathogens can be by-passed through the use of an isolator system. The use of isolators relegates the search for the great variety of potential pathogens to an occasional study of the "nucleus" stock from which the breeders for the colony are taken. These studies must be exhaustive. Routine microbiological monitoring is designed to detect the presence of the common microbes and hence the procedure is relatively simple. The routine test serves to detect a break in the isolation barrier. The detection of contaminants indicates the danger that a pathogen, as yet undetected may have gained entrance.

The isolator system for the production animals is too expensive at present to consider for the majority of laboratory animal uses. However, it seems likely that the cost of this apparatus will continue to be reduced.

## 2. The Transportation of Animals

Two methods of transporting animals have been devised, and have been studied under contract DA-49-007-MD-878 and the grant herein reported. In the first method animals were transported in a private automobile, usually an air conditioned station wagon, in which the isolator was placed and air supplied by a blower operated from the automobile battery. Blowers operating on the battery voltage were used as well as those that operated on 110V AC provided by a converter, which in turn operated from the electrical system of the vehicle.

In the second method, an isolator was placed in a protective box made of wood and metal or glass fiber reinforced polyester. Air was furnished by a battery operated blower, see Figure 2. During the winter months the thermostat was incorporated in the electrical circuit, so that the speed of the blower would be reduced when the temperature of the outside air was 50° or less, to prevent chilling of the animals. As can be seen from Table 5, animals have been shipped as far away as Japan and Europe. Apparently, gnotobiotic animals are not a great deal more susceptible to shipping stress than are conventional animals. This point was not investigated quantitatively, because it was felt that such a study is not warranted at the present stage of development.

Simpler methods of shipping animals have been described (Reynier and Sacksteder). These methods have not been used in the present investigation because it was felt desirable to maintain the animals during transportation with the same degree of security as in the laboratory, and provide an adequate air supply in order to reduce the stress encountered. There are several ways of reducing the cost of shipment via common carrier, one approach is shown in Figure 3. The inner door of the sterile lock has been removed and replaced by flexible film. After the shipper is attached to the receiving isolator the

There seems to be obvious advantages in the development of a system for controlling the environment in which animals are raised, so that these animals can be produced in a sterile environment, or one including pure cultures, or an environment in which little control is exercised over the micro-organisms originally associated with the animals, but that subsequent contamination particularly from the caretaker is avoided.

With the present methods of producing SPF animals, contaminating micro-organisms are added to the microfauna and microflora by the caretakers, pasteurized supplies and errors in quarantine procedures. There does not appear to be a practical microbiological monitoring system for routinely checking for the presence of the great variety of pathogens that can interfere with many of the studies for which laboratory animals are used. The need for such a routine monitoring for pathogens can be by-passed through the use of an isolator system. The use of isolators relegates the search for the great variety of potential pathogens to an occasional study of the "nucleus" stock from which the breeders for the colony are taken. These studies must be exhaustive. Routine microbiological monitoring is designed to detect the presence of the common microbes and hence the procedure is relatively simple. The routine test serves to detect a break in the isolation barrier. The detection of contaminants indicates the danger that a pathogen, as yet undetected may have gained entrance.

The isolator system for the production animals is too expensive at present to consider for the majority of laboratory animal uses. However, it seems likely that the cost of this apparatus will continue to be reduced.

## 2. The Transportation of Animals

Two methods of transporting animals have been devised, and have been studied under contract DA-49-007-MD-878 and the grant herein reported. In the first method animals were transported in a private automobile, usually an air conditioned station wagon, in which the isolator was placed and air supplied by a blower operated from the automobile battery. Blowers operating on the battery voltage were used as well as those that operated on 110V AC provided by a converter, which in turn operated from the electrical system of the vehicle.

In the second method, an isolator was placed in a protective box made of wood and metal or glass fiber reinforced polyester. Air was furnished by a battery operated blower, see Figure 2. During the winter months the thermostat was incorporated in the electrical circuit, so that the speed of the blower would be reduced when the temperature of the outside air was 50° or less, to prevent chilling of the animals. As can be seen from Table 5, animals have been shipped as far away as Japan and Europe. Apparently, gnotobiotic animals are not a great deal more susceptible to shipping stress than are conventional animals. This point was not investigated quantitatively, because it was felt that such a study is not warranted at the present stage of development.

Simpler methods of shipping animals have been described (Reynier and Sacksteder). These methods have not been used in the present investigation because it was felt desirable to maintain the animals during transportation with the same degree of security as in the laboratory, and provide an adequate air supply in order to reduce the stress encountered. There are several ways of reducing the cost of shipment via common carrier, one approach is shown in Figure 3. The inner door of the sterile lock has been removed and replaced by flexible film. After the shipper is attached to the receiving isolator the

flexible film is cut and the animals introduced. The flexible film chamber can then be discarded or a new piece of film sealed in place.

This type of isolator can be made small enough to contain a single animal cage. Gloves are not necessary because the cage can be moved by manipulation through the flexible film walls. Two or three cages can be so handled in one isolator. This reduction in size makes possible to reduce the size of the blower, air filter and the weight of the battery, thus making it possible to design the unit for specific sized shipments.

### 3. Studies with Gnotobiotic Laboratory Animals.

The usefulness of gnotobiotics has been questioned upon the basis that they introduce another variable, since they do not possess "normal flora", and man and the principle domestic animals to whom observations are extrapolated do possess such a flora. This position is untenable primarily because the so-called normal flora is not uniform even within a species, and the relationship between the flora in different species, particularly among animals that live with such greatly different sanitary environments as man and the laboratory animal must depend upon specific effects. With the development of antibiotics and other treatments, certainly the flora of man has been greatly modified. The specific effects of the various components of the microbic flora associated with man and other animals should be useful in the treatment of disease and the maintenance of health. They also should assist in the production of more uniform and controlled laboratory animals.

#### a. The Distended Cecum in the Axenic Animal.

The occurrence of a distended cecum is a most obvious difference between axenic and conventional rodents. This distended cecum is a frequent cause of death (Ward and Trexler, 1958). Inbred rats and mice frequently die because the cecum becomes twisted or may rupture, this occurs most frequently during pregnancy or lactation or upon aging. Observations made at Lobund indicate that while the cecum of the conventional animal may decrease in size with age, that of the axenic animal increases.

Over the years a number of studies have been made at Lobund in an attempt to reduce the cecum size either through the administration of drugs or by changes in diet. No practical solution to the problem has been found to date for the rat and mouse. A search was made for organisms that would reduce the cecum and at the same time could be used as a substitute for the indeterminable flora of the SPF animal. For the past eight years, the cecal size of animals that were accidentally contaminated at Lobund have been measured in my laboratory. No contaminant was found that reduced the cecal size more than 30% without producing disease in the animal. The microorganisms that are readily isolated from cecal contents, lactobacillus, coli, streptococcus, proteus, et cetera, have been associated with mice without producing a significant reduction in the cecum. An association composed of a variety of microorganisms will accomplish such a reduction and the variety is not specifically defined. Animals that are withdrawn from the isolator and placed in an environment in which the microbic population is not controlled invariably show an 80 to 90% reduction within 48 hours. Thus, the environment in which the animal is placed may contain conventional animals or could be a



clean office or laboratory, and about the same reduction will be produced.

Dr. Schaedler of the Rockefeller Institute sent four pure cultures (normal and slow lactose fermenting *Lactobacillus*, *Proteus* and *Enterococcus*) from the NCS colony (Dubos and Schaedler, 1960) and a mouse coli strain. These were associated with axenic mice by placing culture of the food of animals that were fasting overnight. The microbe's presence in the cecum determined microbiologically at necropsy. The pure cultures did not reduce the cecum to within the normal size range though the mixture of cultures did.

Most observations made on laboratory animals are eventually extrapolated to man. For this reason, the cecal reducing organism was sought from a human flora. A group of mice were associated with a fecal specimen from a healthy child. This fecal specimen was introduced into an isolator containing a gnotobiotic mouse colony, within 48 hours the cecal size was within the range of conventional animals. This flora was studied in an attempt to find the responsible microorganisms. After a few months, it was apparent that the cecal size of the isolator reared animals was increasing in size. After six months, the cecum was 60% the size of an axenic animal. For this reason this colony was discarded. A similar control colony which had been started by associating the animals with cecal specimens from mice that had never been caesarian derived, exhibited size comparable to the conventional animal. A series of pure cultures were obtained from these ceca. None of these pure cultures would produce more than 30% reduction in the cecal size. A standard test period of 48 hours was used and an attempt made to find the responsible organisms through a progressive simplification of the flora (Skelly, Trexler and Tanami, 1962). Two strains of *Bacteroides* or a pure culture of *Clostridium difficile* would produce a normal cecal size within 48 hours after being administered to the animal either in food or the drinking water (Table 5). Similar results were obtained from the same species purchased from the American Type Culture Collection #90556. Several other species of the genus were tried without success.

This species has been associated with gnotobiotic mice for three generations of the mice without altering its cecal reducing properties. Dr. Heinz Bauer, working at the Walter Reed Army Institute of Research, has examined these animals histologically and found that the lymph nodes of the gut tract were not stimulated, however, those of the pharyngeal region were. This would indicate that perhaps the organism produces some sort of an infection in the mouth region but not in the intestinal region. Since the organism does not impair rate of reproduction or growth of these animals, at least on gross observation, the use of this microorganism for the production of colonies of animals to be used in biological testing could be considered. In over 400 contaminants found in Lobund Institute, less than 4% were obligatory anaerobes, this would indicate that the presence of a *Clostridium* species would not greatly complicate microbiological testing. The culture media used should be capable of growing aerobic but not obligatory anaerobes.

A series of gnotobiotic Fischer rats were associated with cultures of *C. difficile* in the same manner as with mice. The ceca of these rats were about the same size 15%± as those of inbred mice we have examined (C57, C<sub>3</sub>H and Balb C). The cecal size was considerably reduced (Table 6) and approached the normal range. No attempt was made to isolate organisms from the rat flora.

TABLE 5  
Cecal Size of ND2 Gnotobiotic Male Mice 30-60 Days Old  
Associated with *Clostridium difficile*

Status	Number of animals	Mean cecum size * (range)
Axenic	17	$7.1 \pm 0.88$ (5.6 - 8.9)
Conventional	26	$3.3 \pm 0.80$ (2.3 - 4.9)
Gnotobiotics 2 days after culture administered in diet	8	$3.7 \pm 0.89$ (2.3 - 4.7)
Gnotobiotics 4 days after culture administered in diet	9	$3.4 \pm 0.50$ (2.8 - 4.5)
Gnotobiotics (30 da. old) raised from dams associated with <i>Clostridium</i>	11	$4.1 \pm 0.53$ (3.2 - 5.2)
Gnotobiotics (50 da. old) raised from dams associated with <i>Clostridium</i>	7	$3.9 \pm 0.50$ (3.0 - 4.4)

\* Cecal size expressed as percent of total body weight.

TABLE 6  
Cecal Size of Gnotobiotic Fischer Rats Associated  
With *Clostridium difficile*

Status	Number of animals	Mean cecum size * (range)
Axenic	7	$16.7 \pm 1.29$ (15.1 - 18.2)
Conventional	17	$3.2 \pm 0.10$ (2.5 - 4.0)
Gnotobiotics 10 days after culture administered in the diet	35	$5.2 \pm 0.55$ (3.6 - 6.6)

\* Cecal size expressed as percent of total body weight.

b. A Comparison of the Protection by Pertussis Vaccine to Intracerebral Challenge of *Bordetella Pertussis* in SPF and Axenic Mice.

The potential value of gnotobiotic mice for biological testing was discussed with Dr. C. G. Culbertson, Director of Biological Research, Eli Lilly and Company. The absence of a variable microflora should increase their potential value by providing a more uniform animal but any anomalies of these animals would decrease their usefulness. The mouse test system used to evaluate Pertussis Vaccine gives variable results with some batches of mice due possibly to the presence of endotoxin produced by the intestinal flora. The use of gnotobiotic mice would indicate whether or not other factors were involved.

The LD50 dose of the challenge culture of *Bordetella pertussis* was determined for a strain of ICR mice that were available from the colonies maintained in sterile isolators. The same strain was available from a commercial (A. R. Schmidt and Co.) colony that had been caesarian derived and is maintained in aseptic isolation. This strain of mouse had not been used for pertussis testing but was the only strain available as both axenic and SPF animals. There was no reason to use mice reared in non-isolated rooms since it has been established that they will not give reliable results. Preliminary trials were made to determine the dose range of vaccine as well as challenge culture.

A test run to compare the protective effect of the vaccine in the gnotobiotic and SPF mice was made using mice 12-14 gms. The mice were immunized with Lilly Pertussis Vaccine Fluid diluted 1:13, 1:65 and 1:325 with sterile 0.85% saline. Three groups of 16 mice each of both gnotobiotic and SPF strains were injected IP with 0.5 cc of the respective dilutions. Two weeks after immunization each group was given an intracerebral injection containing 0.03 cc of the challenge culture (100,000 cells). Both strains of mice were kept in isolators. The results are reported in Table 7. Both groups exhibited an exceptional uniform response and showed no significant difference. Apparently gnotobiotic mice can be used in this biological test without prejudice.

c. Collaborative Study with the Indiana University Medical Center, Indianapolis, Ind.

A collaborative program was set up with Professor Dwain N. Walcher, M. D. and James Schaffer, M. D., Department of Pediatrics, Indiana University Medical Center. The purpose of this program was to use surplus germfree mice to investigate their suitability for studies of infant diarrhea and to explore the advisability of placing premature infants in sterile isolators.

This study seemed warranted because there were occasions when larger number of mice were available from our production operation. Isolators for protecting an infant seemed to offer no problem from the design viewpoint. It was felt worthwhile exploring their possible applications. Drs. Walcher and Schaffer were of the opinion that infection contributed to the mortality rate of very small premature infants.

From experience in contaminating germfree animals and the frequent complications that result, it seemed advisable to consider various aspects of maintaining an infant in a sterile environment. It had been observed at Lobund and elsewhere that guinea pigs reared in a sterile environment and accidentally contaminated with an aerobic spore former exhibited from 60 to 90% mortality.

TABLE 7

Immunization of SPF and Gnotobiotic ICR Mice with Various Dilutions of Lilly Fluid  
Pertussis Vaccine Followed 14 Days Later with Challenge Culture  
(100,000 cells Intracerebrally)

Group	Conventional	Axenic
1:13 dil. of vaccine + 100,000 cells challenge	S/T = 13/16*	S/T = 12/16*
1:65 dil. of vaccine + 100,000 cells challenge	S/T = 7/16*	S/T = 7/16*
1:325 dil. of vaccine + 100,000 cells challenge	S/T = 2/16*	S/T = 1/16*
Challenge culture control, 100,000 cells challenge	S/T = 0/10*	S/T = 0/10*
No vaccine, 2000 cells challenge culture	S/T = 5/10*	S/T = 4/10*
No vaccine, 400 cells of challenge culture	S/T = 8/10*	S/T = 7/10*
No vaccine, 80 cells of challenge culture	S/T = 9/10*	S/T = 10/10*

\*S/T = survivors/total number of mice 14 days after administration of challenge culture.

TABLE 8

Mortality of Animals Inoculated with Coxsackie B-Group Virus

Virus Dose	Germfree	Conventional	Germfree and Staph. Monocontamination
10 <sup>-8.8</sup>	10/48	0/48	0/48
10 <sup>-7.8</sup>	10/48	3/48	0/48
10 <sup>-6.8</sup>	7/27	15/48	0/48
Totals	27/123	18/144	0/144

Numerator = Total deaths during experimental interval of 14 days.

Denominator = Total animals in the experimental group.

The aerobic spore formers ordinarily occur in large numbers in the gut tract of the conventional animal. We have observed high mortality of young mice when removed from the sterile environment and placed on clean cages with sterile food and water. The same mice placed on non-sterile food and water exhibited a transient diarrhea with no mortality. Inbred mice such as C3H and C57 exhibited a high mortality when removed from sterile environment and placed in a clean cage with non-sterile food and bedding. This mortality was eliminated if they were placed in a cage that had been dirtied by conventional animals. These observations indicate the importance of a mixed or balanced flora for the health of the animal. An environment with a few species of microbes could be very hazardous.

It seems obvious that an infant would not be obtained without some microbic contamination. If the infant were placed in a completely sterile environment their flora must be compatible with health. Drs. Walcher and Schaffer felt that more information was required regarding the microbial environment necessary for the health of infants. As a preliminary investigation, 24 and 48 hour fecal specimens from three groups of infants were associated with an isolator containing suckling mice. One group came from an infant that was normal born and breast fed. The next group consisted of infants that were caesarean born but breast fed, and the third group, infants that are caesarean born and bottle fed. On several occasions the suckling and adult mice in the group of bottle fed infants exhibited high mortality. A variety of bacteria were found and some indications of the presence of virus. These observations were difficult to repeat and it was felt advisable to discontinue this work until more information was obtained concerning the reactions of gnotobiotic mice to the presence of various microorganisms in the infant flora.

A series of studies were made to determine the response of germfree animals to an experimental contamination with Coxsackie virus. Twenty-four hour suckling mice were inoculated by the intraperitoneal route at Indiana Medical Center. The summary of some of the observations is shown in Table 8. It is interesting that an accidental contamination with staphylococcus organism appeared to induce resistance to the Coxsackie virus. This indicates that perhaps contamination of the young animal or infant with microorganisms is of considerable significance (Schaffer et al., 1963).

#### d. The Removal of Gnotobiotic Mice from a Sterile Environment.

Approximately 400 accidental contaminations have occurred at Lobund during the past 10 years. Aerobic sporeformer contamination of guinea pigs usually produced a fatal disease but obvious signs of disease were rare in other contaminations. The incidence of disease following accidental contamination in the rat and mouse colonies was less than 5%. However two of the four contaminations that occurred during the first two years of operation at Indiana Medical Center resulted in fatal infections. This appears to indicate a difference of the microflora of the rooms in which the isolators were kept.

In order to evaluate the effect of microbic flora a series of axenic mice were removed from the isolator and placed in a hood in one of the laboratories. The mice were put in clean cages with either sterile or non-sterile food, bedding and water. The results are reported in Table 9. Apparently mice that are older are more resistant than younger mice. Inbred strains are more susceptible than

non-inbred (ND strains). All mice that died had the same symptoms, an acute diarrhea with some bloody stools. Upon necropsy all of the animals had an empty intestinal tract and a cecum that was smaller than the conventional animal.

An attempt was made to find the microorganisms responsible for the infection. The cecum was removed from a moribund animal and the contents placed on the food of axenic animals. Deaths occurred as early as 18 hours. Two clostridia were isolated along with a streptococcus and a diplococcus. The disease was not produced by any of the pure cultures associated with the mice in the susceptible age range within the isolator. Several combinations of organisms were also tried without success.

This line of investigation should be continued in order to determine the causative organisms. All of the young mice will survive if placed in a cage in which a "balanced" flora has been established by conventional animals. It would be useful to find a pure culture that would have the same protection.

TABLE 9

Removal of Axenic Mice From the Isolator.

Strain	# animals	age	supplies	deaths
ND-4	40	4-5 wks	non-sterile	none in 4 days
ND-3	57	4-5 wks	non-sterile	none in 4 days
ND-2	21	4-5 wks	sterile	95% in 4 days
ND-2/3	13	4-5 wks	sterile	90% in 4 days
ND-4	19	8-10 wks	sterile	7% in 4 days
ND-4	44	4-6 wks	sterile	89% in 4 days
ND-2	33	4-5 wks	sterile	92% in 4 days
ND-2	22	8-13 wks	sterile	12% in 4 days
C3H/HeN	20	4-6 wks	non-sterile	100% in 3 days
C3H/HeN	20	4-6 wks	sterile	100% in 3 days
C3H/HeN	20	4-6 wks	non-sterile	none in 7 days
+ conventional feces				

#### 4. Isolators for use with large animals and man.

##### a. Large animals isolators

Background: Flexible film isolators have been developed at Lobund Laboratories for use in rearing swine and sheep (Trexler and Smith, 1960). Manipulation within these isolators can be accomplished either through arm length rubber gloves or jackets. The animals have been housed in metal cages in much the same manner as with small laboratory animals (Fig. 4).

##### Present investigation

Studies of isolators for use with large animals were continued because the basic problem of manipulation, introduction and removal of supplies, and transfer from one isolator to another is similar to that presented by the patient isolator.

Studies with small laboratory animals indicate that a gnotobiotic or axenic animal produces an antiserum of higher specificity than a corresponding animal with an uncontrolled microflora. The large animal could be used for producing quantities of such antiserum as well as provide the source of quantities of sterile serum and other products. It is conceivable upon the basis of observations made with mice and Coxsackie virus that the large animal could be associated with an organism which serves as a continuous inoculum to provide quantities of specific antiserum or a source of vaccine production.

As Visiting Professor at Ohio State University assistance was given in the development of a series of isolators with a stainless steel bottom (Fig. 5). The stainless steel base varies in depth from 12" to 24" and provides the bottom portion of the cage for housing large animals (Meyer et al., 1963). These isolators have been used successfully with swine and sheep. The stainless steel base may serve as a support for a cage top upon which experimental materials can be set or may serve as a base of a larger cage extending into the flexible film isolator portion.

At Ohio State University a series of studies were made in order to design an isolator system which could be used for animals of any size including fully grown cattle. These isolators apparently could be made by a logical extension of our present type of equipment provided a means could be found for transferring the animals from one isolator to another.

The two isolators are fastened together, end to end. This can be easily accomplished provided the ends of the isolator contain a flange that is clamped or heat sealed together. The joined ends of the isolators are then sterilized with peracetic acid, either in the liquid or vapor phase. After sterilization the end panels can be cut without introducing contamination. The animals by themselves or in cages are then transferred from one isolator to the other. The opening that has been cut must be sealed in order to provide a complete barrier upon removal of the attached isolator. This transfer system has been demonstrated at Lobund working with some old patient isolators. Patent Application is being prepared.

## b. Patient isolators

Background: Under Contract DA-49-007-MD-878 in collaboration with S. M. Levenson, M. D., of Walter Reed Army Institute of Research, a series of isolators for use in surgery were fabricated in the Lobund Laboratories and by the Standard Safety Equipment Company. These were used on operations with dogs and the design modified for eventual use in human surgery. Assistance was given to the staff at WRAIR in this development work. An isolator in which a patient could be placed was designed and built around a Strykker frame and sent to WRAIR for evaluation.

### Present investigation

Surgical isolator. Following the recommendations made by the team at WRAIR the dimensions of the isolator was changed, various modifications were made in the face piece and the surgical jacket so that units could be obtained that were suitable for use in the operating room. Methods were developed for passing sterile supplies through any clear portion of the isolator wall. The complete plastic units were then ordered through Standard Safety Equipment Company as needed. Four operations were performed at the Walter Reed Army General Hospital using these isolators.

Various methods were devised for reducing the size of the package required for sterilizing the operating chamber. These modifications were first tried at Lobund in working with laboratory animals. The air outlet filter was reduced in size and tried on a jacket isolator in which a colony of gnotobiotic mice were reared. The outlet consists of a simple circular air filter taped in the roof of the isolator. The exhaust air was sent passed through a flat polyethylene tubing which serves as a one way valve.

Ordinarily transfers are made into an isolator through a door in the isolator wall. To reduce the size of the surgical isolator package the transfer door was eliminated by pressing the sterile lock attachment door on the instrument chamber against the wall of the surgical isolator and securing by means of a rubber band placed on from the interior of the surgical chamber. A double coated adhesive tape was used to prevent slippage of the isolator wall on the instrument isolator frame. The sterile lock connection was then decontaminated with peracetic acid as with other sterile locks. After the holding period, the inner door of the instrument lock was removed and a passage way cut in the wall of the surgical isolator. This operation was tried several times at Lobund without introducing contamination. It is essentially similar to the method used to introduce supplies from a sterilizing cylinder or canister.

This method of introducing supplies was further modified at Lobund by covering the exterior door of the instrument isolator with Mylar film. The surface of the Mylar film and of the surgical or receiving isolator is then flood with peracetic acid. The connection was made as before. Since peracetic acid in the liquid phase inactivates resistance force within 30 seconds, the adjacent surface of the isolator and transfer lock are sterilized as soon as they are firmly attached. The quantities of peracetic acid involved are minimal, merely forming liquid films covering the adjacent surfaces. A passage way may be cut between the two isolators so as soon as attachment is made.



The attachment is a little more difficult to make than with the former scheme because the surface of the plastic is wet and does not adhere as firmly. This method was recommended to the Curtiss-Wright Company and has been incorporated in their surgical instrument pod. It provides a method for rapidly introducing sterile supplies without the technical development required for a split seam transfer. To my knowledge this has not been tried at WRAIR.

#### Patient isolators

Three additional patient isolators have been fabricated in the Lobund Laboratories and sent to WRAIR for testing, in addition to the Strykker frame isolator made under Contract MD-878.

A large patient isolator approximately 8' x 8' floor area was made so that a patient could get out of bed and walk up and down for exercise. This isolator contained two jackets, one of which had a skirt that went to the floor. There was a sink that could be equipped with hot and cold sterile water. The nurses at WRAIR found this isolator difficult to use and particularly the long jacket with the skirt to the floor. However, it was decided that such an isolator could be used to take care of the patient provided methods could be designed for providing the variety of supplies and services needed for patient care.

The second isolator was designed for patients confined to the bed. Attention was given to the design of the jackets so that a nurse could make the bed from a position on one side of the isolator. This was accomplished by making the skirt of the jacket large enough to provide the necessary freedom of movement.

The third isolator was made for a patient confined to bed. Arrangements were made to include a table at the head of the isolator for supplies for the patient. The isolator was provided with the means for introducing the patient over the foot of the isolator or from the side. A large pass through lock was provided for the introduction of supplies. This isolator has been under test at WRAIR.

## REFERENCES

1. Dubos, Rene J. and Russell W. Schaedler. 1960. The Effect of the Intestinal Flora on the Growth Rate of Mice and on Their Susceptibility to Experimental Infections. *Jour. Exp. Med.* 111: 407-417.
2. Greenspen, Frank P., Montfort A. Johnsen, and Philip C. Trexler. 1955. Peracetic Acid Aerosols. *Proc. 42nd Annual Meeting of the Chemical Specialties Manufacturing Association, Inc.*
3. Gustaffson, B. 1948. Germfree Rearing of Rats. General Technique. *Acta Pathol. Microbiol. Scand. Suppl.* 73: 1-30.
4. Henthorne, R. D., Capt. VC. 1956. Care and Management of Laboratory Animals. U. S. Gov't Printing Office #0385809, TD Med. 255 ASP 150-12-3.
5. Kuster, E. 1915. Die Keimfreie Zucht von Säugetieren--In E. Adberhalden. *Handbuch der Biochemischen Arbeitsmethoden.* 8:311-323.
6. Levenson, S. M., Bud Tennant, Erving Geever, Lt. Roger Laundry, and Floyd Daft. 1962. Influence of Microorganisms in Scurvy. *Archives of Internal Med.* 110: 693-702.
7. Meyer, R. C., E. H. Gohl, R. D. Henthorne, V. L. Thorp, D. E. Baldwin. 1962. The Procurement and Rearing of Gnotobiotic Swine. *The Third Symposium on Gnotobiotic Technology.* In Press.
8. Reyniers, J. A. 1932 (1933) The Use of Germfree Guinea Pigs in Bacteriology. *Proc. Ind. Acad. Sci.* 42:35-37.
9. Reyniers, J. A. 1946. Germfree Life Applied to Nutrition Studies. *Lobund Rep. No.1:* 87-120. Univ. Notre Dame Press.
10. Reyniers, J. A., P. C. Trexler, and R. F. Ervin. 1946. Rearing Germfree Albino Rats. *Lobund Rep. No. 1:* 1-84. Univ. Notre Dame Press.
11. Reyniers, J. A., and M. Sacksteder. 1958. Apparatus and Method for Shipping Germfree and Disease-free Animals via Public Transportation. *Appl. Microbiology* 6: 146-152.
12. Schaffer, James, Parker R. Beamer, Philip C. Trexler, Gerald Breedenbach, and Dwain N. Walcher. 1963. The Response of Germfree Animals to Experimental Virus Monocontamination. I. Observations on Cocksackie B. Virus. In Press.
13. Skelly, Basil J., Philip C. Trexler, and Junichiro Tanami. 1962. Effect of a Clostridium Species upon Cecal Size of Gnotobiotic Mice. *Proc. Soc. Exp. Biol. and Med.* 110: 455-458.

14. Trexler, P. C. 1959. Progress Report on the Use of Plastics in Germfree Equipment. Proc. Animal Care Panel 9: 119-125.
15. Trexler, P. C. and Louise I. Reynolds. 1957. Flexible Film Apparatus for the Rearing and Use of Germfree Animals. Appl. Microbiol. 5: 406-412.
16. Trexler, P. C. and C. K. Smith. 1960. Nutrition and Physiology of Germfree Ruminants. Abst. 5th International Cong. Nutrition, Washington, 2-6.
17. Ward, Thomas G. and P. C. Trexler. 1958. Gnotobiotics: A New Discipline in Biological and Medical Research. Perspectives in Biology and Medicine. 1: 447-456.

#### PUBLICATIONS RESULTING FROM THIS STUDY

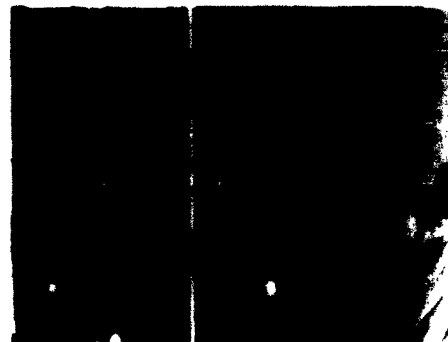
1. Levenson, S. M., P. C. Trexler, O. J. Malm, Maria L. LaConte, R. E. Horowitz, and W. H. Moncrief. 1962. A plastic isolator for operating in a sterile environment. *Amer. Jour. Surgery* 104: 891-899.
2. Skelly, B. J., P. C. Trexler, and J. Tanami. 1962. Effect of a *Clostridium* species upon cecal size of gnotobiotic mice. *Proc. Soc. Exp. Biol. and Med.* 110: 455-458.
3. Doll, J. P., P. C. Trexler, Louise I. Reynolds, and G. R. Bernard. 1963. The use of peracetic acid to obtain germfree invertebrate eggs for gnotobiotic studies. *Amer. Midland Naturalist* 69: 231-239.
4. Walcher, D. N., J. Schaffer, Janet Draper, P. Beamer, and P. C. Trexler. 1960. Viral monocontamination in the gnotobiotic (germfree) animal. *Amer. Jour. Dis. Children* 100: 642.
5. Walcher, D. N., J. Schaffer, Janet Draper, P. R. Beamer, and P. C. Trexler. 1961. The gnotobiotic (germfree) animals' response to viral monocontamination. *Amer. Jour. Dis. Children* 102: 656-657.
6. Walcher, D. N., Janet Draper, J. Schaffer, P. R. Beamer, and P. C. Trexler. 1961. The intestinal flora of infants and children with diarrhea. *Jour. Pediatrics* 61: 306.
7. Doll, J. P. 1962. Rate of carbon clearance in three strains of germfree mice. *Amer. Jour. Physiol.* 203: 291-295.
8. Trexler, P. C. . An isolator system for the control of microbic contamination. 3rd Symposium on Gnotobiotic Technology. *Nat. Acad. Sci.* In press.
9. Trexler, P. C. and B. J. Skelly. . A microbic flora for use with laboratory animals. 3rd Symposium on Gnotobiotic Technology. *Nat. Acad. Sci.* In press..

#### GRADUATE STUDENTS SERVING AS LABORATORY ASSISTANTS

Basil J. Skelly and Philip Vella. Both are candidates for the degree of Ph.D.



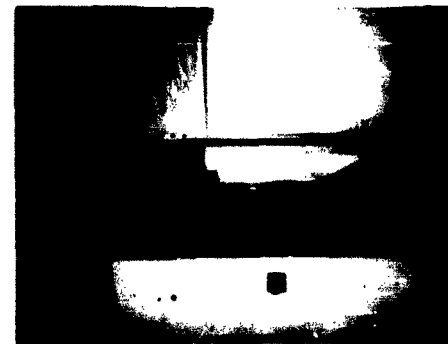
1



1a



2



3a



3b



4



5