DESIGN AND OPERATION OF APPARATUS FOR REARING GERMFREE ANIMALS*

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INTRODUCTION

Rearing germfree animals and using them in experiments is necessarily a problem in biological engineering; without apparatus that will eliminate contamination and at the same time permit animals to be maintained in it there is little point in attempting such experiments. The basic principles involved in the isolation of animals have been discussed elsewhere (Reyniers, 1943). What has not been discussed in any detail is the basic design, operation, and function of germfree apparatus. It is my purpose here to describe one such system, the Reyniers Germfree System II, and the results obtainable from its use.

The need for germfree animals is increasing. Many installations for their production are in operation, and more are presently anticipated. Consequently, it seems proper at this time to examine the steps involved in the operation of germfree equipment and the results obtained in order that the problems inherent in such work may be better understood. The fact that only one type of germfree apparatus is considered is satisfactory, apart from the limitations of space, for my purpose since the operational procedures are common to all such systems.

Until recently there was little point in attempting a critical appraisal of germfree operations, simply because the techniques for rearing such animals were not sufficiently advanced to be reduced to routines, and the necessary degree of certainty relative to contamination had not been attained. As long as the germfree animal remained a laboratory curiosity and the apparatus for working with it was not standardized, there was little purpose in such an analysis. The production of a few germfree animals was merely experimental, and the costs involved were relatively less important than if germfree animals had been available in numbers and widely used.

Highly skilled research personnel is no longer needed to operate germfree apparatus. Some species of animals can be reared germfree in numbers and maintained as colonies by breeding them through generations. Furthermore, this can now be done in a centralized establishment in much the same manner as in the commercial breeding of conventional animals. Shipment of germfree animals by public transportation to other centers is equally feasible (Reyniers and Sacksteder, 1958; Reyniers, 1957). Colonies of germfree animals can be tended by technicians, and the apparatus operated for experiments requires little more effort than does an autoclave. It is time, therefore, to discuss the operation of such equipment in terms of manpower, capacities, maintenance, and output of animals.

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The Reyniers Germfree System II is made to be sterilized *in situ* with steam under pressure. It should be noted that, if the need arises, this same system may be operated with chemical sterilization or by a combination of steam and chemicals. This system has been under development for more than twenty years, and with time and use it has been simplified and standardized. It is commercially available.*

The data given in this report are based upon the operation of three groups of this equipment, one used with rats and two strains of mice, one with guinea pigs, and the other with chickens. The same type of equipment has also been used to rear rabbits, monkeys, chickens, turkeys, dogs, and other animals. However, since the operation of germfree apparatus is essentially the same for any species, there is little point in discussing each species separately.

Basic Design of Germfree Apparatus

The Reyniers Germfree System is made up of a number of individual units called isolators, which can be used either separately or attached to each other to form multiple isolators. Sterilization of the isolator as well as of the supply lock is accomplished *in situ;* that is, the unit it is not sterilized separately in an autoclave or pressure vessel before use.

The basic Reyniers Germfree Isolator (FIGURE 1) is conventionally a cylinder to which are attached: a window, a pair of rubber gloves, a supply lock (also called a food autoclave), inlet and outlet filters for ventilating the isolator, openings for the steam, a drain, electricity, a stand that permits the isolator to be moved from place to place, and a door used to close the open end of the cylinder.

The standard one-man isolator is 28 inches in diameter and 46 inches long. This size is determined by the maximum interior area that can be reached through the rubber gloves from a standing position. The isolators are 42 inches from the floor to midline.

The length and diameter of a germfree isolator may be changed without affecting the basic design. If the isolator is increased in diameter or made longer it must be operated with additional pairs of gloves with a window to each pair. In this way germfree isolators have been built as large as 36 inches in diameter and as long as 12 feet.

The cylindrical shape commonly used permits greater economy in machining the parts and is strongest for the purpose. However, it is possible to use a rectangular or semiround section without changing the basic design. The operation of these isolators is the same regardless of their shape or size.

The standard basic isolator has been modified to four specialized types (FIGURE 1) that experience has shown generally to meet most experimental needs. These specialized germfree isolators are: (1) rearing isolator (RSU-)† used for rearing and breeding animals; (2) examining isolator (REXU-)

* Reyniers and Son, Chicago, Ill.

† The code letters are those of Reyniers and Son and designate the type of germfree isolator; the number following the letters designates the series, which may differ in size or construction details. For example: RSU-400 is a standard stainless steel rearing isolator 28 inches in diameter and 46 inches long.

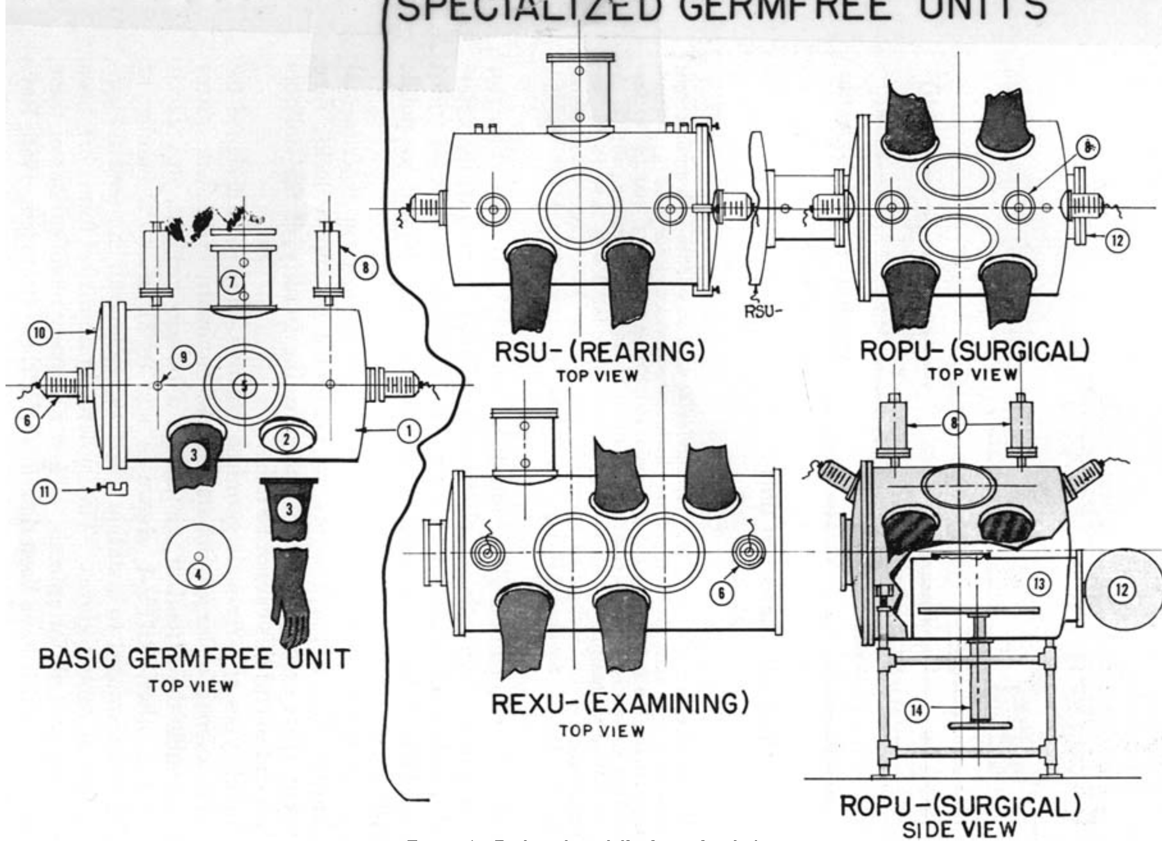

FIGURE 1. Basic and specialized germfree isolators.

Basic isolator: (1) Shell; (2) glove ring for attaching gloves to germfree isolator; (3) rubber gloves; (4) lids for closing glove rings during sterilization (these lids are fitted with a valve for regulating pressure in gloves); (5) window of Pyrex glass; (6) external lamps fitted with ventilating fan and detachable from the light window; (7) double-walled supply lock fitted with 2 quick-opening doors; (8) air filters (traps may be substituted for these); (9) openings in isolator for filters (there may be many such openings on a germfree isolator); (10) end door for closing isolator; and (11) C-clamps for attaching end door.

Specialized isolators: RSU-rearing isolator; general purpose isolator; REXU-examining isolator, intended primarily for experimental work requiring 2 operators and for combining with other germfree isolators to form a system; and ROPU-surgical isolator, intended primarily for cesarotomies; (12) quick-opening door closing off animal compartment on ROPU-surgical isolator; (13) animal compartment closed from the isolator interior by a sheet of cellophane; (14) mechanism for raising pregnant animal into contact with cellophane barrier.

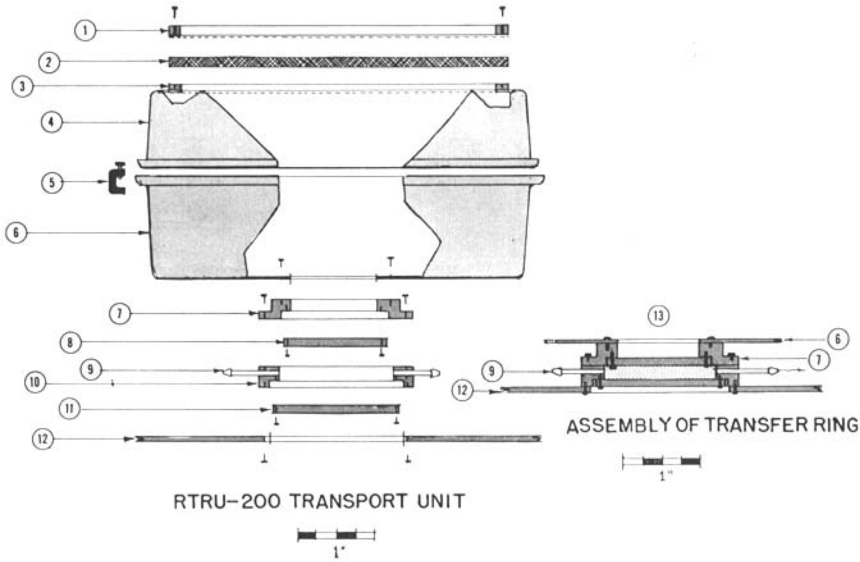

FIGURE 2. Germfree transport isolator. (1) Top frame with one-eighth-inch mesh screen; (2) FG-50 glass-fiber filter mat; (3) bottom frame with protective screen for filter mat, which is compressed between the frames; (4) top of aluminum roasting pan; (5) C-clamps for fastening top to bottom against a rubber gasket; (6) bottom of aluminum roasting pan; (7) A-ring bolted to bottom of roasting pan against a gasket; (8) plate for closing A-ring; (9) pipe for filling span between A- and B-ring with germicide; (10) B-ring, which is bolted to a sterile transfer isolator; (11) plate for closing B-ring; (12) wall of transfer isolator; (13) germicide.

usually made with two pairs of gloves and two open ends*; (3) surgical isolator (ROPU-) mounting two pairs of gloves and intended primarily for cesarotomies, although other surgical procedures may be performed in it; (4) transport unit (RTRU-) in which germfree animals may be shipped by public transportation (FIGURE 2).

Any or all of the isolators mentioned may be joined to form experimental systems (FIGURE 3). If the isolators are connected by supply locks, they may be taken apart or assembled without affecting sterility; if the isolators are attached end to end this cannot be done.

Two general types of large-scale germfree isolators have been designed for use with large animals or large numbers of smaller animals. One such system (RCOL-1) permits the attendant, clothed in a plastic garment, to enter it (FIGURE 4); the other (RSTU-1, shown in FIGURE 5), allows a number of animal pens to be brought to a station where the technician can tend the animals by means of rubber gloves. This system, in a smaller form, has been used as a storage system for animals. There are many modifications of these basic types, all of which have been described elsewhere (Reyniers, 1956, Reyniers, patents).

Over the many years during which this apparatus has been developed it

* This isolator may be attached to other isolators, end to end, or via the supply lock to make a system in which many experiments requiring two or more technicians can be performed.

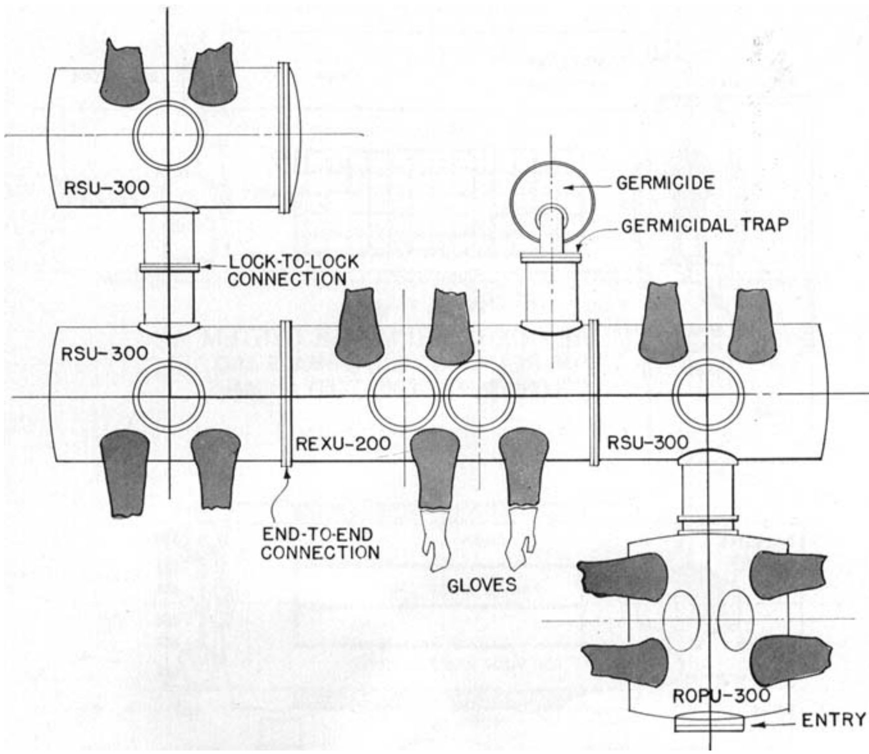

FIGURE 3. Assembly of germfree isolators into experimental systems. The units can be combined to form various combinations involving many operators. The assembly shown is intended to demonstrate combinations by means of supply locks and end-to-end combinations. Entry to the system is by way of the surgical isolator, the various supply locks, and through a germicidal trap. Combinations through the supply locks permit the isolators to be separated without contamination.

has been constructed of brass, of mild steel with painted finishes, of stainless steel, and of aluminum. The metal of choice is stainless steel, which can be polished and is easily maintained. The materials used do not affect the basic design. However, since this equipment has been operated safely for from twenty to twenty-five years, stainless steel has become standard. It is also standard practice to build the shell from 12-gauge (0.081-inch) metal with equally sturdy machined elements. Heavy construction stands up better to the routines of heating and cooling and of moving the equipment from place to place. Weight is not important, since the isolator is moved on ball-bearing wheels. The isolators are easily moved and can be operated by women. Experience has shown that light construction will not withstand the strain of repeated heating and cooling and day-to-day use.

Operation of Germfree Apparatus

All germfree equipment, irrespective of design or kind, is operated essentially in the same way and involves the same steps. Much of the cost of working

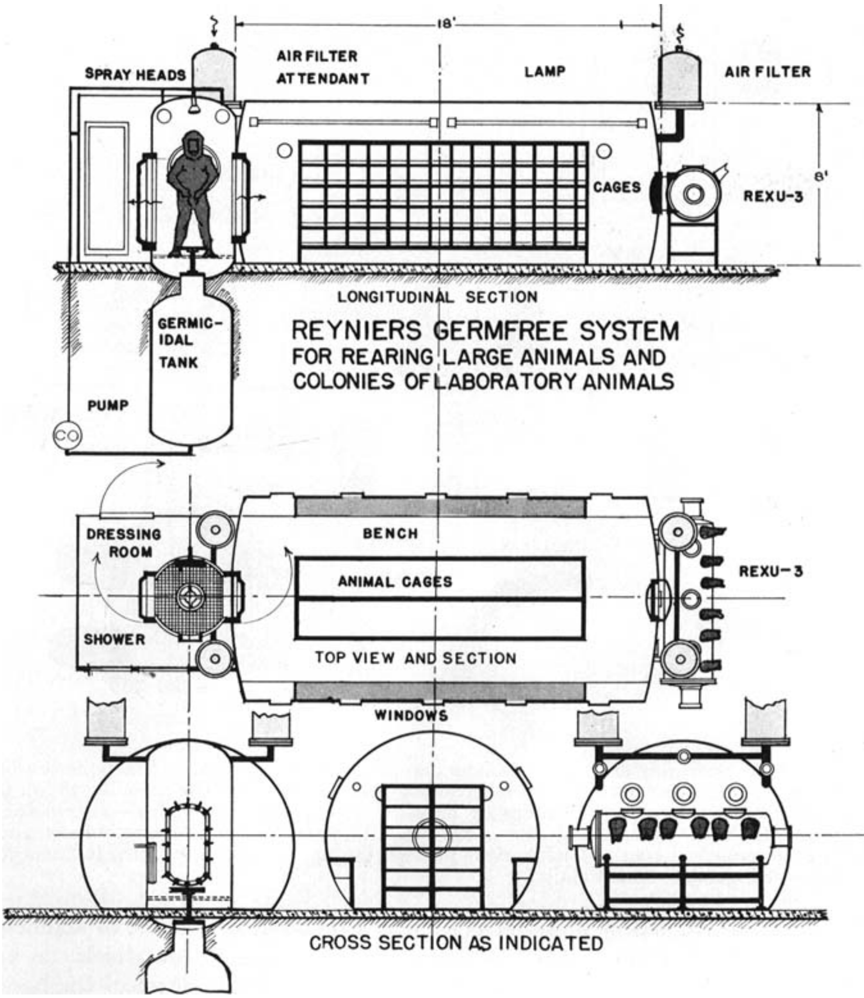

FIGURE 4. RCOL-1. This isolator is intended for rearing large animals or large numbers of animals as a breeding colony, or for using large numbers of germfree animals in experiments demanding the same environment. This diagram illustrates the isolator as it was originally designed. It consists of a tank 18 feet long and 8 feet in diameter, constructed of stainless steel sufficiently heavy to withstand steam sterilization. This tank is equipped with 2 doors, one attached to an REXU-examining isolator and the other to the entry lock. The entry lock is enclosed by a dressing room and can be sterilized with steam. In operation the attendant is sealed into a plastic garment supplied with air inlet and outlet and telephone communication. On entering the lock, the suit is sterilized by sprays of detergent and germicide. It is possible to flood the lock entirely with germicide and so immerse the attendant. Once the suit is sterilized, the attendant enters the main tank and tends the animals. This operation is on one level; because of the lock, quick-acting germicides may be used. The interior of the tank is equipped with standard animal cages. Ventilation is accomplished through large filters, which are in duplicate so that they may be sterilized while the unit is in operation.

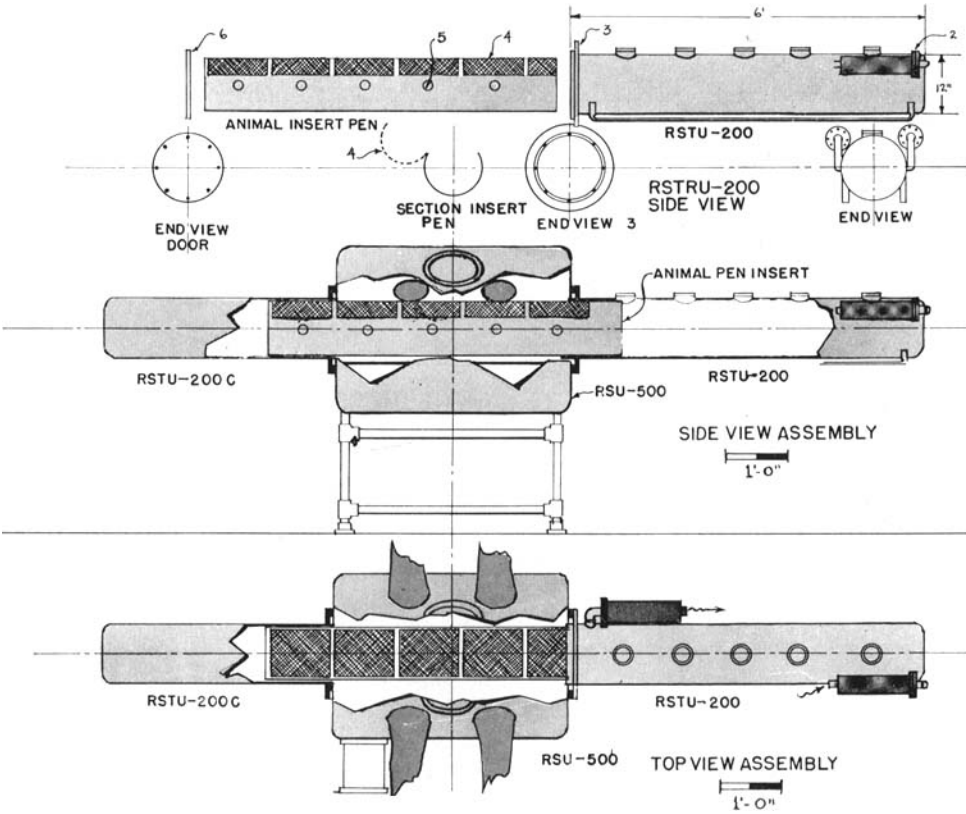

FIGURE 5. RSTU- one-station germfree colony system. This system makes use of cylinders that can be stacked. Each cylinder (RSTRU-200) contains an animal pen insert with separate compartments. In use, the RSTRU-200 isolator is bolted to an RSU-500 isolator with an adapting end door, and an RSTU-200 C-isolator is fastened in the opposite end. The assembly is sterilized. The door to the RSTRU-200 isolator is opened, the animal insert pen is pulled into the RSU-500 isolator and on into the attached RSTU-200 isolator. Pens can be cleaned, animals examined, and food supplies received. The sections of the insert pen: (1) windows; (2) air filter; (3) flange for bolting RSTU-200 to the RSU-500; (4) screen access doors; (5) ventilators; and (6) door for closing RSTU-200 isolator.

with germfree animals is in operation and use of the equipment. If the apparatus is not dependable and an experiment becomes contaminated, the costs are relatively greater. The principal expense is not, as might be thought, in the initial cost of the apparatus, since this may be spread over many years, but in the reliability of the apparatus during use.

Assembling and Testing

At the start or completion of an experiment, a germfree isolator must be disassembled and cleaned. The end door is removed, the contents of the unit removed, and the inner door of the supply lock opened. All inside surfaces of the isolator, the apparatus used in it, and outside surfaces are washed with a detergent. We believe that careful cleaning is an important preliminary to complete sterilization.

When the isolator is reassembled it is routine to replace the rubber gloves and to examine and replace all gaskets that show compression or wear. Valve diaphragms are replaced at each initial sterilization. After several years' use the standard Reyniers Air Filters may be repacked with glass fiber, although we have run these isolators for ten years or more without doing so. On the other hand, the glass-fiber mat or cloth used in the Trexler Air Filters must be changed before each sterilization. When the end door is fastened a little care in drawing it tight against the gasket will avoid leaks. It seems almost elementary to point out that this is best done by equally tightening the clamps opposite each other.

The next step is to test the inner door of the supply lock for tightness by closing both doors and testing the lock with 20 p.s.i. of air. When the supply lock proves to be tight the pressure is relieved, and the inner door opened.

The final test is on the main body of the germfree isolator. This is done by filling the isolator with 20 p.s.i. of air and allowing it to stand overnight. Any decrease in pressure, noted on an attached pressure gauge, indicates a leak that must be located. This is done by painting a viscous soap solution on the gasket edges and valve diaphragms at suspected spots, edges, and pipe joints. If bubbles form the leak can be located and stopped. In most instances this can be done simply by drawing the gasket surfaces tighter. Increasing the air pressure will shorten the time for detecting leaks, just as will the use of electronic leak detectors, but the use of soap solution is readily adapted to routines and has proved to be generally satisfactory.

There are not many places where a leak can develop on a standard germfree isolator. All pipe joints are silver soldered, and the window seal, once it is tight, usually lasts the life of the isolator, or at least for many years, and seldom requires further attention. The glove seals occasionally may show leaks when the gloves are changed, but this can be corrected easily by tightening the holding ring. The end door and outer supply lock door already have been considered.

A check of the records of the operations reported herein shows that very little time has been spent on assembly leaks. For example, in an average series of 15 assemblies only one was found, and this was on an end door. This condition was corrected by tightening the bolts at the point of leak. If proper care is exercised in assembling the isolator there should be little trouble with leaks.

In checking for leaks it is our custom to allow the isolator under test to remain overnight. Since we do not sterilize at night, we have found a satisfactory routine of placing several isolators on test at one time at the end of a working day.

As a matter of record it takes from 4 to 6 hours to disassemble, clean, reassemble, and repair detected leaks in the initial sterilization of a single germfree isolator. While the time can be shortened, we have found that it is best to perform this step carefully, since the success of the experiment depends upon it.

Initial Sterilization

Once a germfree isolator is free of leaks it is ready for sterilization. At this point it is connected to the necessary services through flexible hoses attached to a sterilization cart (FIGURE 6). The services are: steam, drain, vacuum, air, and water. A recorder on the sterilization cart permits a time-temperature record of the sterilization cycle, and this is filed for reference.

Before sterilization an insulating blanket is spread over the entire germfree isolator covering all the exposed surfaces, including the ends and the supply lock, except for an opening over the window. It is necessary to be able to observe the rubber gloves at all times. The insulating blanket is made of two layers of glass cloth between which a pad of glass fiber 2 inches thick is quilted, using glass fiber thread for sewing. The blanket is held in place with straps and drawstrings. The use of an insulating blanket ensures better sterilization and reduces the heat in the room.

Temperatures are taken with thermocouples inserted into the drain and, when the occasion demands, into other areas. FIGURE 7 shows the valves and the germfree isolator at sterilization.

The eight steps in the procedure for sterilization are as follows:

(1) A vacuum of 20 inches Hg is maintained in the isolator for 10 min. Pres-

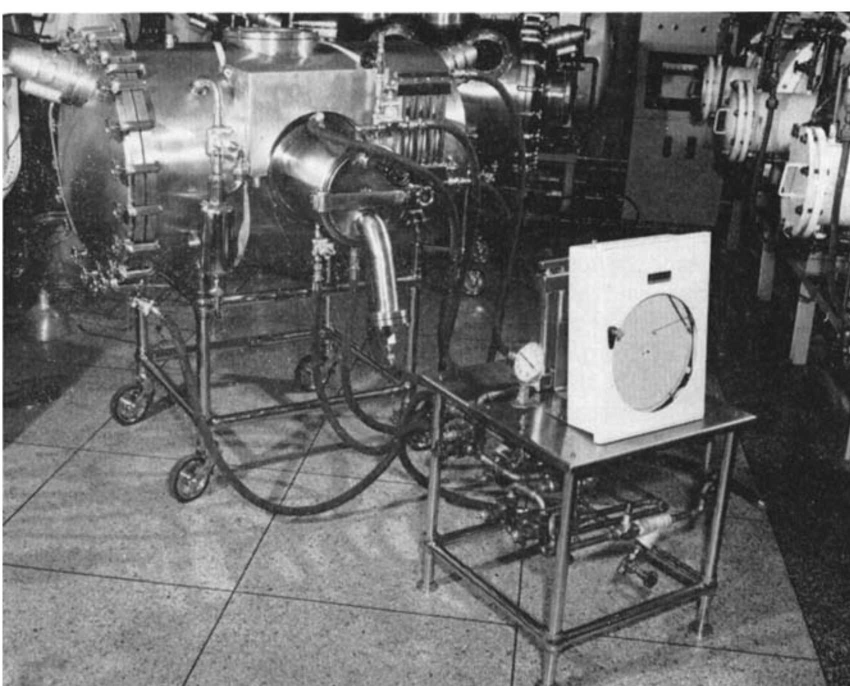

FIGURE 6. Sterilization cart attached to an RSU-300 isolator.

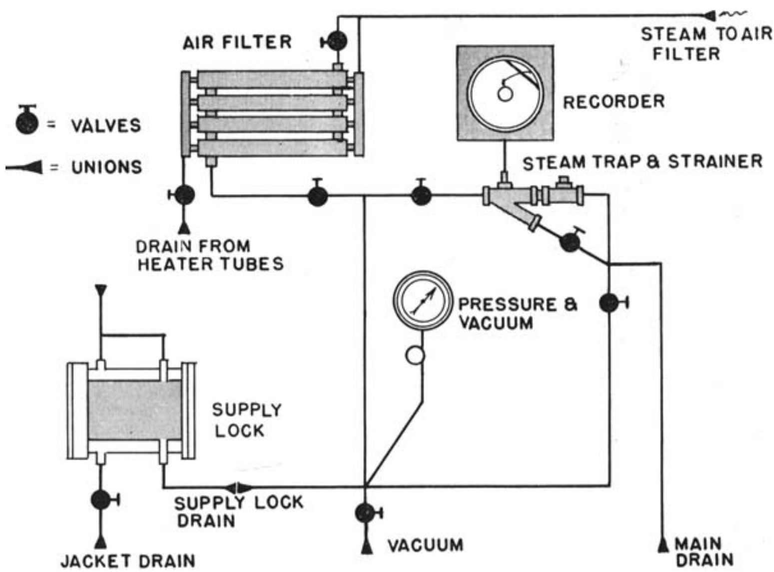

FIGURE 7. Schematic diagram of the piping in the type of sterilization cart used in Group I studies in this report.

sure is equalized on the rubber gloves by small valves in the glove port covers. It is important that the gloves not be allowed to collapse or distend completely at any time during the sterilization cycle. They are best kept partially inflated to prevent creasing.

(2) The vacuum is broken by admitting freely flowing steam into the isolator, to the jacket of the supply lock, and to the air filters until the temperature reaches 212° F.

(3) Steam pressure is now built up to 252° F., always watching the rubber gloves to prevent them from collapsing. When the temperature reaches 252° F., it is held there for 30 min.

(4) At the end of this period the pressure is broken, and a vacuum of 20 inches Hg is created in the isolator. The vacuum is created by steam injectors, since it is necessary to remove the condensate that forms.

(5) The vacuum is held from 10 to 15 min., which is usually adequate to dry out the filters and the isolator interior.

(6) The air filters, which are also sterilized *in situ* with the germfree isolator, are supplied with additional heat through electric heating elements or a steam coil to ensure drying the glass fiber when the vacuum is created; a wet or moist air filter is useless for sterilizing air.

(7) The vacuum is then broken carefully and continuously by admitting air through the filters until the pressure is normal. The insulating blanket is then removed and the isolator, when it is cool, is ready for use.

(8) After sterilization the isolator may again be tested for leaks and, if tight, the glove port covers are removed and the inner door to the supply lock closed.

Placing Animals in the Germfree Apparatus

Animals may be placed into the germfree apparatus as: (1) newborn from a cesarotomy or from eggs that have been incubated to hatching in the supply lock, (2) from another isolator already containing germfree animals, or (3) from a transport isolator.

If newborn mammals are placed in a germfree isolator (RSU-), it must first be attached to a sterilized surgical isolator (ROPU-) by means of the supply lock. The technique for cesarotomy and hand feeding germfree mammals has been described elsewhere (Reyniers *et al.*, 1946). If chicks are to be hatched in a germfree isolator, the eggs are passed through a germicidal trap (FIGURES 8 and 9) and incubated in the supply lock. The technique for hatching and rearing germfree chickens has been described elsewhere (Reyniers *et al.*, 1949*a* and 1949*b*).

If germfree animals are to be transferred from another germfree isolator, the two isolators are attached by means of the supply lock, and the linkage is tested, then sterilized.

Animals may be transported from one place to another by public transportation (Reyniers and Sacksteder, 1958). The germfree isolator used to accomplish this is shown in FIGURE 2 and consists of a box fitted with a free-

FIGURE 8. Germicidal trap attached to a germfree isolator.

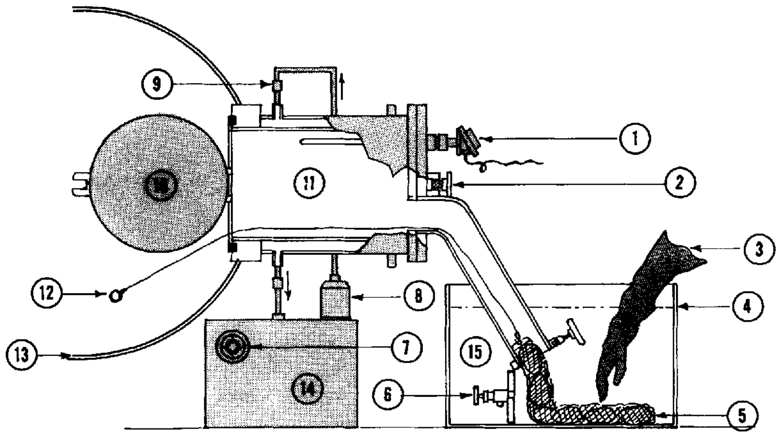

FIGURE 9. Diagram of a germicidal trap attached to a germfree isolator: (1) thermoregulator; (2) standard quick-opening door; (3) rubber gloves for opening passage tube door and inserting eggs or other materials; (4) level of germicide; (5) hen's eggs placed into a nylon mesh tube fastened to the hook of a pull chain; (6) valve on door of passage tube to bleed condensate during sterilization; (7) thermoregulator; (8) pump; (9) union connecting tempered water to outer jacket of supply lock; (10) inner door to supply lock; (11) supply lock; (12) pull chain for bringing materials into the germfree isolator; (13) wall of germfree isolator; (14) water bath for regulating temperature in supply lock; (15) Pyrex or stainless steel vessel.

flow air filter and a transfer ring that forms a germicidal trap. The transport isolator (RTRU-) is first placed in a rearing isolator (RSU-) and sterilized. It is then attached to an isolator containing germfree animals, and the linkage is sterilized. The animals are then removed from the colony isolator and placed in the transport isolator (RTRU-), which is sealed shut and removed from the isolator (RSU-). It may now be shipped by truck, rail, or air. When it arrives at the point of use it is attached to a germfree isolator by the retaining ring. The inner space formed between the bottom of the transport isolator and the top of the receiving isolator is sterilized with germicide. The doors or plates are removed, and the animals are taken into the germfree isolator for experimental use.

Supply Lock Operation

All food, water, bedding, apparatus, and such supplies as syringes, swabs, culture media and surgical instruments are sterilized *in situ* in the supply lock. All wastes, such as soiled bedding, empty containers, and dead or living animals are removed from the germfree isolator through the same device. As previously noted, the supply lock is also used to make transfers from isolator to isolator and may also serve as a passage into the isolator through an attached germicidal trap. With minor variations, the operations performed are essentially the same, so that it is necessary to detail only the procedure for taking food and water into the germfree isolator.

Perhaps it should be noted at this point that a sterilized supply lock is maintained at all times on every germfree isolator in use so that animals may be removed from it. The outer door is never open longer than necessary to place materials into the lock or remove them.

The steps necessary to proper supply-lock operation are as follows.

Preparation of supplies. Presterilized No. 2 milk cans are filled with boiling water through a hole in one end, which is then soldered shut. Screw-top containers are generally avoided because they are difficult to sterilize. If they must be used, then the gasket and cap should be separated before sterilization.

All liquids, such as diets and vitamins are sealed in previously sterilized (dry heat) glass ampules. The surface of the ampules should be washed in a detergent before sterilization. Dry nonpelleted diet is loosely packed in cloth bags, which are flattened to one-inch thickness and laid on wire screen to ensure penetration of steam. Wood shavings, used as bedding, may be packed in the same way and in the same type of bags used for diets. Pelleted diets are sterilized on a wire screen. All materials, such as wood shavings, that will withstand repeated sterilization should be presterilized.

Presterilization. It is especially important to remember that in germfree work all living organisms, not only in the food or other supplies, but also on the surface of all containers, the inside of the supply lock, inner surfaces of doors, and the contained air, must be killed. In order to ensure sterilization the exposed surfaces of the inside of the supply lock are carefully washed with a detergent, and 2 per cent alkyl aryl quaternary ammonium chloride (Hyamine) is sprayed on the gasket surfaces of the doors. In other words, before the supply lock is packed it is carefully washed. Care in this detail ensures successful operation over long periods of time and through many hundreds of supply-lock passages.

The carrier (FIGURE 10) into which the supplies are packed fits into the supply lock, leaving a space of about one-quarter of an inch around it. The bottom is perforated, and a wire screen separates it on the long axis. Previous to use this carrier is soaked in a hot detergent and carefully scrubbed with a brush to free it of all debris, after which it is soaked in 2 per cent Hyamine.

Sterilization. After the supplies are packed into the carrier it is placed in the supply lock and the outer door closed. A vacuum of 20 inches Hg is created in the lock and held for 10 min. to test for tightness. Free-flowing steam is then admitted to the jacket of the supply lock until a temperature of 212° F. is registered. Steam is now admitted to the interior of the lock until a temperature of 212° F. is reached. At this point the drain is throttled down and steam pressure built up in both the jacket and the isolator interior to 252° F. for 25 min. At the end of the sterilization cycle a vacuum of 20 inches Hg is created in the supply lock, and steam is allowed to circulate at 252° F. in the jacket for 10 min., which is usually sufficient to dry out the contents. At the end of the drying cycle the negative pressure is relieved with air passed through an air-sterilizing filter mounted on the sterilization cart. Immediately after cooling, the inner door of the supply lock may be opened and the contents removed.

During sterilization of the supply lock it is necessary to avoid heating the

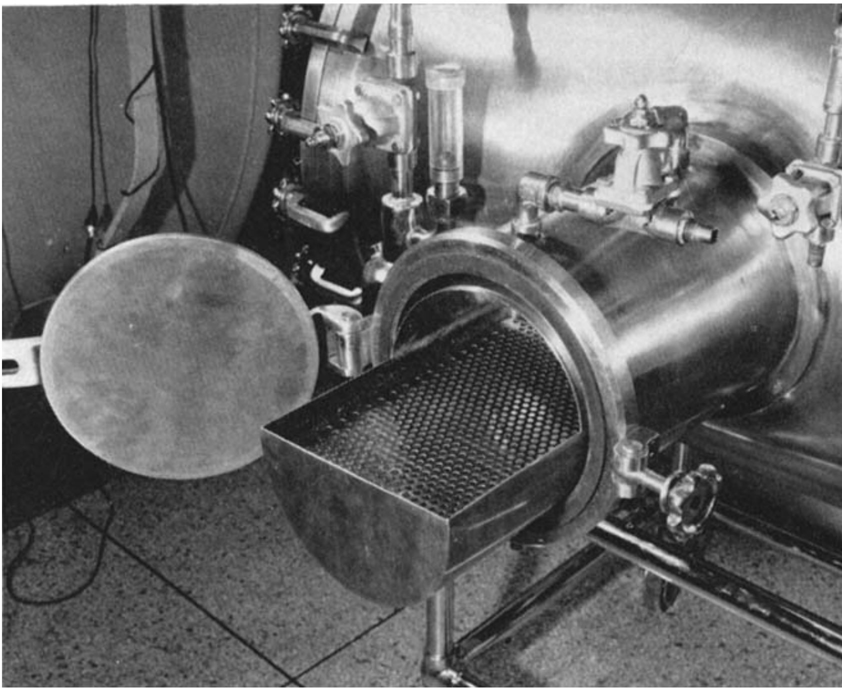

FIGURE 10. Supply lock, showing insert.

interior of the germfree isolator. This can be accomplished by passing a cooling stream of water over the outside wall of the germfree isolator about 2 inches from the outside casting that fastens the food autoclave to the wall of the isolator. Additional protection is obtained by using an insulating cap that fits over the inner door of the supply lock. This is made in the same way and of the same materials as the insulation blanket already described. It is sterilized at the time of initial sterilization or by passing it through the supply lock. With this protection and the normal ventilation (3.5 cubic feet per minute) of the stainless isolator, there is less than 1° C. rise in temperature at a distance of 6 inches from the inner door. At the position of the animals there is no appreciable temperature rise. As far as can be determined, repeated sterilizations of the supply lock have no effect on the animals in terms of growth and reproduction. This certainty as to the harmlessness of the sterilization process to the condition of the animals leaves little doubt as to the effectiveness of the procedures employed.

One point should be noted. Before opening a sterile supply lock to the outside it should be tested with air pressure to determine the tightness of the inner door. Air is supplied to the lock through sterile air filters mounted on the sterilizing cart.

Passage of Heat-Labile Materials into Germfree Apparatus

There are occasions when it is necessary to pass materials into the germfree apparatus without subjecting them to heat. This can be done by the use of a liquid germicidal trap, or by the use of germicidal vapors (ethylene oxide, peracetic acid; see Trexler and Reynolds, 1957) in the supply lock. In those instances when viruses are not important it is also possible to pass liquids through ultrafine filters attached to the germfree isolator and sterilized *in situ*. Examples are the passage of fertile eggs, antibiotics, pure cultures of microbes, animal sera, and some food supplements. In most instances the germicides are used to sterilize the surface of the containers holding the heat-labile material. We have used radiation (Luckey *et al.*, 1955) to sterilize food supplements and antibiotics. These materials are placed in a double plastic bag, which is passed into the germfree isolator through the liquid germicidal trap.

In the work reported herein only the liquid germicidal trap has been used. This trap (FIGURE 8) consists of a stainless steel tube 4 inches in diameter, welded at an angle into a supply lock door. The bottom end of the tube is fitted with a hinged lid that can be closed against a gasket by a screw acting on a bar. The door is furnished with a small valve for bleeding condensate during sterilization. The germicidal trap is used as follows: (1) the trap is fastened into place on a supply lock and tested for tightness; (2) the sterilization procedure is the same as already described; (3) after sterilization and drying, the bottom end of the 4-inch tube is submerged into germicide; and (4) in the case of fertile eggs the germicide of choice is 2 per cent $HgCl_2$ and the eggs are placed in a nylon mesh tube immersed in the germicide. The door on the end of the 4-inch tube is opened by hand below the surface of the germicide. A rubber glove protects the hands. A hook on the end of a stainless steel chain passing into the germfree insolator is fastened to the nylon tube containing the eggs, which are then pulled into the germfree isolator. The eggs are then taken from the mesh tube and incubated. The lid on the end of the germicidal trap is closed as is the inner door of the supply lock. The details of egg incubation have been described elsewhere (Reyniers *et al.*, 1949).

Sterilization of Air and Ventilation of Isolator

Insofar as can be determined, sterilization of air has caused us the least trouble. If the glass fiber air filters become wet, contamination will occur, but when this happens it is the fault of poor maintenance. Beginning with a complicated air-sterilization system in the early 1930s, we have simplified it to its present form.

Several styles of air filters have been used on the equipment described in this study. All, however, are essentially the same inasmuch as they all depend upon glass fibers and *in situ* sterilization with steam, followed by drying in a vacuum. The operation of these filters requires little attention and, as can be seen from the results reported, we have run such filters continuously for at least nineteen months without resterilization.

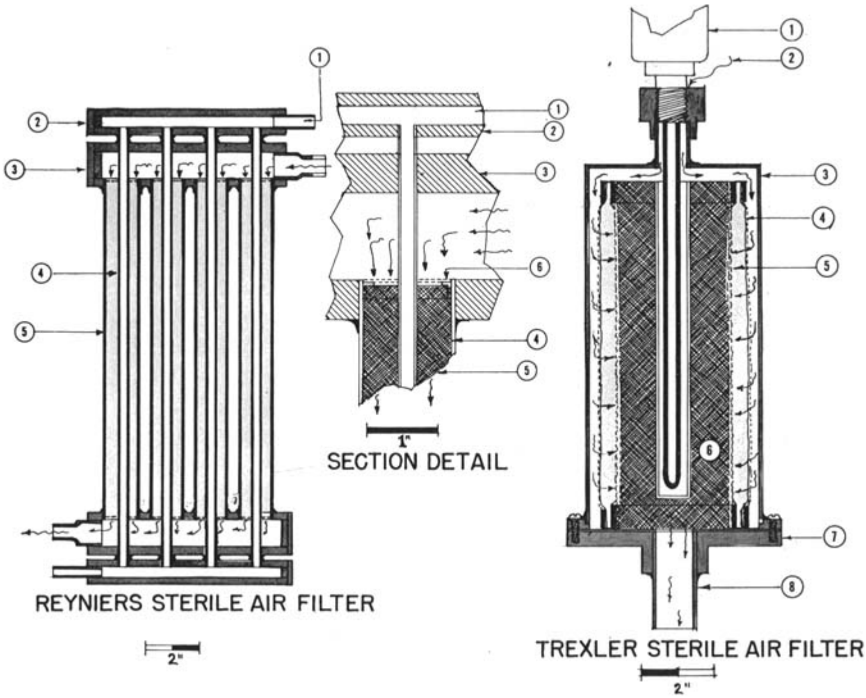

FIGURE 11. Design of air filters for use on germfree isolators. These air filters are sterilized *in situ* with steam under pressure and dried by heating in a vacuum. Left: cross-section of Reyniers Sterile Air Filter; (1) inlet for steam to heat glass fibers after autoclaving; (2) manifold for heating pipes; (3) manifold for filter tubes; (4) glass fiber, packed evenly and to a standard weight per volume; and (5) filter tubes. Center: cross-section detail of Reyniers Filter: (1) steam inlet; (2) manifold for heating tubes; (3) manifold for filter tubes; (4) filter tube; (5) glass fiber; and (6) screens at both ends of filter tube. Right, cross-section of Trexler Filter: (1) electric heating element for use during drying after steam sterilization; (2) T-inlet for air; (3) outer shell of filter; (4) wire screen; (5) two or more layers of glass fiber filter mat (AAF-FG-50) through which air must pass; (6) inner screen around which filter mat is wrapped; (7) base to which cover shell is bolted; and (8) inlet to isolator.

The Reyniers Air Filter offers more resistance to the flow of air than does the Trexler type, which employs a sheet or pad of glass fibers (FIGURE 11). On a basis of use, both seem to be equally effective. On the isolators where an outlet trap has been employed; infective material should not be used. These outlet traps were designed to offer less back pressure than the filters, hence more air can be passed through the unit without inflating the gloves. The preference in this work has been for an outlet filter.

It is important to use a clean air supply for ventilating the isolator. This may be accomplished by cleaning the air in a series of steps (FIGURE 12). The raw outside air is first passed through "roughing out" filters (commercial glass-fiber filters), then to a blower. Compressors that depend upon oil seals should be avoided. The air from the blower is conditioned, that is, brought to a controlled humidity and temperature by a series of heat exchangers and

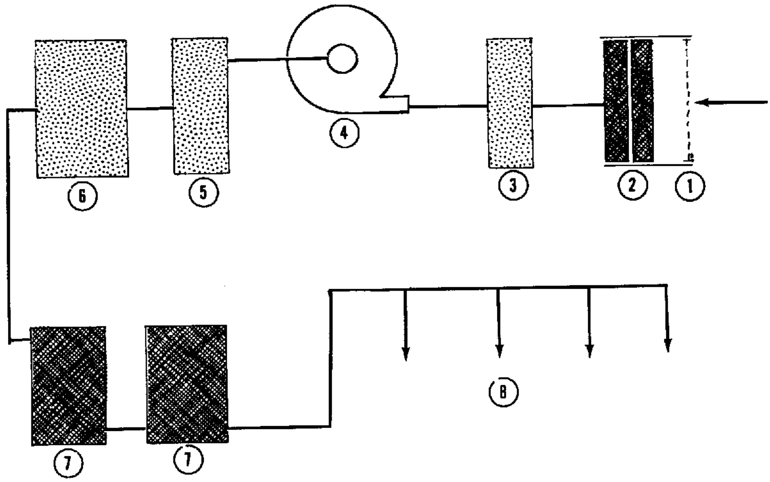

FIGURE 12. Schematic diagram of air supply to germfree isolators: (1) screen taking coarse particles and insects out of the system; (2) standard commercial glass fiber filters for roughing out the air supply; (3) preheater; (4) pressure blower (compressors with oil or water seals should be avoided); (5) exchanger for humidity control; (6) mixing plenum for temperature and humidity control; (7) large glass fiber filters that can be sterilized and dried *in situ* (these are in pairs to permit one or the other to be sterilized without interfering with the operation); (8) manifold to germfree isolator filters.

refrigeration, after which it may be passed through another series of filters, hence to the filter mounted on the germfree isolator. If the secondary filters are used, they should be in duplicate and capable of being sterilized and dried *in situ.* The secondary filters have not been used in the work reported, since tests of the air at the germfree isolator outlet show it to be very low in microbial count.

The amount of air passed through a germfree isolator will vary with the size and kind of isolator, the number of animals, the resistance of the outlet filter or trap and, particularly, the manner in which the animals are housed within the isolator. For most of this work using RSU-300 or -400 isolators, 1.5 to 3.0 cubic feet per minute of air has been used. This air flow does not overly inflate the gloves.

Sterility Checks on Germfree Apparatus and Animals

No attempt is made to detail the tests used, since this subject has been discussed elsewhere (Reyniers, *et al.*, 1946, 1949a, 1949b). The routine examinations have been primarily for bacteria, but from time to time in the course of the work described herein other tests have been made for protozoa, viruses, fungi, and parasites.

Cotton swabs in test tubes plugged with cotton are taken into the isolator through the supply lock. Samples of freshly expressed feces, waste materials, water, and animal surfaces are gathered on the swabs, and these are removed from the isolator. They are cultured in thioglycollate broth incubated at

25°, 37.5°, and 55° C. for 24, 48, and 170 hours. Microscopic examination is made from wet mounts of the incubated broth and from the fresh samples , and stained slides are also examined. If further culturing is necessary this is done from the microscopic tests.

From time to time (averaging monthly in Group I studies) an animal from each isolator is sacrificed in the germfree isolator, and cultures are made of the intestinal tract, blood, liver, spleen, lymph nodes, and other tissues. These cultures are subject to microscopic and biochemical examinations.

From time to time the animals are especially examined for protozoa and, by blind passages of lungs and other organs for viruses.

It should be remembered that experience has shown that, if contamination occurs in a germfree isolator, all animals in that isolator will be contaminated, which makes microbiological examination more certain. Moreover, there is no competition from other microbes and from the environment that includes the animal. Finally, the animal itself is an excellcnt culture medium.

Housing and Maintaining Germfree Animals

There are certain practical limits imposed by the technique as to the numbers of animals of any given species it is possible to house and maintain in a germfree isolator. This is not entirely a matter of space.

It should be pointed out that the position of the operator is fixed, and there is only a certain area that can be reached in which objects can be handled directly. If the same isolator contains two or more pairs of gloves arranged in opposite or aligned positions, the isolator can be made larger, but the area covered by each individual remains the same. The coverage for an individual can be increased if objects can be brought to his position by means of extension devices. In such instances the animals must be housed in movable cages. The number of animals that can be housed in a single germfree isolator is also restricted by the need for sterilizing everything brought into contact with the animals. Such limiting factors include the size of the supply lock, the storage space available, and the length of time food can be stored.

At present food can be stored at isolator temperatures for from ten to fourteen days after sterilization before its nutritional value deteriorates. Water and wood shavings are limited in amount only by storage space. Even if large quantities of food and water are sterilized and refrigerated in a separate germfree isolator, then passed by a supply lock transfer to an isolator containing animals, this will not entirely satisfy the demands because, in the operation of an isolator, there is a need for frequent passages. If large rooms are built to house many animals, the gain may not be commensurate with the difficulties of entry and maintenance and the loss of a large number of animals if contamination occurs.

The RSU-100, -200, and -300 isolators used in this study represent the maximum size that can be managed by one individual from a standing position in which the entire arm can be used. The isolator is 28 inches in diameter and 46 inches long, with a floor space of 24 inches by 46 inches. The floor space is limited by the fact that the floor must be below the supply lock inner door

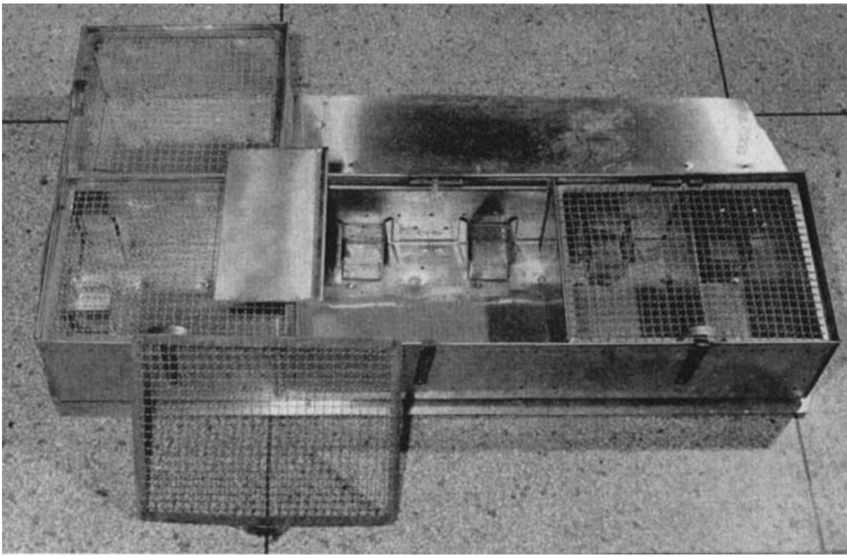

FIGURE 13. Mammal pens and floor for RSU-300 isolator. Removable partitions form compartments. Holes in side of pen furnish ventilation.

so that it can be opened. The supply lock is located on the midline, since experience has proved this to be best; in this position it is directly opposite to the operator. The space below the floor is used as a storage space for food, bedding, and other supplies.

Rats are housed in an L-shaped run (FIGURE 13), 44 inches wide and 7 inches deep, that can be divided by partitions into 3 or more compartments. Mice are housed in square glass jars 8 inches by 8 inches by 10 inches (FIGURE 14) fitted with screw-top screen lids. Chickens are housed in pens (FIGURE 15), 21 inches by 12 inches by 14 inches high. Guinea pigs are allowed the use of the entire floor space for one week after birth and are then caged in a rat run.

In a standard germfree isolator (RSU-300), it is possible to maintain 14 jars of mice, 5 adults to a jar (70 adults); 28 adult rats; 8 adult Bantam or 4 standard-breed chickens; or 10 adult guinea pigs. Unused floor space of 288 sq. in. minimum is necessary to permit manipulation of the supply lock door and general operations involved in reaching the subfloor storage space and stacking supplies. Because it is necessary to examine the animals through the window from above, it is inadvisable to stack the cages. This causes overcrowding and difficultly in working inside the isolator.

The RSU-400 isolator is 36 inches in diameter by 56 inches long, has a floor area of 34 inches by 54 inches, and is furnished with 2 pairs of gloves situated opposite each other. This isolator has been used only with mice because movable cages are necessary. In this isolator 25 mouse jars (125 adults) can be operated easily.

The REXU-3 isolator is 28 inches in diameter by 108 inches long, and is furnished with 3 pairs of gloves on the same side of the isolator. This unit

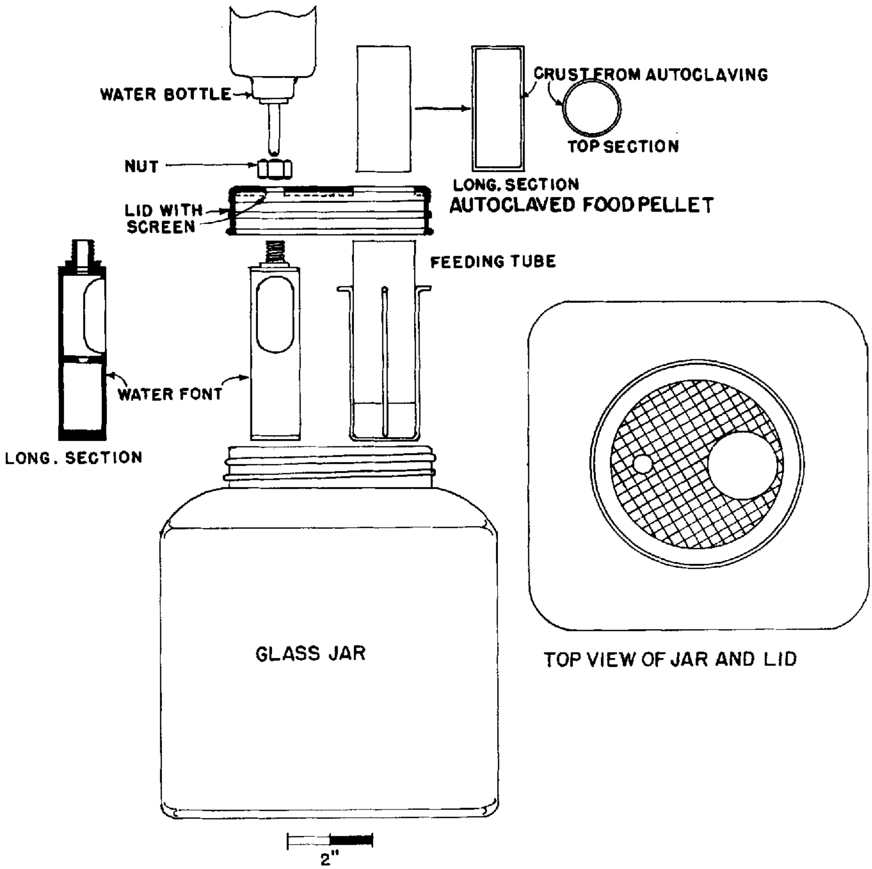

FIGURE 14. Schematic diagram of mouse jar, showing water font and feeding tube. On autoclaving, the compressed food pellet (2½ inches long and 1½ inches in diameter) forms a crust.

in which we have maintained chickens only, will easily house 16 Bantam or 8 standard-breed adults. Since the floor space is 26 inches by 106 inches, it can house as many as 34 mouse jars (170 adults) and approximately 50 adult rats. This isolator is equipped with 2 supply locks.

The RESU-1 isolator is 28 inches in diameter and 66 inches long, has a floor space of 26 inches by 64 inches and has been used only for guinea pigs in this study. Two pairs of gloves on opposite sides of the isolator and 4 supply locks permit a maximum of service. In this isolator, twelve adult guinea pigs can be maintained.

The amount of supplies that can be taken into a germfree isolator at one time is limited by the size of the supply lock. Since all standard supply locks are 9 inches in inside diameter and 15 inches long, it is possible to calculate

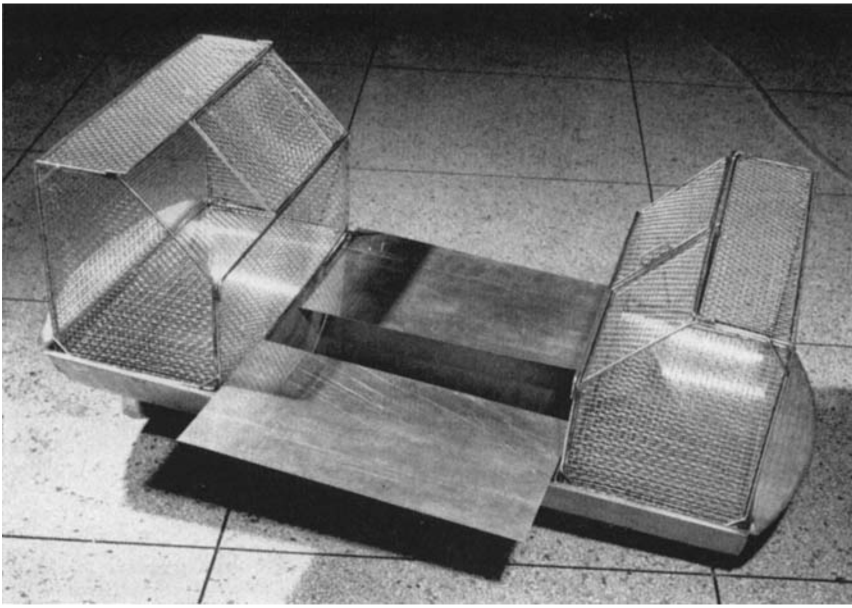

FIGURE 15. Chicken pens and floor for RSU-300 isolator. The center compartment is for storage of supplies, and the wire cages set over compartments to collect waste. These pens also can be used for guinea pigs and rabbits.

the number of passages necessary to maintain the species of animals housed in each type of germfree isolator. The size of the supply lock has been determined through experience as the most usable. Doors larger than 12 inches in outside diameter (the size needed for a standard supply lock of 9 inches inside diameter) are difficult to hold tight with a simple bar closure and take up too much floor space. When more supply-lock space is necessary, a second detachable lock can be fastened to the first, thus increasing the capacity without increasing the diameter. The accessory lock can be removed after use.

The solid food used for rats and mice is pelleted by compression into the Lobund pellet (Reyniers and Sacksteder, 1958), which is 1.5 inches in diameter and 2.5 inches long and weighs about 66 gm. (FIGURE 14, upper right). This pellet is made to be sterilized (autoclaved), at which time a crust forms around it so that it is not fragile and there is very little (about 2 per cent) waste when fed. The pellets may be fed from a tube to avoid soiling by the animals.

The water cans hold 400 ml. of water and are 3 inches in diameter and 4 inches long. Wood shavings are put up in packages of 120 gm., sufficient for one mouse jar, and are 4 inches by 8 inches by 2 inches thick.

A supply lock will hold at each loading as many as 12 cans of water (4800 ml.,) 60 pellets of food, (about 3900 gm.) or 12 packages of shavings (1450 gm.). The water and bedding are storable without deterioration, but the food should not be stored in the isolator for more than 2 weeks. Since other supplies must be taken into the isolator and, because any given passage may contain mixed supplies, the following average number of supply-lock passages per month have been made in the studies reported: RSU-300 isolators (mice), 12 passages; 8 passages for RSU-400 isolator (mice); 8 passages for the REXU-1 isolator (guinea pigs); 12 passages for REXU-3 isolator (Bantam chickens); 12 passages for RSU-300 isolator (rats); 8 passages for REXU-100 isolator (guinea pigs); and 18 passages for REXU-3 isolator (standard-breed chickens). In actual practice, which the figures given above represent, different experiments involving different species of different ages and numbers will demand a varying number of food autoclave passages per month. The numbers of rats, mice, chickens, and guinea pigs that can be maintained in the various isolators used in the study are summarized in TABLE 1, and the numbers of weaned rats and mice (21 to 25 days of age) that can be conservatively produced in an RSU-300 isolator are given in TABLE 2.

In operating germfree equipment, special attention has been paid to the proper care and use of the rubber gloves attached to an isolator. Each time an isolator is placed into operation, new gloves should be used; this is cheap

TABLE 1
OPTIMUM NUMBER OF ANIMALS MAINTAINED IN A STANDARD RSU-300 GERMFREE ISOLATOR*

Species	Average no. of adults per isolator†	Average no. of supply-lock passages per month‡
Rats	28	12
Mice	60–70	12
Bantam chickens	6–8	12
Standard chickens	4–6	14
Guinea pigs	8–10	8
Rabbits	2–3	8

* Floor space 24 inches by 46 inches.
† These numbers may be increased if younger and smaller animals are held, or by overcrowding by 20 per cent.
‡ An average of supply-lock passages would include food, water, bedding, instruments, and general and special supplies.

TABLE 2
NUMBER OF WEANED (21- TO 25-DAY-OLD) RATS AND MICE PRODUCED PER YEAR PER GERMFREE ISOLATOR RSU-300*

Species	No. of breeding adults/isolator		Average no. of young/litter	Percentages weaned 21-25 days	No. young weaned/ isolator/year
	Males	Females			
Rats	4	6	7.4†	80‡	240–250§
Mice	6	15	8.2†	80‡	320–340§

* Dimensions: 28 inches in diameter by 46 inches long.
† May be increased by selective breeding.
‡ May be increased by selection.
§ These figures are based on the removal of the young from the isolator at weaning and the maintenance of vigorous breeding stock.

insurance for an experiment. Each time the gloves are used and after each sterilization they should be washed, dried, and powdered. Careful inspection and reinforcement of rough spots are also necessary. Before an operator dons the gloves, his arms are covered with stockinette or he puts on a clean gown, and his hands are covered with clean, close-fitting cotton gloves. All operations inside the isolator that involve opening or closing the supply lock door or manipulating the animal cages are done only after putting on a pair of cotton work gloves over the rubber-covered hands. Using these techniques we have had very little trouble with punctured gloves. Furthermore, all mice are handled with long forceps with rubber-covered tips. Larger animals are handled with the work gloves already mentioned. Only in those instances where dissection of an animal or procedures demanding more sensitive touch are involved have the rubber gloves been uncovered. Finally, as a matter of good practice, access to the isolator should be limited to a minimum number of individuals. This is more important than the number of times the gloves are entered.

Man Hours Involved in Operations

It is extremely difficult to prescribe the man hours necessary for operating germfree equipment, because this depends to a certain extent upon its use. If the purpose is merely the rearing of animals, this is one thing but, in the studies reported, the purpose was experimental, requiring daily observation of each animal. In the Group I studies the animals were bred to a specific pattern. However, time approximations can be given for some operations.

In my experience it is better to assign a number of germfree isolators, together with the necessary help to operate the equipment, to a single investigator. In the Group I studies the equipment was operated by one technician, who has been able to maintain the necessary operations for ten isolators and to give limited assistance in the task of feeding. In this instance the individual concerned was responsible for disassembling an isolator, cleaning, testing, and sterilizing it, making all isolator-to-isolator transfers, all supply-lock passages, and other necessary maintenance. The food, water, and supplies came from central services and were packaged when received.

In Group II studies the maintenance operations were performed from central services. However, the equipment was under the direction of a single investigator.

Many of the operations necessary to maintain germfree equipment are performed seldom; others, such as feeding and caring for the animals, are done more frequently. The fact that a germfree isolator may be run for as long as 2 years before the animals are changed and the isolator disassembled spreads the actual hours of labor. Also, several isolators may be operated at the same time; therefore each would require less individual attention. The same thing may be said of supply-lock operations. These are factors that must be taken into account in any estimate of the time required to operate germfree equipment. Nevertheless, as a matter of information, the man-hours necessary in the various steps involved in the operation of the equipment are summarized in TABLE 3.

TABLE 3
APPROXIMATE MAN-HOURS NECESSARY FOR THE OPERATION OF GERMFREE ISOLATORS

Operations	Man-hours*
Disassembling and cleaning a germfree isolator	2
Assembling and testing for leaks (presterilization)	2
Initial sterilization	2
Supply-lock passage steam sterilized and vacuum dried	2
Isolator-to-isolator transfer via supply locks, including testing	2
Germicidal passage via liquid trap into isolator	2

* These times are average for the operations described in this report and are ample for careful work. Time can be cut by duplicating some operations such as, food autoclave passage or by taking short cuts if the attendant risk of contamination is accepted.

Cost Factors

The cost of working with germfree animals is governed by the apparatus, the installation, and the operations necessary to maintenance. The same can be said for any experimental work requiring specialized apparatus and techniques. If the purpose is to produce germfree animals as well as to use them experimentally, a different installation is necessary than if it were intended to use animals produced elsewhere. By the same token, if the interest in germfree life is continuous rather than casual, this will also influence cost. The point is that the cost of installation will depend upon the extent of the anticipated work. The cost of operation will remain about the same per isolator per day, regardless of the kind of apparatus used. In any case the situation is changing: it is entirely feasible today to produce animals (rats and mice) from breeder stock obtained from a central source and to continue production in a few germfree isolators set aside for the purpose. This is an added convenience as compared to complete dependence on a central service for all experimental animals.

The cost of metal germfree apparatus lies largely in the fabrication, which includes welding, machining of parts, assembly, grinding, and polishing. Fabrication costs are about the same regardless of the thickness of metal used in the shell. From a manufacturing point of view the use of light, (16-gauge) metal adds to the fabrication costs. From the point of view of service life there is no advantage to a lighter shell, as can be attested in our experience with the 16-gauge REXU-1 series of isolators, all of which collapsed after several years of use and required reinforcement with heavy bands. Since all isolators are mounted on rubber tires and ball-bearing wheels, the weight of the isolator is not important. Again, the best metal in our experience has been stainless steel as far as service life and maintenance costs are concerned. The saving in initial costs made possible by using mild steel is more than offset by the increased costs of maintenance and dependable function. Finally, the difference in costs between an unfinished (that is, with unpolished and unground welds) stainless steel isolator and a finished isolator is relatively small, and may not be worth the difference in view of the factor of ease of maintenance. The cylindrical shape for pressure apparatus is probably the best and is certainly the cheapest to machine and fabricate as compared to boxes and semiround shapes, but this is a matter of choice.

The Reyniers germfree isolators have been stripped down to the barest essentials consistent with the work for which they are intended. It is probable that, in time, manufacturing costs will be cut with economy in details of construction and with volume, but the changes in the basic isolator will not be great. Consequently isolators purchased today will function, like autoclaves, for years to come, and new isolators will be essentially the same. The cost of the present isolators may be written off over an anticipated twenty-five years. Changes that may be expected will be in size and kinds of accessories, but accessories may be added to any isolator. Isolators highly specialized to certain purposes have been and will be built. At present the cost of a standard germfree isolator is about the same as that of an autoclave.

The use of steam, heat, and chemical, or cold disinfection is a matter of choice. Both steam and chemicals can be used in operating metal isolators. Autoclaving food and supplies will probably continue to be the method of choice because it is safest. This being the case, permanently attached supply locks are necessary. If chemical disinfection is used in the initial sterilization of germfree isolator in an effort to utilize materials other than metal (Trexler and Reynolds, 1957), this again is a matter of choice. However, regardless of the method used to produce sterilization, the essential operations involved—for example, in supply-lock passage—remain the same for any equipment.

Where initial costs of apparatus are important, as may be the case with investigators who have a transient interest in germfree life and who depend upon receiving germfree animals from a production center, there are many devices useful to the purpose. These range from isolators to the so-called "dry box," in the construction of which a variety of materials have been used. Such equipment is useful for short-term experiments, for example, hemorrhagic shock, so that it has its place in the scheme of things. For the investigator, the problem lies in a decision as to the extent of the work and the kind of experiment undertaken. As already pointed out (Reyniers, 1957), there are many devices that can be constructed in the laboratory in which limited work with germfree animals can be done; accordingly, there is no reason why anyone interested in this field of work cannot enter it.

The costs of producing germfree animals has declined steadily with improved apparatus and better routine methods. The cost to the investigator has been minimized by the fact that breeding stock may be shipped from a production center to the investigator and a local colony instituted. Apart from the initial cost of apparatus, which is comparable to initial costs of setting up any specialized laboratory, the cost of maintaining a colony of germfree animals compares well with the costs of producing disease-free or "clean" animals. Researchwise, there is no way of comparing the costs of using germfree animals with those of using conventional animals, since it must be presumed that conventional animals will not answer the purpose or they would be so used. Since there are, at present, no commercial centers for rearing and shipping germfree animals, any costs would have to be estimated on an experimental basis, just as the costs of antibiotics in the initial stages of development could be estimated but had no real basis until these products were produced commercially.

Finally, the initial costs of setting up a germfree installation will vary according to the kind and amount of work to be done. In some instances, one or two isolators may satisfy the needs; in other cases more isolators will be necessary. If cesarean sections are to be performed and the animals reared by hand-feeding, specialized isolators are necessary. This seems obvious. At any rate, on the basis of the information given, some estimate can be reached concerning the numbers and kinds of germfree isolators necessary. The costs of these isolators must be added to that of a proper air system and quarters, as in the case of any germfree system.

Maximum Time a Germfree Isolator Can Be Operated Continuously

In the past it has been a matter of routine to remove animals from one to another isolator about every 6 months. In the Group I studies this routine has been lengthened to 10 months. However, in the interests of determining the maximum time that a single isolator can be maintained before contamination in routine operations, the RSU-400 isolator was selected for test. This isolator is of mild steel, with a baked enamel finish, and is 36 inches in diameter and 56 inches long. It is equipped with standard Trexler Air Filters on inlet and outlet, and has two pairs of gloves and one standard supply lock.

This isolator was set into operation on August 8, 1956, with 21 mouse jars and a full complement of mice. It has been maintained without changing the animals to date (May 1958), and is still in operation. During this period more than 180 supply-lock entrys have been made. The animals and isolator have been maintained constantly and have tested free from contamination.

Such information is of considerable practical value, since it demonstrates that fewer isolators are necessary if their dependability is increased. By the same token, less time is needed to maintain the animals, and there is less risk in transfer to another germfree isolator. Finally, a greater sense of security and dependability may be expected with the use of steel equipment maintained by steam sterilization.

Number of Germfree Isolators Needed

The number of germfree isolators necessary to an installation will depend upon the kind of work for which they are used. There are instances in which one isolator may suffice if germfree animals can be produced commercially and shipped to an installation. If, on the other hand, the experiments are of long range (several months to several years) and greater numbers of animals are required, more germfree isolators will be necessary. To enable the investigator to estimate his needs, the following information should be useful.

A germfree isolator such as the RSU-300 (28 inches in diameter and 46 inches long) occupies a floor space of 24 inches by 46 inches, and will accommodate 12 mouse jars, or approximately 60 adult mice. In it a pen arrangement may be used for rats (FIGURE 13), which will accommodate 16 to 20 adults. The same isolator with 2 chicken pens (FIGURE 15) will accommodate 4 adult chickens or 8 adult Bantams.

With the RSU-300 isolator used only for breeding animals, from which the young are removed at weaning to other isolators, it is possible to rear the following numbers of germfree mice and rats.

Rats. Assuming 6 breeding females and 4 adult males per isolator with a litter size of 7.4 and about 80 per cent weaning, approximately 240 to 250 weaned animals can be produced per year.

Mice. Assuming 15 breeding C3H females and 6 adult males per isolator with an average litter size of 8.2 and about 80 per cent weaning, approximately 320 to 340 animals can be reared per year. These figures are approximately the same for the Swiss mice.

It is possible, of course, to obtain more young from a single RSU-300 isolator if greater care is taken to select the best breeding stock and by putting more animals into the isolators. While we have not been especially interested in rearing a maximum of weanling animals, we have found that the above numbers can be obtained, as well as maintained, with a single isolator.

The figures given for the number of adult animals that can be held in a germfree isolator can be increased if younger animals are used. The problem is not only the maximum number that can be housed in a single germfree isolator, but also the number that can be supplied with food, water, and bedding, since these supplies must be sterilized and also stored until used. With the larger isolators, proportionally greater numbers of young can be produced and brought to weaning.

It is not possible to state exactly the number of isolators necessary to an installation without knowing the kind and extent of the operation. If the intention is to perform cesarotomies and to hand-rear the young, it will be necessary to have an ROPU-200 isolator with one or more RSU-300 isolators in which hand feeding can take place. If chickens are to be used it will be necessary to have germicidal traps and incubation isolators, both of which are accessories. There are other accessories, such as special interior equipment, deep-freeze units, vacuum sweepers, external water sterilizing tanks, end-to-end connecting links, homogenizers, filtering units, and accessory supply locks.

A complete installation has been variously estimated (Reyniers, 1957) as: 1 ROPU-200, 1 REXU-200, 10 RSU-300, 1 RGTRPO with RIX-200 incubator, and 1 accessory supply lock, with the necessary interior equipment, such as animal pens. This installation permits work with chickens and small mammals that are obtained by cesarotomy, hand-reared, and produced as a colony, together with 8 to 10 isolators for experimental purposes. However, as has been pointed out, it is difficult to determine the needs of individual groups of scientists; more isolators may be added as needed.

Results

Germfree Apparatus Used in this Study

Two groups of Reyniers germfree isolators, respectively designated as Group I and Group II, have been used in the results here reported. This equipment

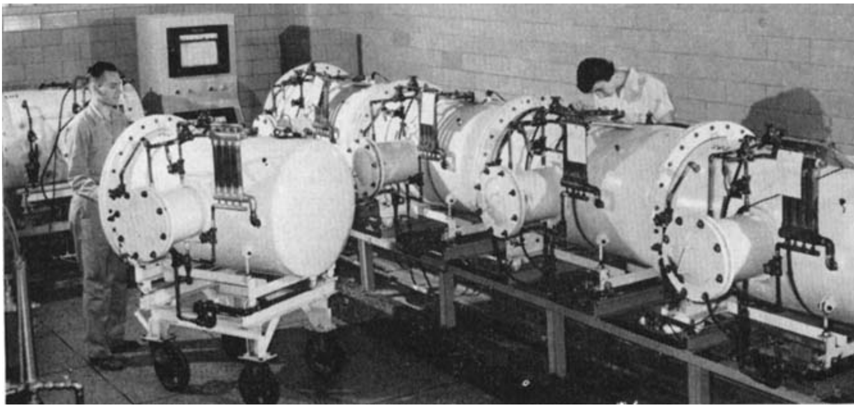

FIGURE 16. Types of isolators used in these studies. The RSU-200 isolators are mounted on a rack. They are moved about on the truck as shown.

was made in the Lobund Institute shops and must be considered developmental. No attempt was made to polish the welds or to finish this equipment completely. Some of it is made of stainless-clad steel; some of mild steel, with painted surfaces.

Group I apparatus. This consists of the following germfree apparatus: 5 RSU-300 rearing isolators; 1 RSU-400 isolator; 1 REXU-3 isolator attached to the end of the large-scale colony apparatus (this is a three-man isolator); 2 RSU-100 isolators and, from time to time, 1 or 2 RSU-200 isolators. The apparatus is shown in FIGURE 16. The RSU-200, -400, and the REXU-3 isolators are mild steel, painted inside and outside; the RSU-100 and 300 isolators are stainless-clad steel.

The ventilation systems vary somewhat in design on some of the isolators, but the air filters are all of glass fiber. On a few isolators a germicidal trap has been used on the outlet; the rest of the isolators are equipped with standard Trexler outlet filters. The series 100 isolators (stainless steel) were placed in operation in 1950; the series 200 isolators (mild steel) in 1950; the series 300 (stainless steel) in 1953; the series 400 isolator (mild steel) in 1955; and the REXU-3 isolator (mild steel) in 1950. These isolators have been used continuously since these dates.

All germfree isolators except the RSU-400 are operated from racks; the latter is operated from its own stand. This means that the isolators on racks are operated from a central control panel, while those on stands are operated from a sterilization cart. Experience has shown that the latter method is more certain, since long supply lines are difficult to maintain. Also, by operating from a sterilization cart, the number of valves on the germfree isolator may be cut to a minimum since they need not be duplicated on each isolator inasmuch as they are on the cart.

All doors, windows, and sterile locks are sealed against rubber gaskets. The end door gaskets may be changed only during the disassembly, but those on the supply lock may be changed at any time. Since this is a critical area, such changes are more frequent. The Saunders-type valve is used almost exclusively on this equipment. The diaphragms are changed on inspection when necessary.

Group II apparatus. These germfree isolators were used exclusively for rearing guinea pigs and for using these animals in studies on amebiasis (Phillips *et al.*, 1955). The following kinds and numbers of germfree isolators were used in these studies over a 5-year period: one REXU-1 isolator (stainless steel) equipped with 2 pairs of gloves and 4 supply locks, and 3 series RSU-200 isolators (mild steel). The RSU-200 isolators are of the basic design already described. The REXU-1 isolator is on its own stand, but is fixed in position; the RSU-200 isolators are on racks (FIGURE 16). The REXU-1 isolator was commercially made of stainless steel with polished welds and finishes. It was made of light-gauge (16-gauge) metal and collapsed after several years of use. It was necessary to reinforce it with heavy metal bands welded into place. This isolator was placed in operation in 1946 and the RSU-200 isolator in 1950.

One other isolator, the ROPU-2 operating unit (mild steel) has been used. It was placed in operation in 1950 and is still in use on the average of twice weekly.

Group I Operations: March 1955 to October 1957

This operation has been concerned with cancer studies in germfree C3H and Swiss mice, white rats, and Bantam and standard Leghorn chickens. Because of the nature of the study it has been necessary to breed the mammals and to hold some of them for their life span as well as to conduct experiments of shorter range. For this reason only the mammal operation is considered. In this operation within the period of 31 months there has been one contamination in an RSU-200 isolator containing C3H mice. This was probably due to a warped outer supply-lock door that leaked during sterilization and became contaminated when a vacuum was created in the lock.

A résumé of the operation is given in TABLE 4. All the isolators involved were operated by Robert Sieczko.

In an effort to determine how long a germfree isolator could be operated without changing the animals, the RSU-400 isolator was placed in operation with a full complement of C3H mice on July 6, 1955, and was still in operation without contamination on March 4, 1958. During that period there were more than 180 supply-lock passages.

The oldest C3H mice are 900 days plus, the Swiss mice are 900 days, and the rats 600 days plus. We have had no occasion to hold chickens (they were not bred and were not held) more than 12 months, and have had this phase of the work under our direct supervision since May 1957. During that time 60 chickens have been hatched in 5 experiments. We had no contaminations in this short run.

There have been no failures due to glove breaks, through the air filters, or because of failure of the apparatus, except as noted.

TABLE 4
GROUP I OPERATIONS OF GERMFREE ISOLATORS WITH C3H MICE, SWISS MICE, AND RATS
(March 1955 to October 1957)

Total number of germfree isolators used	6
Total number of C3H mice reared and held	375
Number of germfree isolators used for C3H mice	3
Total number of germfree Swiss mice reared and held	327‡
Number of germfree isolators used for Swiss mice	2
Total number of albino rats reared and held	65‡
Number of germfree isolators used for albino rats	1
Total number of supply lock passages	1210
Total number of isolator to isolator passages	64
Total number of initial isolator sterilizations	32
Total number of contaminations due to apparatus failure*	1
Oldest animals in days and species held in the isolators	600 albino rats 900 C3H mice 900 Swiss mice
Number of generations from start of operations†	4 albino rats 8 C3H mice 10 Swiss mice

* This is the only failure in these experiments and was due to a warped outer supply lock door which failed when a vacuum was created in the lock following sterilization.

† This period is from 1953.

‡ The figures given for numbers and kinds of animals are not production numbers and must not be confused with the capacity of these isolators to produce young. These are adult animals that breed and rear their young in the isolators and are held for their life span.

Group II Operations: August 1952 to November 1957

This work was conducted at Lobund Institute by Bruce P. Phillips of the National Institutes of Health, Bethesda, Md., and has been confined to rearing germfree guinea pigs for use in studies on amoebiasis. Since this has been largely an experimental study and involves cesarean operations, the results are of interest and supplement those of Group I operations.

Three germfree isolators were used in this operation, one REXU-1 examining isolator and two RSU-200 isolators. The ROPU-2 isolator was used for the cesarotomies. The results of this study are shown in TABLE 5. The operation of this equipment was performed by the service division of Lobund Institute, and over that period of time 5 individuals were involved in the operations. However, these isolators were under the direct supervision of Phillips, and all feeding and experimental work was performed by him or by a technician under his direction. The cesarean operations were largely performed by technicians from the service division of the Institute.

Group III Operations: June 1957 to April 1958

The work done by this group was confined to chickens using RSU-100, -200, and -300 isolators operated for cancer studies. Successive experiments are reported. The results of these studies are shown in TABLE 6.

The total number of isolators used for chickens never exceeded 4 in the studies reported. The number of days for each experiment was dictated by the demands of the experiment. The single contamination occurred because of

TABLE 5

GROUP II OPERATIONS OF GERMFREE ISOLATORS WITH GERMFREE GUINEA PIGS*
(August 1952 to November 1957)

Total cesarotomies	298†
Total number guinea pigs from cesarotomies	963
Total number of experiments	114
Total number of experiments germfree	98
Total number of experiments contaminated	16
Causes of contamination:	
Failure due to surgery	1
Failure due to intended marginal sterilization of new experimental dietary regimes but not to apparatus	11
Failure of apparatus (gloves)	2
Failure due to unknown causes	1
Failure due to contaminated amoeba	1
Total number of times the germfree operating isolator (ROPU-2) was used	115
Total number of initial isolator sterilizations	230
Approximate total number of supply lock passages	465
Approximate total number of germicidal trap passages	110
Age of oldest animals	14 months

* These results were made available by Bruce P. Phillips, National Institutes of Health' and are from work he did while stationed at the Lobund Institute. His experiments were with germfree guinea pigs and amoebiasis.

† Multiple cesarotomies into germfree isolator.

TABLE 6

OPERATION OF REYNIERS GERMFREE ISOLATORS (RSU-100, -200, -300) WITH CHICKENS*
(June 1957 to April 1958)

No. of experiment	No. eggs set	No. chicks hatched	Time (days) of experiment	Status of chicken	No. of lock sterilizations
1	15	15	180	GF†	4/mo.
2	12	10	79	GF	4/mo.
3	20	14	150	GF	4/mo.
4	20	12	127	GF	4/mo.
5	17	8	60	GF	4/mo.
6	15	11	92	GF	4/mo.
7	15	10	81	GF	4/mo.
8	15	4	5	Contam.‡	0
9	7	6	100	GF	4/mo.
10	15	14	170	GF	4/mo.
11	14	10	160	GF	4/mo.
Totals	180	128 (71%)	Av. 117 Days	10—GF 1—Contam.	160

* These experiments were concerned with induced tumorigenesis and this will explain the termination days of each experiment.

† Germfree.

‡ Contamination brought in with eggs. This was a poor hatch and poor quality of egg shells.

failure to sterilize the shells of a poor clutch of eggs. The poor quality of these eggs is further borne out by their low hatch in the germfree isolator. No failures were due to apparatus or during an experimental run.

Keeping chickens in germfree isolators makes it necessary to stretch a supplementary screen of surgical gauze over the outlet filter from inside the isolator. This filter must be changed daily because the dust and feathers shed by chickens will plug the glass fiber outlet unless the preliminary screen is used.

If an isolator is not overcrowded with chickens and if the watering devices are watched, no problems of moisture inside the isolator will be encountered at the normal rate of air flow. The numbers of chickens shown in TABLE 6 are young chickens and from single hatches. Multiple hatches in a single isolator should be avoided. Each hatch should be held separate until sufficiently tested for contamination (usually 21 days), at which time hatches may be combined if these are not contaminated.

SUMMARY

Apparatus for rearing germfree animals has been standardized in design and construction to a point where it can be produced commercially, thus making it available to investigators. The operation of this apparatus and the techniques for rearing the animals can be reduced to dependable routines so that experimentation is possible. Because this is the present status, it is now possible to examine the operation and function of the apparatus with some degree of certainty.

Only the Reyniers germfree apparatus has been considered in this report, because it is the only apparatus that we have used and because it has been developed over a period of 25 years. Because the device has become standardized in design and use an analysis of its operations becomes meaningful.

The basic design of this apparatus is considered. Essentially, it consists of a series of isolators that can be connected for transferring germfree animals and carrying them through generations without contamination. The animals can be held through their complete life span. This apparatus is made to be sterilized *in situ* with steam under pressure and, while it may be used with chemical sterilization, the preferred method is heat. Because the device is made to be used with steam pressures, it is strongly constructed and will stand continual use for years.

Two groups of apparatus, sufficiently standardized for the purpose, are considered. Each group was directly under the supervision of a single investigator. Group I consisted of 6 germfree isolators operated by a single technician (a single technician can properly operate 10 isolators). This group of isolators was used for cancer studies, primarily with mice and rats. Mice were bred to the tenth generation, as were rats, and held for more than 900 days. Group II studies involved only guinea pigs, and included the recording of cesarotomies, rearing, holding, and experimenting on the animals. The two experimental groups cover the essential operations necessary to obtaining, rearing, and using germfree animals.

The eight basic operations involved in rearing germfree animals are: (1) disassembling, assembling, and testing the germfree isolator for leaks; (2) initial sterilization of the isolator; (3) placing germfree animals in the isolator; (4) supplying the animals with sterilized food, water, bedding, and necessary instruments through supply-lock operations; (5) passage of heat-labile materials and embryonated eggs into the germfree isolator; (6) sterility checks on the

animals and isolator interior; (7) sterilization of air for ventilation; and (8) housing and maintaining the animals.

The results of these studies are discussed. From the results it is possible to conclude that a germfree isolator may be operated without contamination for at least 21 months continuously. Rats and mice may be reared germfree for at least 10 generations and for as long as 900 days. The operations are no more expensive in terms of manpower or time than with any kind of germfree apparatus. In the Group I operations one contamination occurred due to a warped outer supply lock in more than 2 years of continuous service, involving over 1200 supply-lock passages, 64 isolator-to-isolator transfers, and more than 700 animals. In the Group II operations 114 experiments resulted in 16 contaminations: 11 due to experiments involving marginal sterilization of new type diets; 1 because of surgery; 2 due to apparatus failure (punctured gloves); 1 to a contaminated inoculum; and 1 to causes unknown. Over 900 guinea pigs were used in 5 years of operations, and some animals have been held for 14 months. These studies were performed with apparatus made in the institute shops and must be regarded as developmental.

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