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Procurement and Maintenance of Germ-Free Swine for Microbiological Investigations[1]

R. C. MEYER,[2] E. H. BOHL, AND E. M. KOHLER

Department of Microbiology, The Ohio State University, Columbus, Ohio

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ABSTRACT

MEYER, R. C. (The Ohio State University, Columbus), E. H. BOHL, AND E. M. KOHLER. Procurement and maintenance of germ-free swine for microbiological investigations. Appl. Microbiol. **12**:295-300. 1964.—Germ-free swine were routinely procured by both hysterectomy and hysterotomy (Caesarian section). By means of light-weight portable equipment, piglets could be obtained and transported to the laboratory (without contamination) over distances in excess of 100 miles. The isolators employed in rearing were constructed of stainless steel and flexible plastic film. At weekly intervals, fecal swabs and waste from the floor of the isolator were cultured on blood-agar and in thioglycolate broth, as well as being examined microscopically for the presence of bacteria, yeast, and fungi. The presence of pleuropneumonia-like organisms (PPLO) and viruses in such material was not demonstrable, either by the use of enriched PPLO media or primary porcine-kidney cell cultures. Tissues, body fluids, and cecal contents of piglets sacrificed specifically for microbiological examination were also negative for PPLO, viruses, bacteria, yeast, and fungi. Prenatal infestations by ascarids were not observed. Nutritional problems related to rearing of germ-free piglets, such as hypoglycemia, were not encountered, and the use of an autoclaved commercial sow's milk replacer proved quite satisfactory. The temperature to which piglets were subjected during the first few days of life, however, was very important. The isolator design and application of gnotobiotic techniques to the procurement and rearing of a large germ-free animal such as the pig proved feasible and less difficult than anticipated.

Although theoretical considerations, principles, and basic techniques essential to rearing small gnotobiotic animals have been available for many years, it is only quite recently that developments and modifications in equipment have permitted the use of large germ-free animals in laboratories and situations where a few years ago it would have been highly impractical, if not impossible.

The present work with germ-free swine at Ohio State University stems primarily from studies and interests in the enteroviruses and intestinal flora of this domestic animal. It is worthy of note, however, that the pig possesses numerous physiological characteristics desirable in an experimental animal. With reference to man, similarities exist relative to their nutrition, hematology, and the anatomy of their respective vascular systems, skin, eyes, and intestinal tract. In this context, the pig, while not presently recognized as such, could very well prove to be a useful and adaptable experimental animal for a wide variety of biological studies.

The first reports on the experimental use of germ-free swine in an isolator system were by Landy, Growdon, and Sandburg (1961) in a study on sterile bile peritonitis and by Weide et al. (1962) in a study of hog cholera.

The following is a description of procedures and equipment which, in our experience, have proven feasible for the procurement and rearing of gnotobiotic swine.

MATERIALS AND METHODS

Germ-free piglets were routinely obtained by the standard techniques of hysterectomy and hysterotomy (Caesarian section).

Hysterectomy. Healthy, pregnant sows of known breeding date were selected for surgery on or near the 112th day of gestation. The sows were washed with a stream of warm water from a hose, suspended by their rear legs, and their abdomen cleaned with a strong germicidal solution of Clorox (Proctor & Gamble Co., Cincinnati, Ohio). The sows were anesthetized with CO_2 by placing a large canvas bag over the head and employing a standard CO_2 fire extinguisher (Fig. 1).

Proceeding quickly, an incision was made down the midline, and the uterus was removed and introduced into a sterile stainless-steel isolator through a germicidal trap containing a strong Clorox solution.

By working through rubber gloves in the sides of the surgical isolator, the piglets were removed from the uterus, separated on the basis of sex, and passed into one of two attached transfer isolators (Fig. 2) where the piglets were washed in a 10% solution of Weladol (Pitman-Moore Co., Zionsville, Ind.), dried, and their umbilical cords ligated and cut.

As a clean, sterile surgical unit was employed for each sow, the transfer isolator was sealed off and disconnected after each operation. To reduce the time interval between operations, two surgical units were employed; while one was in use, the other was in the process of being cleaned and sterilized for the next operation.

Depending upon the size of the litters, male piglets from

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[2] Present address: National Cancer Institute, National Institutes of Health, Bethesda, Md.

two or three sows were placed into one transfer isolator but kept separate in large plastic trays. When the isolator was full, it was disconnected and prepared for transport back to the laboratory.

Hysterotomy. This procedure was previously reported in some detail elsewhere (Meyer et al., 1963).

In essence, the procedure consisted of attaching a plastic surgical isolator to the skin and flank of an anesthetized sow (Fig. 3). By working through the rubber gloves attached to the sides of the isolator, an incision was made with an electric cautery through the floor of the isolator and integument of the sow. The uterus was exposed and opened, and the piglets were passed directly into an attached transfer isolator where they were washed, dried, and their umbilical cords ligated and cut as discussed under hysterectomy. In this procedure, unlike the hysterectomy, a surgical repair of the sow was possible, and in such cases was routinely performed.

Transport. Light-weight, flexible-film isolators, equipped with an auto battery-powered d-c air blower, were employed in transporting the piglets from procurement locations. These units were each designed to carry 15 piglets and to fit into the rear of a standard six-passenger station wagon (Fig. 4). Sterilized in advance, these units contained all the instruments and various items needed to perform routine hysterectomies. Upon returning to the laboratory, the piglets were transferred into special rearing units designed to hold two piglets.

Rearing. The isolators used for rearing were composed

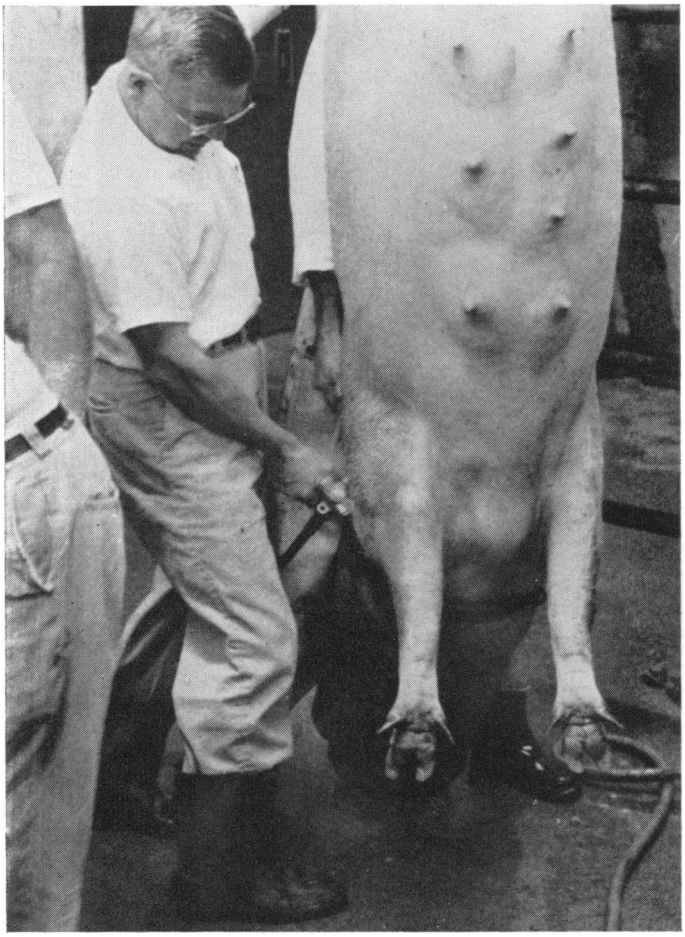

FIG. 1. *Sow being anesthetized with CO_2 fire extinguisher.*

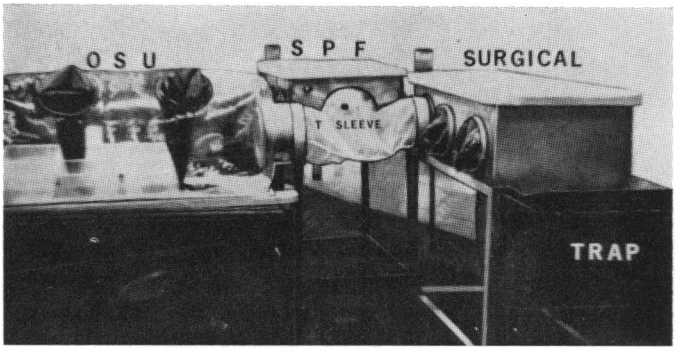

FIG. 2. *Metal SPF surgical isolator with germicidal trap on right and attached flexible film and SPF transfer isolator.*

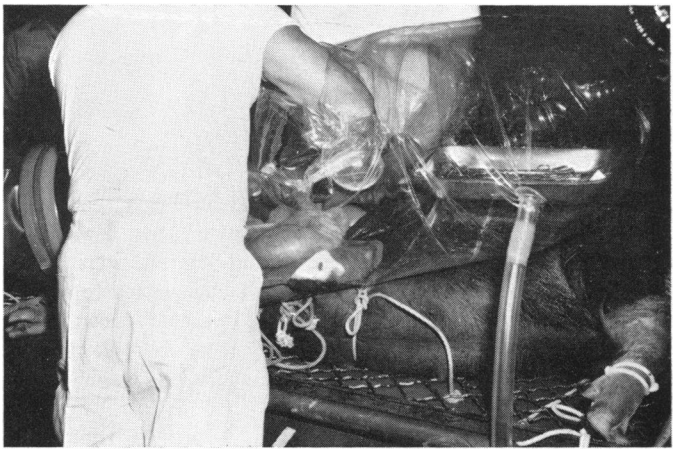

FIG. 3. *Plastic surgical isolator attached to flank of sow.*

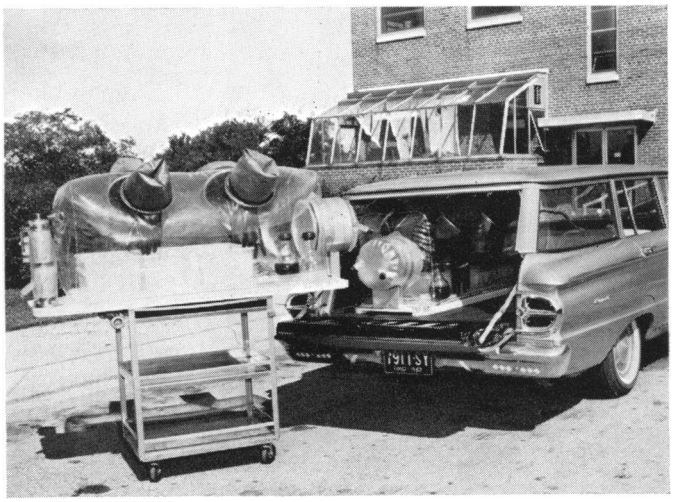

FIG. 4. *Light-weight flexible-film transfer isolators ready for transport.*

of leak-proof, stainless-steel, rectangular tubs, 22 by 28 by 24 in. (55.8 by 71.1 by 61 cm), fitted with a flexible plastic canopy, a 12-in. (30.5-cm) port, and two pairs of rubber gloves (Fig. 5).

Each tub or metal portion of the unit contained a removable false bottom, partition, feeders, retaining grid, and shelf of stainless steel (Fig. 6).

The canopy was fabricated of polyvinyl chloride. The air blower and filters were similar to those described by Trexler and Reynolds (1957).

When it was necessary to hold a pig beyond a 3-month period, they were transferred into the large Pen-tub isolator (Fig. 7).

The temperature for the piglets was maintained between 32.2 and 35 C for the first 4 days. Thereafter, it was lowered over a period of 2 to 3 days to 21.1 to 23.9 C.

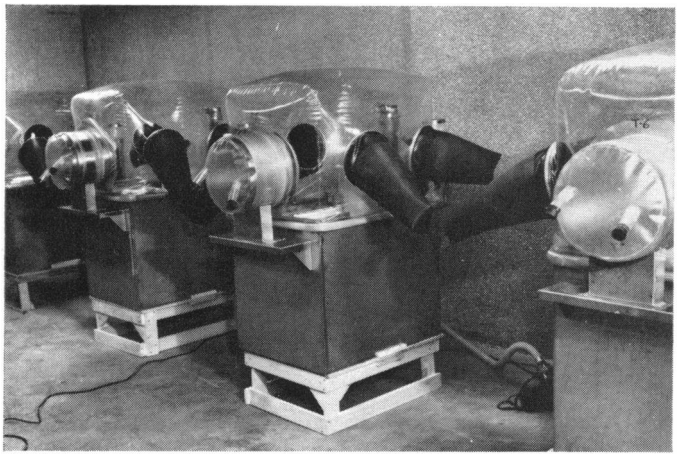

FIG. 5. *Small tub isolators employed in rearing germ-free swine.*

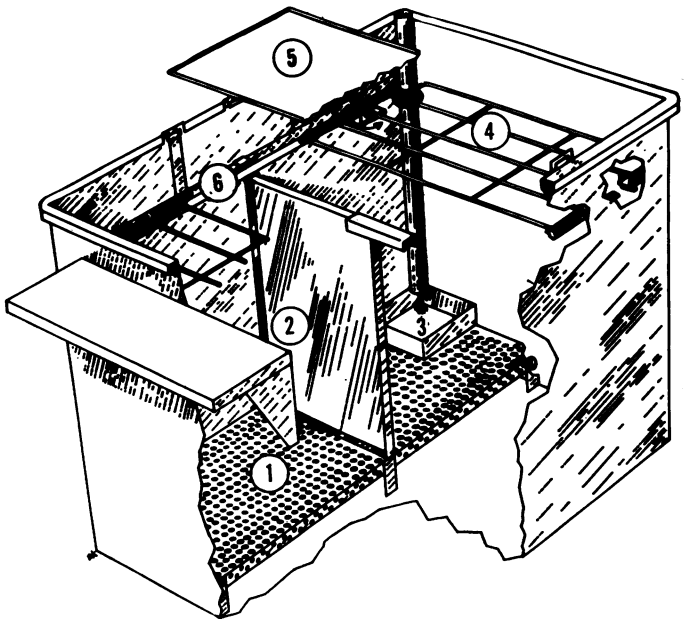

FIG. 6. *Cut-away diagram of small tub unit showing removable (1)f alse floor, (2) partitions, (3) feeders, (4) retaining grids, (5) shelf, and (6) retainer bar.*

During the winter months, chilling of the piglets in transport could be prevented by modifying the air-intake blower and hose so as to utilize the warm air from the car heater.

In the first several rearing experiments, the temperature of the entire isolator room was raised to the desired level by means of electric space heaters. This practice, however, was discontinued wh n it was found that if a temperature of 23.9 C was maintained in the isolator room a proper temperature within the isolators could be achieved by placing an ordinary goose-neck lamp with a 100-w bulb against a metal side of the isolator, thus circumventing the need to raise the room temperature to uncomfortable levels.

All pigs were fed three times daily at 8:00 AM, 3:00 PM, and 8:00 PM. The ration originally employed was a modification of that described by Young and Underdahl (1951), and consisted of autoclaved, homogenized, cow's milk, fortified with minerals, dextrose, eggs, yeast extract, vitamin D, cod liver oil, and agar (Meyer et al., 1963).

The present diet consists of commercial powdered sow's milk replacer [pig milk replacer #601 (without aureomycin); Land O'Lakes Creamery, Minneapolis, Minn.]. The diet was prepared in 22.7- to 26.5-liter lots in a 10-gal (37.9-liter) milk can and, after thorough mixing, was dispensed into 2-liter Square Pak bottles (American Sterilizer Co., Erie, Pa.) in 1,500-ml quantities (Fig. 8). The bottles were then capped, and the neck and cap were wrapped with paper prior to autoclaving. Sterilization was accomplished by autoclaving for 35 min at a pressure of 15 psi.

Determination of germ-free status. Microbiological moni-

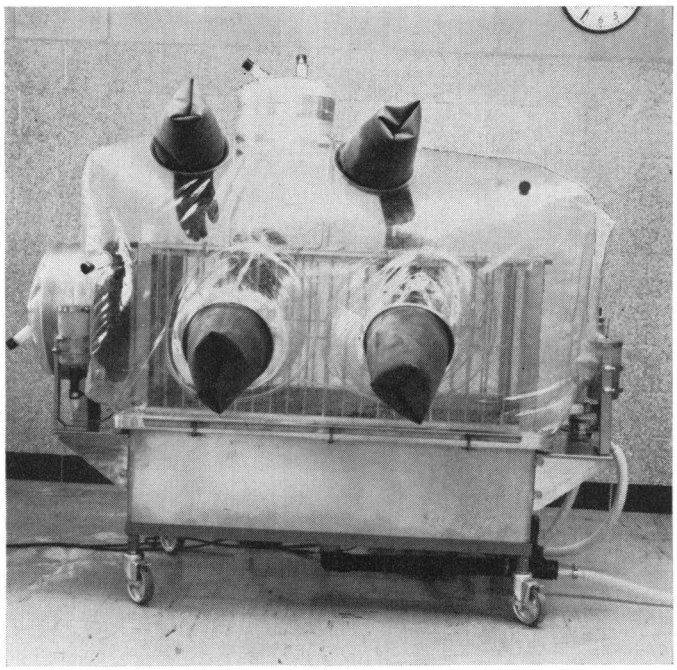

FIG. 7. *Large Pen-tub isolator.*

toring was initiated at the time of birth. At weekly intervals, rectal swabs and material from the floor of the isolators were cultured and examined microscopically for bacteria, yeast, and fungi. Blood-agar and fresh thioglycolate broth were employed as culture media, with incubation temperatures of 20 and 37 C. All such cultures were held for a minimum of 21 days before being judged negative and discarded.

Piglets from several different litters were killed between 2 and 8 weeks of age, and their tissues, body fluids, and cecal contents were examined for bacteria, fungi, pleuropneumonia-like organisms (PPLO), and viruses. The technique used for attempted isolation of PPLO was that described by Lecce (1960). Porcine-kidney cell cultures were employed in checking for viruses.

To determine possible prenatal infestations by ascarids, fecal material was collected from a piglet on 4 consecutive days, pooled, and examined by the $NaNO_3$ flotation technique (Koutz and Rebrassier, 1959). Intestinal-cecal contents and the tissues of animals sacrificed in the course of studies were also examined.

Sterilization and preparation of isolators. After each use, the isolators were dismantled and thoroughly cleaned. The stainless-steel tub was steam-cleaned with special attention given to the corners. All removable metal parts, such as the feeders, false bottoms, etc., were steam-cleaned, wrapped in brown paper, and baked at 180 C for 2 hr. The clean canopy, sterilized air filter, etc. were replaced on the tub, and the unit was inflated and freon tested for leaks. If found free from leaks, a spray gun was passed inside, and the interior was sprayed with 3% peracetic acid.

The presterilized items, such as the removable metal parts, were unwrapped, sprayed with peracetic acid, and passed into the isolator for assembly. All items were handled with rubber gloves. The units were then sealed, inflated with an aerosol of peracetic acid, and allowed to stand overnight. The next morning the Mylar seal on the air filter was broken, and the unit was put into operation. To be sure they were dry and free from noxious residues, all freshly sterilized isolators were operated a full 24 hr before any piglets were introduced into them.

Removal of wastes. To remove wastes from the isolators while in operation, the end of the unit opposite the drain was raised 5 to 8 cm. The end of the drain tube was placed into a clean bucket containing a small amount of a 3% phenol solution (Fig. 9). While submerged in the phenol solution, the rubber stopper was removed, and the wastes were allowed to drain into the bucket. When the bucket was full, the drain tube was tightly clamped off near its connection to the tub by a simple wooden clamp fashioned from two 1.27-cm wooden dowl rods. The end of the drain tube was then removed from the bucket. Where more than one bucket was needed for complete drainage, the end of the drain tube was transferred into another bucket, the wood clamp was removed, and drainage continued. When drainage was complete, the wooden clamp was again put in place, sealing the tube. The end of the tube was removed from the bucket, and the tube was thoroughly flushed out with 3% peracetic acid. The rubber stopper was rinsed, sprayed, replaced, and taped into place. After 1 hr, the

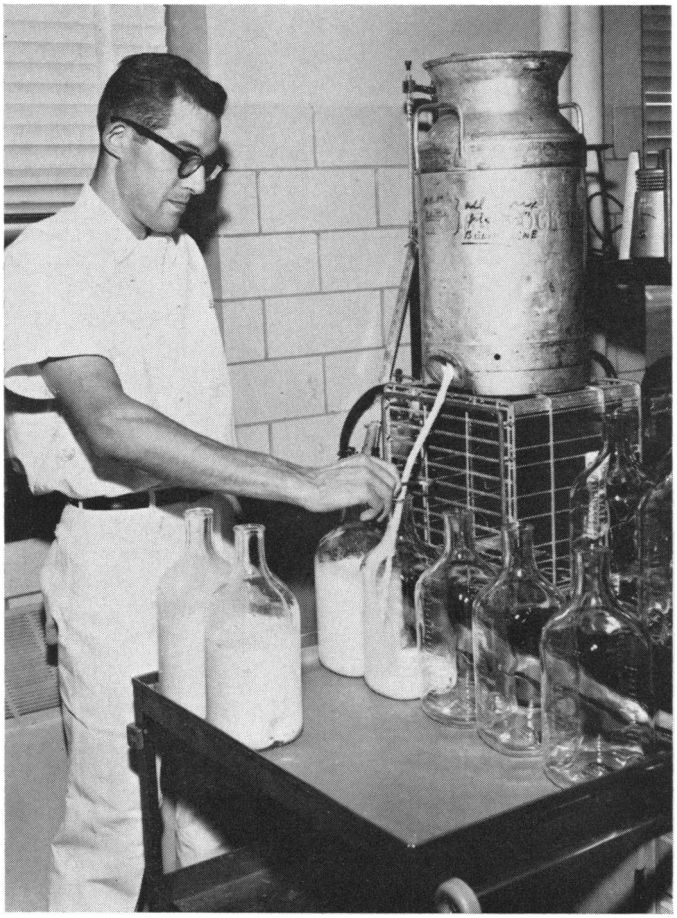

FIG. 8. *Freshly prepared diet being dispensed into 2-liter square paks.*

FIG. 9. *Drain tube submerged in 3% phenol just before removal of stopper.*

wooden clamp was removed, and the unit returned to normal operation.

RESULTS AND DISCUSSION

The outlined procedures have resulted in 19 consecutive germ-free litters: 4 by hysterotomy, and 15 by hysterectomy. Although the hysterotomy was the first surgical technique employed, unlike Landy et al. (1961) we found the hysterectomy to be by far the most convenient. This was true largely because of the availability of pregnant sows at a commercial "specific-pathogen free" (SPF) swine laboratory in Liberty, Ind. With the cooperation of B. G. Eaton and associates at the S. P. F. Laboratories, Inc. of Liberty, Ind., it was possible for us to obtain a large number of young boars "germ-free" from numerous operations, dispensing with the problems associated with: (i) locating, purchasing, and transporting pregnant sows with known breeding date to our laboratories; (ii) variability in the size of litters and the obtaining of sufficient piglets to make the operation worthwhile; and (iii) the postoperative care and disposition of convalescing sows.

Although Dr. Eaton and his associates were primarily interested in procuring and maintaining SPF stock for repopulation purposes, it is noteworthy that, with very few modifications in procedure, piglets could be obtained germ-free on a production basis.

With the ready availability of young boars in Indiana, transportation problems were solved with the plastic, flexible-film isolators and a station wagon (Fig. 4). A conventional 12-v car battery and d-c air blower proved more than adequate, and enabled us to operate and transport such units routinely on round trips, well in excess of 200 miles, while carrying a total of 25 or more piglets from Indiana to the laboratory without contamination. Care was taken, however, to see that the battery was well charged beforehand.

In rearing the piglets, the smaller tub isolator with its removable partition adequately housed two piglets for 1 month (Fig. 5). Usually by that time one of the piglets would have been sacrificed and, with the removal of the partition, there was sufficient space to accommodate the remaining piglet up to 3 months of age. In those cases where it was necessary to hold an animal beyond the 3-month period (Fig. 10 and 11), it did not prove difficult to transfer it to a larger Pen-tub isolator.

The temperature during the first few days of life was very important, with the optimum between 32.2 and 35 C. Animals that had been severely chilled or merely subjected to the ordinary room temperature of 21.1 to 23.9 C for the first few days of life were noticeably retarded.

During the winter months, the car heater in the station wagon was sufficient to prevent chilling of the young when the air intake and blower were modified to use the hot air from the heater.

The use of the goose-neck lamps to heat the metal sides of the isolators proved of value, making the isolator room a more comfortable place in which to work during the period when elevated temperatures were needed.

The original fortified cow's milk diet employed at the start of our studies was both expensive and time-consuming to prepare.

For these reasons, it was decided to try a powdered com-

FIG. 10. *Germ-free Poland-China boar (58.9 kg) in fifth month.*

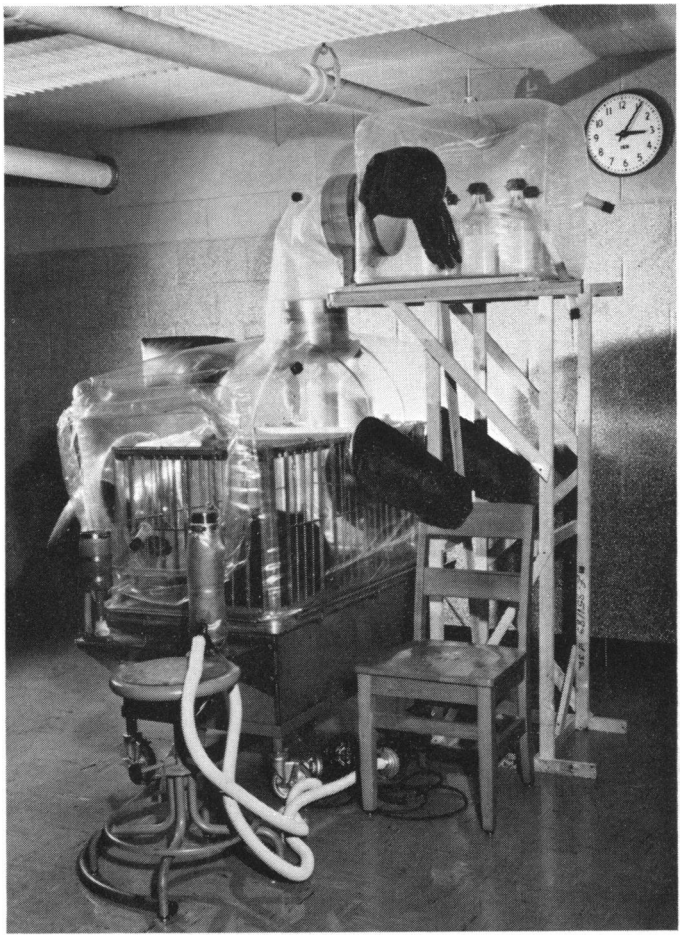

FIG. 11. *Large Pen-tub isolator in operation with small transfer isolator containing diet.*

mercial sow's milk replacer. While some coagulation occurred during the sterilization of the commercial material, it was no greater than that observed with the earlier diet. Upon cooling, all that was necessary to resuspend any coagulated material was a little vigorous shaking before it was passed into the isolator, followed by additional shaking just prior to dispensing to the piglets.

During the period in which the piglets were maintained on this material, no nutritional problems were encountered, and hypoglycemia was not observed. Besides the advantages of lower cost and greater convenience, one could control the total solids-liquid ratio of the diet more easily and accurately.

Since our previous report and change to a hysterectomy procedure, microbial contaminants commencing with surgical removal have not been demonstrable in the 15 litters obtained by hysterectomy. Routine bacteriological monitoring prior to the experimental use of the piglets has been negative. Numerous piglets which had been employed as germ-free controls, and sacrificed at 2, 3, and 6 weeks of age, were negative for bacteria, fungi, PPLO, viruses, and ascarids. In these cases, if such contaminants were present, they could not be demonstrated by conventional techniques. In checking out our procedures, equipment, and diet, however, no attempt was made to hold a pig germ-free longer than 6 months.

The accumulation of urine and excrement during the first 2 to 3 weeks of life was not excessive. Even when the units contained two piglets, as was the normal case, drainage did not become necessary until the fourth week. Thereafter, however, drainage became more frequent and was routinely performed as needed.

With the diets employed, the fecal materials (stools) were semisolid, and blockage of the drain was not encountered. A diameter of 2.5 cm in this respect appears satisfactory.

With the larger isolators safely accommodating germfree swine into the 45- to 68-kg range, the equipment's design and basic gnotobiotic procedures have been proven adequate for the procurement and rearing of large germfree animals such as swine.

ACKNOWLEDGMENTS

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