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Gnotobiotes

Standards and guidelines for the breeding, care, and management of laboratory animals

A Report of the
Subcommittee on Standards for Gnotobiotes
Committee on Standards
Institute of Laboratory Animal Resources
National Research Council

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PREFACE

The widespread use of gnotobiotic animals, either germfree or intentionally associated with micro-organisms, makes it essential that standards and guidelines be developed to assure their uniform quality. A substantial number of germfree animals are produced at facilities that are not controlled by the investigator. This monograph gives recommendations for the production, characterization, and transport of germfree rats and mice. Short sections on the production and maintenance of germfree guinea pigs and poultry have been added, although these species are not yet generally available, and much less experimental experience with them has been accumulated.

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I STANDARDS AND CHARACTERISTICS OF GERMFREE RATS AND MICE

A. DEFINITION

The germfree animal may be defined as a gnotobiote that is free of all demonstrable microbial associations as determined within the limitations of the detection procedures available (see Chapter V).

Animals can be raised with a good assurance that they are free from bacteria and related forms, including yeasts, fungi, protozoa, and metazoan parasites. The viral status of these animals is open to question because latent viruses may be present but remain undetected.*

B. STANDARDIZATION AND CHARACTERIZATION

Germfree mice and rats, the most widely used germfree animals, are now readily available from commercial sources. As guidelines for standardization, only general characteristics can be considered, and the detailed analysis of the biologic properties of organs and tissues must devolve on the investigator. For his guidance, the researcher can use the extensive studies published in recent years on the anatomy, physiology, immunology, and pathology of germfree animals (1).

*According to the methods currently recommended in Section V.B., page 30, for virus detection, so-called germfree mouse colonies carry a leukemogenic virus, and some mouse strains also carry a mammary tumor virus. However, these or similar viruses have not yet been found in the rat by similar test procedures. Mice carrying leukemogenic or mammary tumor virus will have to be so designated according to the nomenclature adopted for designation of various gnotobiotic lines of mice (2).

To characterize germfree mice and rats in a uniform and comparable manner, the data indicated in the remainder of this section should be made available by the producer.

General characteristics of animal colonies

The following information should be made available:

- Designation of species, inbred or noninbred stock.
- Breeding system.
- Average weight and litter size at birth.
- Average weight and litter size at weaning. If litters are intentionally reduced, this should be stated.
- Detailed composition of diet and sterilization procedures.

Rate of growth

To establish growth curves and their confidence limits, a number of steps must be standardized:

1. Each age group should consist of a minimum of 25 males and 25 females.
2. Animals should be weighed at weekly intervals from the time of weaning until mice and rats are 10 and 16 weeks old, respectively.
3. Weighing should be done on nonspring balance scales at the same time of day. Weight should be recorded within 1/2 gram for mice and to the nearest gram for rats.
4. If rearing procedures are changed, new curves should be compiled.
5. The weight of animals supplied according to such growth curves should not differ by more than 1 standard deviation from the plotted group mean.

Cecal weight to body weight ratio

Since the cecum of germfree rodents is generally enlarged, and because this enlargement affects physiology and metabolism of the animals, quantitative knowledge about the size (expressed as weight) of the cecum is essential. In the collection of such data, the following guidelines should be observed:

- Anatomically, the cecum is the portion of the bowel that extends from the ileocecal valve to the beginning of the rugal folds of the colon.
- Ceca should be clamped, removed, and weighed immediately with

all contents. The weight should be expressed per 100 grams of total body weight.

- Mice should be sampled at 10 weeks (4 weeks optional), and rats at 16 weeks of age (4 weeks optional).
- Separate data should be obtained for every strain and stock. Since diet affects cecum size, diet composition or a suitable reference should be shown.
- Separate data should be compiled for two groups, one of 25 males and one of 25 females.

All data supplied according to Section B should be redetermined at least every 2 years, and whenever there has been a change in diet.

C. ADDITIONAL CHARACTERISTICS

The findings outlined below are generally present but may be modified by genetic and dietary variables.

Intestinal contents

The contents of the enlarged cecum are usually liquid. Germfree mice tend to defecate well-formed pellets. Rat feces may be soft and resemble those typical of diarrhea, although the animals are and remain healthy.

Changes in other organs

The protection of germfree animals from interactions with viable environmental micro-organisms is reflected in the low level at which their cellular and humoral defense mechanisms operate (3). The morphologic expression of this relative hypoactivity is particularly discernible in organs and tissues that, in conventionally reared animals, are most intimately exposed to the effects of a microbial flora, i.e., the gastrointestinal tract, the lymph nodes, and the spleen (4). The germfree state of the animal thus tends to modify size, cell composition, and metabolism of these organs and affects their products; e.g., the serum gamma globulin level of germfree animals is lower than that of their conventional counterparts (5).

II PRODUCTION, CARE, AND MAINTENANCE OF GERMFREE RATS AND MICE

A. FACILITIES AND EQUIPMENT

An individual desiring to work with gnotobiotic animals should be familiar with the husbandry of that particular species in the conventional environment (6). The following standards are based on this assumption and on present-day knowledge and experience in the care and maintenance of gnotobiotic mice and rats. They apply to populations of experimental animals, as well as to those of production colonies.

Room environment

The facilities, equipment, and husbandry procedures shall be designed and operated so as to afford maximum environmental control and optimal comfort and welfare for the animals. The cages, feeders, and waterers shall be so designed and fabricated as to afford maximum comfort for the animals, to make the food and water readily available, and to make cleaning and sterilization practicable and efficient.

A desirable floor plan for extensive germfree work should consist of:

1. a work area for assembling and sterilizing the isolators
2. an area for maintaining the isolators with animals
3. a laboratory area for the routine monitoring of the gnotobiotic environment

An office and diet-preparation area may be incorporated in the floor plan.

The room environment for maintaining gnotobiotic isolators should meet the standards established for housing conventional laboratory rodents. The structure should be insect-proof and rodent-proof, and the walls and floor should be moisture-proof. Lighting should be uniform, with the same light–dark cycle throughout the year. Ventilation should rapidly remove any fumes caused by chemical sterilization, and the climate should be controlled as specified below.

Temperature The generally accepted animal room temperature of 21°–27°C (70°–80°F) may need to be adjusted downward to keep the isolator temperature between 22° and 26°C (72° and 78°F).

Humidity The relative humidity (RH) should be kept at the human comfort level of 40–60 percent. However, when room air is used to ventilate the isolator, 40–50 percent RH is recommended.

Ventilation The room-air changes should be sufficient to remove rapidly any fumes generated during chemical sterilization. Ten to fifteen air changes per hour are recommended. Head masks with fresh-air ventilation should be available to protect personnel exposed to dangerous levels of chemical fumes.

Germfree equipment (7,8)

Complete exclusion of environmental microbes requires an absolute barrier. The successful operation of the isolator depends on the maintenance of that barrier at all times. There are two general types of isolators available, metal and plastic (Figure 1). Some metal units are built to withstand internal steam pressure of 20 psi (1,406 g/cm^2) (9,10). Others are generally placed in a large autoclave for initial sterilization (11).

The flexible-film isolator (12) is now the most widely used unit. It is usually made of flexible laminated vinyl, must be chemically sterilized, and is readily adapted to specific needs. Another type, made from a large tube of nylon, tied at each end, can be sterilized in an autoclave (13). Plexiglass isolators and disposable flexible-film units have also been developed. Many of these are light enough to be stacked two or three high on a rack, a feature that conserves floor space.

A special cylinder for sterilizing food and supplies is generally used with the heat-sensitive isolators. It should be designed with a large fil-

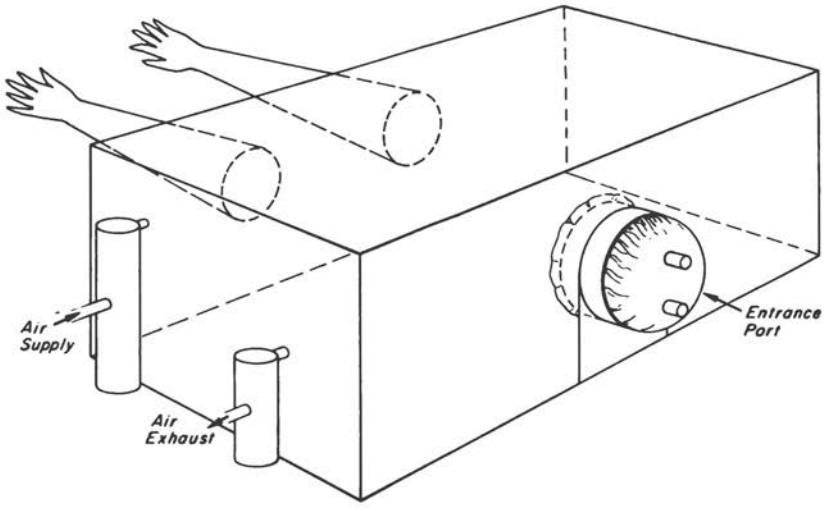

FIGURE 1 Diagram of the type of isolator in general use for germfree animals. This type is constructed either of metal or of plastic.

tration area to facilitate air removal in a high-vacuum autoclave (14). Alternatively, the cylinder may be fitted with a drain tube vented to the atmosphere for removal of air and condensation during sterilization without the benefit of a vacuum (15).

Sterilization

All equipment, food, bedding, water, and air used in the isolator must be absolutely sterile. The methods and conditions employed are determined by characteristics of the individual items.

Steam under pressure is the best-known method of sterilization. It is particularly suitable for porous items that are heat-stable. Every area that can conceivably harbor microbes must be brought into direct contact with steam. Exposure time is related to the temperature used. It is recommended that the *least accessible portion* of the load (the center of the packages) be exposed for a minimum period of 15 minutes at 121° C (250° F). Higher temperatures and shorter exposure periods may be used after careful testing to ensure absolute sterilization. Standard package

size and density of diet, bedding, and other materials are of primary importance to assure that the steam penetration time will be constant and predictable.

Dry heat has been used for sterilization of the air supply for the isolator (10,11).

Peracetic acid (CH_3COOOH) is widely used on heat-sensitive, nonporous materials, especially the flexible-film units. This acid is used in a 2 percent solution with a wetting agent (detergent) (12). Other chemicals may be used for special situations, e.g., hypochlorites, iodophors, or quaternary ammonium compounds in the liquid trap to introduce newborns obtained by hysterectomy, or $HgCl_2$ to introduce eggs under sterile conditions prior to hatching.

Ethylene oxide (ETO) may be used to sterilize nonwettable heat-sensitive items. Sterilization time is dependent on the temperature, humidity, pressure, and concentration of ETO. ETO may react chemically with bedding and dietary components to produce toxic or undesirable compounds. Because of its flammability and toxic hazards, routine use of ETO for sterilization should be restricted to the commercially available gas mixtures, which contain not more than 20 percent ETO (16).

Fiber glass filters are commonly used for sterilization of the air supply. They should function as absolute filters.

Membrane filtration of liquids can be used to avoid exposure to heat, provided these membranes are absolute filters.

Irradiation by gamma rays or electron-beam sources may be used to sterilize diets or other special items. Dosages employed vary from 2.5 to 6×10^6 rads.

Internal environment

Temperature The internal isolator temperature is a function of the room environment and should be maintained between 22° and 26°C (72°-78°F).

Humidity The isolator is subject to condensation of moisture in cases of overloading, inadequate ventilation, or both. Air entering the isolator should be below 50 percent RH and preferably above 40 percent RH.

Air Supply The isolator should have 12 to 20 air changes per hour and a positive pressure of 3–5 in. (8–13 cm) of water. Air may be supplied from a central source or from individual blowers for each unit. A turbine-type air compressor is recommended for a central air-supply system because the oil-piston type tends to atomize oil into the air-supply lines.

An air-diffusion isolator (8) is not subject to loss of ventilation in the event of power failure. However, this type has the disadvantage of fewer air changes per hour and lacks the protective positive pressure that could help prevent contamination should small breaks occur in the barrier.

Emergency Safeguards Adequate provisions for the maintenance of air pressure within the isolator in the event of power failure or mechanical failure must be provided with not more than a few minutes' interruption in the air supply. Collapse of unsupported film isolators may eventually result in suffocation of the animals, but the more immediate danger is that the animals may be able to reach and damage film or gloves. This may be prevented temporarily by plugging air conduits with rubber stoppers. The operation of individual isolator air supplies requires only an emergency power supply. A central air system should have a second turbine compressor for standby air supply.

Graphic recording of the temperature and pressure is recommended. An audiovisual alarm system should be incorporated in a central air system to be actuated by a drop in line pressure in the event of either loss of power or mechanical failure. Similar alarm systems should indicate undesirable fluctuations in the temperature of the air supply. For individual isolator air systems, continuous graphic monitoring of the room environment is recommended.

Caging and interior equipment

Equipment A basic list of equipment for an isolator may include cages with secure lids, water bottles and food hoppers, protective cloth gloves for the rubber gloves, an extra door gasket or cap closing ring, long rubber-tipped forceps, hemostats, scissors, a towel, gauze sponges, a two-quart can for holding instruments, a covered four-quart diet can, a

spoon, culture tubes, paper bags, and moisture-resistant bags for dirty bedding.

Cages shall be fabricated of a smooth corrosion-resistant material. They shall be impervious to liquids and easily sterilized. Materials considered acceptable include plastics, stainless steel, and glass. Galvanized metal becomes corroded and is not recommended because trace-metal contamination may influence experimental results.

Cage dimensions are usually limited by the size of the entry port. The minimum area for a female mouse and litter is 50 in.^{2} (325 cm^2); for a female rat and litter 150 in.^{2} (970 cm^2). In many circumstances more space per animal may be needed.

Table 1 lists the recommended floor space per animal for mice and rats according to weight groupings (6).

Miscellaneous recommendations

Freon tests for minute leaks are recommended to ensure the integrity of the barrier system.

Each unit should be equipped with its own operations log to maintain a chronological record of every procedure involving the unit from the time it is assembled and sterilized. Such records are conveniently kept

TABLE 1 Amount of Floor Space Recommended per Animal for Caged Mice and Rats

Category Number	Weight (g)	Space per Animal in.^{2} (cm^2)	Maximum Population Per Cage
MICE			
1	up to 10	6 (40)	40
2	10–15	8 (50)	30
3	15–25	12 (75)	20
4	over 25	15 (95)	16
RATS			
1	up to 50	15 (95)	50
2	50–100	17 (110)	50
3	100–150	19 (125)	40
4	150–200	23 (150)	40
5	200–300	29 (185)	30
6	over 300	40 (260)	25

in metal hospital-chart holders identified by the isolator number. They should also contain notes for routine maintenance, e.g., glove replacement. Breeding-performance records may be kept in the same chart holder.

Due to the limited space available inside the isolators, paper and folding containers are recommended for diets and bedding, and for the transport of animals between isolators linked by a sterile passage.

No ether should be used inside an isolator because it may explode when static sparks occur. Fluothane (bromochlorotrifluoroethane) is recommended as a volatile, nonflammable anesthetic.

B. DIETS, BEDDING, AND WATER

General recommendations

The complete formula for commercially produced diets should be provided, listing all the ingredients and their concentrations, including preservatives, antioxidants, and other additions. The date of production should be clearly indicated. The manufacturer should guarantee that the diet is:

1. Within the normal acceptable limits of naturally occurring hormone activity
2. Free of additives containing drugs, hormones, antibiotics, or any other substance that may create abnormal physiological conditions or interfere with investigative procedures
3. Free of salmonella on the basis of statistically selected samples
4. Free of rodent and vermin contamination
5. Free of all unrendered meat scraps or fish meal that may contain pathogens

Fortification of diets

Diets for germfree animals must contain more than normal requirements of certain nutrients to compensate for the heat-sterilization loss of vitamins (especially certain B vitamins and vitamins A and D) and of the nutritive value of protein (reduction in available lysine, methionine, arginine, and tryptophan). They must also provide required nutrients, which in conventional animals would be available through microbial

synthesis in the gastrointestinal tract (17). An example of such a diet is L-485, an inexpensive diet that has been extensively tested (18) and can be commercially produced (see Table 2). Supplementation with specific amino acids rather than increased total protein content should be considered as a means to compensate for loss in protein quality. Increasing the total protein content of the diet will result in a greater consumption

TABLE 2 Composition of Diet L-485 for Rats and Mice

Ingredient	Amount per kg	
DIET		
Ground yellow corn (maize)	590	g
Soybean oil meal (crude protein 50 percent)	300	
Alfalfa meal (dehydrated; 17 percent protein)	35	
Corn oil (once refined)	30	
NaCl	10	
$CaHPO_4 \cdot 2H_2O$	10	
$CaCO_3$	5	
Lysine (feed grade)	5	
Methionine (feed grade)	5	
B.H.T. (butylated hydroxytoluene)	0.125	
Trace mineral mix	0.25	
VITAMIN MIX		
A	26,000	IU
D_3	1,000	IU
E (α tocopherol acetate)	225	mg
K_3 (menadione)	90	
Riboflavin	30	
Pantothenic acid	285	
Niacin	65	
Choline chloride	2,000	
B_{12} (0.1 percent trituration in mannitol)	2	
Thiamine HCl	65	
Pyridoxine HCl	20	
Folic acid	10	
Para-aminobenzoic acid	50	
TRACE MINERAL MIX (commercial)		
Mn as manganous oxide	65	mg
Fe as ferrous carbonate	20	
Cu as copper oxides	2	
Zn as zinc oxide	15	
I as calcium iodate	1.3	
Co as cobalt carbonate	0.6	

and excretion of water, causing humid conditions and thereby limiting the number of animals that can be housed in an isolator of a given size.

Steam sterilization (17)

Actual procedures will depend on the equipment available. Three factors are of general importance:

1. A presterilization vacuum, whenever possible, of at least 20 in. Hg will assist steam penetration of the diet in a clave or cylinder vented to the atmosphere. A vacuum of 28 in. Hg or more is recommended when the supply cylinder is not vented to the atmosphere.

2. Use of the shortest sterilization phase that will ensure total sterility, with an added safety margin dictated by equipment and skill. Temperatures measured at the inner core of the diet should reach at least 121°C (250°F). At that temperature the actual sterilization phase should last a minimum of 15 minutes. With higher sterilization temperatures, sterilization times will be relatively shorter.

3. A poststerilization vacuum will speed the reduction of temperature of the diet. This will avoid unnecessary heat destruction of nutrients. However, the design and performance of the apparatus must be adequate to avoid leaks during this stage of the operation.

In steam sterilization of diets, the goal is to avoid both incomplete sterilization and unnecessary nutritional damage caused by excessively prolonged heating. Although some nutrient loss is unavoidable, quite acceptable results may be obtained by manipulation of:

a. Technical procedures such as temperature, time presterilization and poststerilization vacuum, and pellet size.

b. The water content of the diet. An increase in water content leads to better recovery of B vitamins after sterilization (19). For solid diets, a water content up to 25 percent, or as high as proves to be compatible with the storage quality of the diet, is recommended. A change in water content of the diet should be followed by a new test of the rate at which the diet reaches sterilizing temperature.

Radiation sterilization (17,20)

Techniques and dosimetry will depend on equipment and type of radiation. Although, in general, radiation sterilization is considered to result in less destruction of nutrients, it is at present recommended that diets

to be sterilized by irradiation be fortified in the same way as diets to be sterilized with steam.

Test for sterility

To monitor sterility achieved with any specific sterilization procedure, the use of *Bacillus stearothermophilus* spore strips* is recommended. The strips should be embedded in the core of the diet.

Estimation of nutrient loss during sterilization

As a useful check on the loss of vital nutrients, determination of acid-extractable thiamine as an indicator of the recovery of thiamine added to the diet is recommended (21). A recovery of less than 25 percent indicates severe impairment of general nutritional quality of the diet. With adequate equipment and care, recoveries of 50 percent or more should be achieved.

Storage of solid diet

Because of the generally high cost of germfree experimentation, extra care should be taken never to use diet that has decreased significantly in nutritional value. It is recommended that (a) nonsterilized diet always be stored under refrigeration, and never for longer than one month, and that (b) storage time of sterilized diet inside the isolator should be one week or less and must never exceed ten days.

Bedding

Bedding should be changed at least once a week. It is recommended that bedding material be easy to sterilize and not readily eaten by the animals. It should not yield toxic compounds as a result of the sterilization procedure. Dustfree white pine chips (sawdust) and shavings are recommended. Basswood and poplar shavings or crushed corn cobs are acceptable. Diatomaceous products, cedar, resinous woods, and hardwoods are not recommended. Ethylene oxide sterilization should not be used until the question of possible formation of harmful compounds has been clarified.

*Commercially available.

Water

Drinking water must be sterilized. It may be autoclaved in square pack flasks, Mason jars, or tanks attached to the unit. A small air space should be left inside each container.

C. PRINCIPLES OF CESAREAN DERIVATION OF GNOTOBIOTES

The success of any cesarean operation is keyed in part to having the pregnancy advance to full term. This is particularly true of animals with short gestation periods, where the fetus may gain 20 percent of its weight in the final 24 hours before parturition. Timed matings are reasonably successful, but with animals yielding large litters (rats and mice) it may be helpful to wait for the female to deliver the first offspring before proceeding with the operation. In guinea pigs, the most satisfactory method is to select females for surgery by measuring the spread of the pubic bones (23).

The cesarean-derived young must be delivered into a germfree environment before they take their first breath of air. They may be taken directly from the mother by hysterotomy, through an incised sterile barrier membrane into a sterile isolator, or by hysterectomy, through a germicidal trap into a sterile isolator. The usual surgical preparation of the female prior to the cesarean operation includes removal of abdominal hair and cleansing and disinfection of the operative site. Anesthesia is accomplished preferentially by dislocation of the cervical vertebrae in rats and mice, although an abdominal midline local anesthetic or general anesthesia may also be used without incurring serious levels of fetal depression and mortality. With guinea pigs, surgery is generally performed after prior sedation and under local anesthesia.

Delivery of the young by hysterotomy through a barrier membrane requires special isolator equipment. The Reyniers stainless-steel surgical unit (24) has a built-in horizontal metal divider that separates the upper and lower compartments of the unit. The divider contains a circular port covered with a mylar plastic film to maintain the integrity of the upper compartment. The female, prepared for surgery, is placed in the lower compartment with the abdomen pressed against the mylar. All surgical instruments are in the upper compartment, and the surgery is performed in this sterile area. An incision is made through the plastic and skin with an electrocautery or scalpel. The self-sterilizing electro-

cautery blade is preferred for skin incisions. The edges of the skin and mylar are clamped together and reflected. A sterile drape is placed over the abdomen to cover the cut edges of the skin, and warm disinfectant (benzalkonium chloride 1:1,000) is applied to the exposed fascia before opening the abdominal cavity. Extreme caution must be exercised to avoid cutting into the bowel. The insertion of a pair of forceps or hemostats between the peritoneal wall and the viscera may be helpful. The uterus is then opened and the young removed. The fetal membranes are removed, and the umbilical cord is clamped and cut. The young are gently dried and massaged to stimulate respiration. They are then transferred to a rearing unit to be foster-nursed or hand-fed. Another sheet of mylar may be secured over the surgical port and the procedure repeated with as many as 5 or 6 females without serious risk of contamination. Cesarean delivery may also be accomplished using a plastic isolator or glove bag as a surgical unit. The exterior surface of the isolator floor is presterilized and brought into contact with the animal's abdomen, thus serving the same purpose as the mylar sheet described above. Following the operation the slit in the plastic barrier can be closed with sterile tape and the surgical procedure repeated on additional gravid females.

Delivery of the young by hysterectomy is more common when plastic isolators are used (25). The uterus is aseptically exposed and clamped just anterior to the cervix. The excised uterus is transferred into the germfree unit through a liquid germicidal trap. Once inside the isolator, the young are delivered as rapidly as possible to prevent aspiration of fetal fluids. Normally they are dried and breathing well before the umbilical cord is clamped and cut. The infants are then given to the foster mother or hand reared.

Hysterectomy may be used successfully for mice, rats, and swine. In guinea pigs, however, hysterotomy is preferred, since a high mortality occurs if as much as two minutes elapses between the severance from the maternal blood supply and delivery inside the isolator.

D. BREEDING SYSTEMS IN GNOTOBIOTIC COLONIES

Inbred strains

The usual brother X sister mating system employed in conventional breeding colonies can also be used in gnotobiotic colonies. For details

of proper implementation of such a breeding system references 26–30 should be consulted.

Noninbred stocks

True random breeding includes some matings of siblings and of first cousins. Although such matings are normally avoided in noninbred breeding colonies, the resulting mating system does not decrease the rate of inbreeding to the maximum extent possible. In order to maintain a noninbred colony under a known *minimal* rate of inbreeding, a mating system such as described in Section D, "Mating system for minimal inbreeding," should be used.

Gnotobiotic Colonies Any system of minimal inbreeding (28,30) can be used. However, if a small long-term germfree breeding colony includes an effective breeding population of less than 15 pairs (resulting in a rate of inbreeding of 1 percent or more per generation), the colony manager should consider maintaining inbred strains, unless he obtains new breeding stock from a noninbred production colony either annually or every three to four generations. The effective breeding population consists of those animals whose offspring constitute the next generation of breeding stock.

Comparable Conventional and Gnotobiotic Colonies If it is desired to maintain both conventional and gnotobiotic colonies for comparative purposes, their similar genetic constitutions may be maintained by introducing cesarean-derived litters from the effective breeding population of the conventional colony into the gnotobiotic colony. This appears to be the method of choice for those producers who place most emphasis on the production of nongnotobiotic rats and mice but has the disadvantage of preventing the establishment of a microbiological pedigree that would simplify microbiological monitoring (see Section V). Ideally, this could be done by using litters from specific matings as breeding stock for the conventional colony and using the next litter from each of these matings as breeding stock for the gnotiobiotic colony. If this procedure is followed every second or third generation, the genetic constitutions of both colonies should remain very similar, provided, of course, that the same mating system is used in each colony.

Alternatively, litters from the effective breeding population of the gnotobiotic colony may be used to establish or replenish the conven-

tional colony. The genetic consequences will be identical, provided the same procedures are followed.

Minimal inbreeding

The chief problem in any noninbred colony, gnotobiotic or conventional, is the maintenance of a constant pattern of gene frequencies in the population. Any finite population, whether maintained by true random breeding or by a system of minimal inbreeding, will eventually become inbred. However, the rate at which this process occurs can be controlled by use of different mating systems.

In true random breeding, the rate of inbreeding, when there are equal numbers of male and female parents, varies with the size of the effective breeding population as $1/2N$, where N is the total number of parents. The rate of inbreeding can be halved to approximately $1/4N$ by using a system of minimal inbreeding within the effective breeding population. Inbreeding within a period of time can also be retarded if new breeding stock is selected only from the later litters of each dam. This procedure also makes possible improved selection for high productivity within the effective breeding population.

The chief requirement of the mating system for minimal inbreeding is that each mating unit of the effective breeding population contribute the same number of offspring to the subsequent generation of breeding stock.

Mating system for minimal inbreeding

Mating units, as used in this breeding system, can be either single matings or groups of matings and must be established in numbers that are a power of 2 (4, 8, 16, 32, 64, and so on). Within each group of matings, selection can be made for productivity. However, it is important that each such group of matings contribute the same total number of offspring to the subsequent generation of breeding stock. If each breeding cage consists of one male mated with more than one female, the system of minimal inbreeding can still be applied. In this case, all the offspring from that cage should be dealt with as though they were produced by one pair of parents.

Robertson's mating system for maintenance of a minimally inbred breeding colony (28) can be adapted to breeding colonies of any size. This system, based on single-pair matings, is illustrated in Table 3.

This illustration is limited to 16 mating units. However, the system can be used for any number of mating units that is a power of 2.

E. RECORD-KEEPING (31)

Proper records shall be kept for the animals and for the maintenance of the isolator. The animals' records should determine the efficiency of the operation and the biological performance of the animals. The isolator records should maintain a chronology of events related to the isolator to assist in locating a breach of the barrier if contamination occurs.

TABLE 3 Robertson's System of Minimal Inbreeding

Male from Mating Unit Number	+	Female from Mating Unit Number	→	Mating Unit Number (Next Generation)
1	+	2	→	1
3	+	4	→	2
5	+	6	→	3
7	+	8	→	4
9	+	10	→	5
11	+	12	→	6
13	+	14	→	7
15	+	16	→	8
2	+	1	→	9
4	+	3	→	10
6	+	5	→	11
8	+	7	→	12
10	+	9	→	13
12	+	11	→	14
14	+	13	→	15
16	+	15	→	16

III DERIVATION AND HUSBANDRY OF GERMFREE GUINEA PIGS

A. CESAREAN DELIVERY

It is impractical to maintain breeding colonies of germfree guinea pigs, especially where the popular 2 X 4-ft germfree isolator is being utilized. The size of the animals limits the number of breeders per unit, the gestation period is long (55–67 days), and the average litter size is small. Hence, it is economically more feasible to obtain the animals by cesarean delivery. Germfree guinea pigs of good quality can be obtained by hysterotomy or hysterectomy of healthy dams. The young require no suckling or hand-feeding and are easily reared to maturity, at which time they will reproduce. Methods for cesarean derivation are presented in Section II C.

Careful selection of the animals for hysterotomy is important because it is imperative to perform surgery on the pregnant dams when they are close to term. Timed breeding is not a satisfactory procedure because the gestation period may vary appreciably, even within a single strain. The most satisfactory method for selecting the donors to ensure full term of the neonates is to palpate the pubic bones of the dams by the method described by Phillips *et al.* (23). The change in width of the junction between the pubic bones is an accurate indicator of the time of parturition.

The method of performing the cesarean delivery will vary with the type of equipment available. Hysterectomy and introduction of the

uterus with its neonates into the isolator via a germicidal trap (which is quite satisfactory for rats, mice, and swine) is dangerous for guinea pigs. The newborn often expire before the procedure can be completed. For this reason, the pregnant female is usually sedated and given a local anesthetic prior to surgery.

B. HOUSING

Three or fewer young animals may be housed in a variety of plastic or metal cages measuring 9 X 12 in. or 10 X 15 in. and provided with cloth towel bedding. This reduces the perineal suckling that occurs in larger groups. The animals may be placed on wood shavings or other bedding when they reach 3–4 weeks of age. Although the animals will ingest some bedding material, this usually has no deleterious effects. The occasional hair-eater should be isolated, for he may strip all those within reach. Extensive hair loss impairs the health of the animals.

C. DIETS

Cesarean-delivered guinea pigs begin to eat during the first 12–15 hours of life. Food in flat dishes and drinking water either in open-top containers or bottles with drinking tubes should be provided when the animals are several hours old. Nutrition is best provided by a combination of two distinct diets, one of which is optimal for the neonates, and the other, for animals 3 weeks of age and older. For neonates a semisolid diet, such as diet L-445 fortified with vitamin supplement VA-2 (23), as indicated in Table 4, should be diluted with water to the consistency of heavy cream and placed in the food dishes twice daily. After the third day, less water is added to the diet. Quantities that the animals will consume within approximately 1 hour are provided in the morning and late afternoon. In addition, when the animals are 3–4 days old, a dish of pelleted diet, such as L-477 (32), supplemented with vitamin supplement VC-2 is kept in the cage at all times (Table 4). When the animals are about 10 days old, the feeding of the semisolid diet is limited to once daily, then gradually reduced in quantity, and discontinued at about 21 days of age. The animals then consume L-477 pellets and water *ad libitum*.

TABLE 4 Composition of Diet L-445 and Diet L-477 for Guinea Pigs

Ingredient	Amount per kg	
DIET L-455		
Rolled oats	80	g
Ground Purina Laboratory Chow	80	
D-Glucose (anhydrous)	8	
Active dry yeast	12	
NaCl	4	
Boiling water	816 ml	
Supplement VC-2	1 ml/day[a]	
DIET L-477		
Ground whole wheat	320	g
Ground yellow corn (maize)	145.5	
Rolled oats	220	
Lactalbumin	100	
Technical casein	78	
Alfalfa meal (dehydrated; 17 percent protein)	20	
Desiccated liver	20	
Corn oil	30	
$CaCO_3$	11	
$CaHPO_4 \cdot 2H_2O$	4	
CH_3COOK	9	
$Mg(C_2H_3O_2)_2 \cdot 4H_2O$	9.5	
I-inositol	0.5	
Supplement VC-2	1 ml/day[a]	
Oil-Soluble Vitamins		
A	8,000	IU
D_2	1,000	IU
E (mixed tocopherols)	380	IU
E (α tocopherol acetate)	100	mg
K_3 (menadione)	100	mg
Corn oil carrier q.s.	20	g
Mineral Mix 24		
Fe $(C_6H_5O_7)_2$	0.6	g
$MgSO_4$	4.0	
Iodized NaCl	5.3	
$MnCO_3$	42	mg
CuO	25	
ZnO	25	
$CoCl_2 \cdot 6H_2O$	4	
NaF	0.22	
MoO_3	0.15	
KBr	0.1	
Na_2SeO_3	0.1	

TABLE 4 (continued)

Ingredient	Amount per kg	
B Vitamins		
Thiamine HCl	30	mg
Riboflavin	15	
Nicotinamide	25	
Niacin	25	
Calcium pantothenate	150	
Pyridoxine	10	
Pyridoxamine 2HCl	2	
Biotin	0.5	
Folic acid	5	
Para-aminobenzoic acid	25	
B_{12} (0.1 percent trituration in mannitol)	125	
Choline Cl	1,000	
Cornstarch carrier	1,087.5	
SUPPLEMENT VC 2[a]		
Ascorbic acid	1	g
Thiamine HCl	0.05	g
Distilled H_2O	100	ml

[a]Dispense in 1-, 2-, or 5-ml ampoules. Add 1 ml per animal either to estimated daily food intake or to estimated daily water intake.

D. CHARACTERISTICS

Germfree guinea pigs derived from several strains of healthy breeders and reared on the above regimen have shown good growth, excellent appearance, and normal physical activities. Cecal distension occurs but only rarely affects the health of the animals. Anal prolapse is more serious and occurs in varying incidence in different groups of germfree guinea pigs with an average occurrence of 5 percent.

IV DERIVATION AND HUSBANDRY OF GERMFREE POULTRY

The research worker intending to use a particular species of poultry should become familiar with the general husbandry practices associated with the rearing of that species (33, 34). The material covered in these standards is intended as a guide for the rearing of limited numbers of birds from surface-sterilized eggs under germfree conditions.

A. ENVIRONMENTAL CONDITIONS

Temperature

The eggs should be hatched at 37.5° C and the chicks brooded at 35°–37° C for the first week. The temperature should be reduced 3° C each week until it reaches 22°–24° C.

Relative humidity

A relative humidity of at least 70 percent should be maintained within the isolator during the final stage of incubation of the eggs and hatching. A relative humidity of 50–60 percent is ideal for the rearing of chicks.

Ventilation

Room ventilation should be sufficient to avoid the accumulation of fumes and odors in the room. The isolator should have a positive pressure of 3–5 in. of water and sufficient air exchanges to prevent the accumulation of moisture and odors within the isolator.

B. EQUIPMENT

Isolators

The isolator commonly used to rear gnotobiotic mammals may be readily adapted to hatch and rear gnotobiotic chickens. It is frequently advantageous to hatch the chicks in a small portable isolator that can be conveniently moved into a 37°C room and later to transfer the chicks to a larger isolator at 2–3 weeks of age.

Caging

The floor used for hatching should consist of a 1/4-in. mesh screen or be covered with toweling to prevent the birds from slipping or from other accidents that may cause injury. After 3–5 days, a solid floor covered with bedding may be provided, or the chicks may be placed on a 1/2-in. mesh floor.

Space and feeder requirements

The space and feeder requirements for the species of poultry most frequently used in gnotobiotic studies are listed in Table 5.

TABLE 5 Space and Feeder Requirements for Three Species of Gnotobiotic Poultry

Age weeks	Space ft^2 (cm^2)	Height in. (cm)	Feeder Length in. (cm)	Waterer Length in. (cm)
Chickens				
Up to 4	0.5 (450)	6.0 (15)	1.0 (2.5)	0.5 (1.3)
4 to 12	1.0 (900)	9.0 (23)	2.0 (5.0)	0.5 (1.3)
Adult	1.5 (1,350)	21.0 (53)	3.0 (7.5)	1.0 (2.5)
Turkeys[a]				
Up to 6	0.5 (450)	14.0 (35)	2.0 (5.0)	1.0 (2.5)
6 to 8	1.0 (900)	18.0 (45)	4.0 (10.0)	1.0 (2.5)
Coturnix coturnix japonica[b]				
Up to 2	0.1 (90)	4.0 (10)	0.5 (1.3)	0.25 (0.6)
Adult	0.14 (125)	7.0 (18)	1.0 (2.5)	0.5 (1.3)

[a]Investigators planning the use of mature birds should investigate the space requirements of the adult animals of the strain under consideration.
[b]Also known as Japanese quail.

C. PROCUREMENT OF EGGS

Unsoiled fertile eggs should be obtained from flocks that are free of *Salmonella pullorum* disease and are being fed a ration that does not contain antimicrobial agents. Eggs from flocks free of other salmonella, mycoplasma, and congenitally transmitted disease are recommended. The eggs should be trap-nested, gathered frequently, and stored at 10°–13° C for not longer than 7 days before incubation. The eggs should not be cleaned or sanded.

D. TREATMENT OF EGGS

Preincubation treatment

If eggs from flocks free of mycoplasma are not available, the eggs may be treated with tylosin tartrate as follows:

1. Incubate the eggs at 37.5° C for 3 hours.
2. Immerse the eggs in a solution containing 1,000 ppm of tylosin tartrate for 15 minutes at 7° C.
3. Incubate the eggs at the required time and temperature.

Incubation condition

	Chicken	Turkey	Coturnix
Temperature (°C)	37.5	37.5	37.5
Days to pip	19–21	26–28	16–17
Relative humidity (percent)	70	70	70

Decontamination procedures

Either of the two procedures outlined hereafter may be used to produce gnotobiotic poultry. The use of $HgCl_2$ has less tendency to reduce hatchability, but it requires a liquid germicidal trap, and the bacteriostatic effect may be reversed if the excreta of the newly hatched birds get in contact with the egg shells. The alternative procedure, using peracetic acid, utilizes standard gnotobiotic and laboratory equipment, but excess exposure to the fumes of peracetic acid may kill the embryo.

Procedure A

1. Select clean eggs that are within one day of hatching.

2. Wash and brush the eggs thoroughly in a detergent solution at 35°-37° C.

3. Place the eggs in a nylon net sack.

4. Immerse the eggs for 5 minutes in a 2 percent solution of $HgCl_2$ at 35° C in a liquid trap connected to an isolator. Gently agitate the sack to remove trapped air bubbles.

5. Pull the sack containing the eggs into the isolator and allow them to dry. Continue to incubate the eggs at 37° C.

6. After hatching, separate the chicks from the egg shells as soon as possible to avoid possible toxic effects or reversal of the bacteriostatic effects of $HgCl_2$.

TABLE 6 Composition of Diet L-289F2 for Chickens and Turkeys

Ingredient	Amount per kg	
DIET L-289F2		
Ground yellow corn (maize)	490	g
Soybean oil meal (minimum crude protein 44 percent)	233	
Alfalfa leaf meal	28	
Wheat flour middlings	93	
Casein (plain)	70	
Fish meal	23	
Meat scraps	23	
NaCl (iodized)	5	
$CaCO_3$	17	
$CaHPO_4$	15	
$MnSO_4 \cdot H_2O$	0.25	
Vitamin A	10,000	IU
Vitamin D_3	25,000	IU
Vitamin E (mixed tocopherols)	190	IU
Vitamin K_3 (menadione)	50	mg
Thiamine HCl	70	mg
Riboflavin	7	
Choline Cl	1,000	
Ascorbic acid	200	
Calcium pantothenate	31	
Niacin	16	
Niacinamide	20	
Pyridoxine HCl	7	
Inositol	200	
Biotin	0.1	
Folic acid	4	
Vitamin B_{12}	0.1	

Procedure B

1. Select clean eggs that are within one day of hatching.
2. Wash and brush the eggs thoroughly in a detergent solution at 35°-37°C.
3. Wash and brush the eggs in a 33°-35°C solution at 2 percent peracetic acid adjusted to pH 6.0 with NaOH, and place in entry lock.
4. Seal entry lock and spray lock and eggs lightly,* using a freshly made 2 percent solution of peracetic acid (pH 6.0) containing 0.1 percent of a wetting agent such as sodium alkylarylsulfonate, and place the eggs into the entry lock. Use a stainless-steel grid or basket to support the eggs above the pool of acid that is collected at the bottom of the door.
5. Allow the eggs to remain in the entry lock for 25 minutes.
6. Dry the eggs with a cloth. Place them in the isolator and continue incubation at 37°C.

E. DIET

The diet may be varied according to the special requirements of the experiment. Table 6 shows a diet (L-289F2) that has been used to rear germfree chickens to maturity. In this formulation the calcium phosphate level has been adjusted to obtain eggs satisfactory for the production of second-generation gnotobiotic chickens.

*High-velocity spray should be avoided.

V MICROBIOLOGICAL MONITORING

A. INTRODUCTION

The following procedures are recommended for the microbiological monitoring of germfree animals. The problem of monitoring is considered separately for the producer and for the user of gnotobiotes. The producer has the obligation of supplying a microbiologically pedigreed animal to the user. As the producer, he maintains a continuously reproducing colony on which repetitive and exhaustive tests can be run and from which accumulating data establish a microbiological pedigree over a period of time and into successive generations.

The user who receives a shipment of microbiologically pedigreed gnotobiotes ordinarily needs to test only for subsequent accidental contaminations resulting from breaks in the isolator barriers or from inadequate sterilization of the isolator or its contents. These contaminants are usually ubiquitous micro-organisms found in dust, soil, air, food, and on skin surfaces. They are generally easy to detect because they grow readily in the animal and on the accumulating waste at the bottom of the animal pens. They are readily cultured when inoculated into common laboratory culture media (35).

The producer also faces these problems and must also determine whether his animal stock is free from more esoteric micro-organisms and obligate parasites that may be harbored in the latent state for long periods of time. These forms may be transmitted vertically from the conventionally reared progenitors of the gnotobiotic colony but may

remain latent and undetected until activated or found by more sensitive tests. At best, the germfree state can be defined only in terms of the test methods that have been applied at any particular time.

The microbiological pedigree may be compiled from tests at the laboratory facilities of the producer, by work at consultant laboratories, or by users whose expertise in a specific area of microbiology or parasitology may add useful data to the pedigree of the colony from which the experimental animals were derived.

The introduction of first-generation gnotobiotes, cesarean-derived from conventional stock, into a previously existing pedigreed gnotobiotic colony (in order to maintain genetic similarity between gnotobiotic and conventional animal colonies) destroys the microbiological pedigree, unless the cesarean derivation was from animals known to be free from specifically defined agents. For example, the progeny of germfree rats previously shown to be free of polyoma virus could no longer be assumed to be free of this agent if the new stock derived from polyoma-positive conventional rats were introduced into the isolator system. Evidence for the absence of polyoma virus would have to be re-established.

The proposed methods that follow may be beyond the capabilities of the routine control microbiology laboratory. It is precisely for this reason that microbiologically pedigreed animals should be made available by animal producers so that the user need apply only the simpler tests as recommended for routine use under "Routine Methods" (Section V.C.).

B. DETAILED METHODS–RATS AND MICE

Gross observations

Any visible anatomical or behavioral abnormality may have a microbiological etiology. Animals that die in the isolator should be necropsied, and the cause of death should be determined where possible. Lesions suspected of being caused by infectious agents should be prepared for histopathological examination and should also be cultured for recovery of possible etiologic agents.

A microscopic examination should be made for ectoparasites, such as lice, fleas, and mites. These forms are not likely to be present in gnotobiotes.

Direct microscopic examination of sample materials from the isolator

- Stained fresh fecal smears
  1. Gram stain (36) for general observation of bacteria and fungi
  2. Heidenhain's Iron Alum Hematoxylin (HIAH) stain (30) for protozoa
  3. HIAH stain for protozoan cysts after concentration by zinc sulfate flotation (37)
- Stained gastrointestinal contents at sacrifice or death (stomach, small intestine, cecum, and colon)
  1. Gram stain for bacteria and fungi
  2. HIAH stain for protozoa
  3. HIAH stain for protozoan cysts
  4. Machiavello stain (36) for rickettsiae
  5. Ziehl or Kinyoun's acid-fast stain (36) for mycobacteria
- Stained accumulated waste (feces, urine, diet, water)
  1. Gram stain
- Stained blood smears and organ imprints
  1. Giemsa stain (37) for protoza and Bartonellaceae
  2. Machiavello stain for rickettsiae
  3. Acid-fast stain for Mycobacteriaceae
- Wet mounts of fecal and cecal contents, intestinal wall scrapings, organs, and tissues to look for:
  1. Motile and nonmotile bacterial forms
  2. Mycological forms
  3. Protozoa
  4. Helminths (adults, larvae, and ova)
     a. Direct wet mount
     b. Wet mount after concentration by flotation and centrifugation
     c. Pressure plates of tissues and organs (e.g., diaphragm) for larval cysts (38)
     d. Sediments from chopped organs and tissues digested in artificial gastric juice (pepsin + HCl) for 24 hours (38)
- Histopathological examination of stained tissues
  1. Bacteria by Brown and Brenn (B & B) modification of the gram stain (39)
  2. Mycologic forms in Gram (B & B) stained and in Hematoxylin and Eosin (H & E) stained preparations (37)

3. Protozoa in H & E stained preparations
4. Helminths in H & E stained preparations
5. Viral inclusion bodies in cells stained with H & E stain

Detection of micro-organisms by cultivation procedures

The inoculation of samples into culture media may be done in the following ways:

Media may be taken directly into the isolator and inoculated *in situ*

or

Samples may be transferred from the test isolator to an attached isolator that has been stocked with the appropriate media

or

Samples may be removed from the isolator in sterile containers and aseptically transferred to appropriate media

Bacteria

Samples from living animals

1. Freshly voided feces
2. Accumulated waste at the bottom of the pen

Samples from sacrificed animals

1. Contents from the various anatomic levels of the alimentary tract
2. Organs and ground tissues: liver, kidney, spleen, lungs, gonads, brain, blood, segments of alimentary tract
3. After cesarean delivery of a new litter, samples of the uterus, placenta, and one or more fetuses, if desired

Media

Fluid thioglycollate medium*

This is an all-purpose medium that supports the growth of many fastidious bacteria and provides both aerobic and anaerobic zones for meeting the oxidation-reduction potential requirements for growth.

A.C. medium* (ascorbic acid medium)

An all-purpose medium similar to fluid thioglycollate medium

*Commercially available.

that substitutes ascorbic acid for thioglycollic acid as the reducing agent to provide anaerobiosis. Since thioglycollic acid is inhibitory to some fastidious anaerobes, it is recommended that the A.C. medium be substituted periodically in the test routine for the fluid thioglycollate medium.

Brain-heart infusion agar* with 5 percent sterile defibrinated blood* added aseptically

Blood from a number of different animal species may be used. It is recommended that the species source be changed from time to time to check for the possible presence of substances inhibitory for certain fastidious micro-organisms.

Sabouraud's dextrose medium*

The agar or liquid medium may be used for cultivation of yeasts and molds. In most cases, fungi will appear more rapidly on Sabouraud's medium than on the media mentioned previously. However, since the other media also support growth of yeast and molds, it is not necessary to include Sabouraud's medium in the routine culture procedures, except as a periodic check.

Other media

Other media may be employed at the discretion of the investigator, particularly when the microscopic examination of samples reveals the presence of microbial forms that are not recovered by the above culture techniques.

Incubation temperatures

Incubate replicate cultures at the following temperatures to meet the growth requirement of various micro-organisms:

1. 20°-25°C
2. 35°-37°C
3. 55°C

Oxygen requirements

Replicate cultures in deep tubes of fluid thioglycollate or A.C. medium, incubated at the various temperatures in the ordinary incubator. Provide graded aerobic and anaerobic zones to meet the oxygen tension requirements of a variety of bacteria.

Replicate petri dish cultures of the solid media should be in-

*Commercially available.

cubated aerobically and anaerobically at the temperatures mentioned above. A 90–95 percent nitrogen gas atmosphere containing 10–5 percent carbon dioxide is recommended for anaerobic incubation.

Time of incubation

Three weeks should be allowed for observation before a culture is considered to be negative. Examine all tubes and petri dishes periodically. Observe carefully for the development of slow-growing and fastidious organisms, which may cause only a slight turbidity in broth or develop minute colonies on the surface of solid media. Petri dish cultures, particularly those incubated at 55°C, are subject to excessive dehydration and should be observed frequently during the early days of incubation. Humidified incubators will aid in delaying desiccation of the agar.

Mycoplasma (PPLO)

Recommended samples

1. From live animals

feces	ear swab
oral swab	urine
nasal swab	blood
conjunctival swab	

2. Sacrificed or dead animals

intestinal contents	spleen
blood	brain
trachea	synovial fluid from joints
lung	ear

Media

The following growth medium has been used successfully in a number of laboratories for the cultivation of fastidious *Mycoplasma* strains.

1. Autoclaved basal medium (7 parts)

Beef heart infusion	5.00 percent
Peptone	1.00 percent
Glucose	1.43 percent
NaCl	0.50 percent
Phenol red	0.002 percent

2. Filter-sterilized 25 percent fresh yeast-extract solution* (1 part)

3. Sterile horse serum (2 parts)*

Mycoplasma growth agar

Same as "Media" above, but add 1.25 percent agar* to the basal medium before autoclaving.

Cystine trypticase agar (CTA)*

CTA medium is recommended for the cultivation of L-forms.

Inoculation procedures

1. Inoculate the fluid medium and make blind transfers at weekly intervals for a minimum of three transfers.

2. Make transfers from (1) above to the surface of *Mycoplasma* growth agar and observe at 30–100 magnification for the development of typical *Mycoplasma* colonies.

3. Make agar block transfers from (1) above to fresh *Mycoplasma* agar plates.

4. Check suspect colonies with Dienes stain (40) to observe and verify colony type.

Incubation temperatures

20°-25°C

35°-37°C

Oxygen requirements

Aerobic

Anaerobic (90–95 percent nitrogen with 10–5 percent carbon dioxide)

Incubation time

Observe at 3–5 day intervals for periods up to 30 days

Serology

Check serum for the presence of complement-fixing or growth-inhibiting antibodies against appropriate murine strains of *Mycoplasma*.*

1. *Mycoplasma pulmonis*
2. *Mycoplasma neurolyticum*
3. *Mycoplasma arthritidis*

*Commercially available.

Viruses

A number of methods employed for the detection of viral contamination are listed below. In some instances the methods may overlap, but these are complementary rather than mutually exclusive.

1. The development of an abnormality not attributable to any other micro-organism, injury, or nutritional deficiency should be suspected as having a viral etiology.

2. Cytopathologic effects (CPE) or the detection of virus-like inclusions in pathologic or normal tissues by standard histopathologic techniques could indicate the presence of viruses.

3. Detection of virus-like particles by electron microscopy in normal or pathologic tissues could indicate the presence of viruses (41).

4. Detection of serum antibodies by hemagglutination-inhibition, complement-fixation, and virus-neutralization tests against known viral agents* for the host animal could indicate prior or current exposure to virus: polyoma, Sendai, Reo 3, Theiler's mouse encephalomyelitis (GD VII), mouse hepatitis virus (MHV), mouse adenovirus, pneumonia virus of mice (PVM), new-born mouse pneumonitis (K virus), minute virus of mice (MVM), Kilham rat virus, lymphocytic choriomeningitis virus (LCM), Simian virus (SV-5), and Toolan's H-1 virus. Sera from animals at various ages should be tested.

5. Test susceptibility of germfree animals to standard strains of viral agents to detect tolerance or resistance to the virus (42).

6. Check for CPE in tissue cultures derived from germfree animal tissues.

7. Check for induction of CPE in homologous, isologous, or heterologous tissue cultures by inoculation with tissue extracts and excretions from germfree animals (42).

8. Check for induction of overt disease by inoculation of host tissue extracts and body excretions into newborn, weanling, and adult germfree and conventional animals. This should include weekly blind transfer of tissue extracts from animal to animal for at least 3 passages.

9. Examine spontaneous and chemically induced tumors by electron microscopy for the presence of virus-like particles. Examine primary as well as transplanted tumors. Tissue culture of tumor tissue should also be examined in this manner (42).

10. Apply stress agents such as cortisone and sublethal doses of x ray to activate latent viruses (e.g., leukemia virus of mice) (42).

*Commercially available.

Rickettsiales

Rickettsiae

The presence of rickettsiae normally would be detected in the culture and microscopic methods used for the detection of viruses in Section B, page 30. Rickettsiae are probably not important in gnotobiotic animals in the absence of arthropod vectors. However, *Coxiella* may be important.

C. ROUTINE METHODS–RATS AND MICE

The above procedures represent a tremendous work load, which certainly could not be adapted to routine day-to-day or week-to-week use. The concept of establishing a microbiological pedigree for animal colonies assumes that once sufficient negative data have been accumulated to reasonably establish the absence of a particular group of organisms, the animals and their progeny will remain free of these contaminants. It is further assumed that should a break in the barrier occur, or should sterilization be inadequate, the resulting contamination would generally involve readily detectable bacteria and fungi.

Once the pedigree for the microbiologic status of a colony has been established, the producer and the user can rely on the much simpler procedures of monitoring for the common bacterial and fungal contaminants. Essentially, this would involve the following sections selected from the preceding outline.

Gross observations

Direct microscopic examinations

Gram stain of fecal smears for bacteria and fungi

Gram stain of intestinal contents (small intestine, cecum, colon) at sacrifice or death

Gram stain of accumulated wastes (feces, urine, diet, water)

Wet mounts of feces to look for:

1. Motile bacteria
2. Yeasts and molds
3. Protozoa (active or encysted)
4. Helminths (adults, larvae, ova)

Detection of micro-organisms by cultivation in nutrient media

Bacteria

Samples from living animals

Freshly voided feces

Accumulated waste at the bottom of the cage

Samples from sacrificed animals

Contents from alimentary tract

Organs and tissues

Media

Fluid thioglycollate medium

Ascorbic acid medium

Brain-heart infusion agar with 5 percent defibrinated blood

Sabouraud's dextrose medium (solid or liquid)

Incubation temperatures

Replicate cultures incubated at room temperatures, 37°C and 55°C respectively

Oxygen requirements

Fluid thioglycollate medium or ascorbic acid medium for aerobic and/or anaerobic organisms

Replicate plates of blood agar incubated aerobically at the above-mentioned temperatures and another set incubated anaerobically at the same temperatures.

Incubation time

Observe all media for a minimum of three weeks before recording negative results.

NOTE: It is important to recognize that setting up routine monitoring procedures tends to make the detection protocol static and unimaginative. The microbiologist must be continually aware of the necessity for the development of new methods for the detection of micro-organisms and he must apply them to the existing animal colonies for specific evaluation.

D. MICROBIOLOGICAL MONITORING–GUINEA PIGS

The microbiological monitoring of the gnotobiotic guinea pig for bacteria and bacteria-like forms, fungi, protozoa, and helminths does not differ from the procedures recommended for rats and mice.

Viral agents of the guinea pig have not been extensively studied, and its viral flora is largely unknown. A number of guinea pig diseases are associated respectively with symptoms of paralysis (meningomyeloencephalitis), respiratory disease, or marked emaciation. However, the direct association of these diseases with specific viruses has not always been possible (43). Similarly, pneumonic syndromes have been described in "germfree" guinea pigs (44,45), but again, the direct presence of a suspected viral etiologic agent was not demonstrated.

A salivary-gland virus (46) has been found to be present in conventionally reared guinea pig colonies. The virus is asymptomatic in the naturally infected stock, but death can ensue when the salivary gland extract from infected guinea pigs is injected intracerebrally into young cavies. While the salivary gland virus is considered unimportant from an animal husbandry standpoint, it assumes academic importance within the definition of gnotobiology and should be sought for in gnotobiotic guinea pigs. Since the viral flora of the guinea pig has not been well documented, a serological screen for antibodies against specific viruses cannot be put on a rational basis at present.

In summary, the recommendations for the microbiological monitoring of gnotobiotic guinea pigs can follow the recommendations for other rodents, except that the search for specific circulating antibodies as an indicator of previous viral infections is not yet feasible.

E. MICROBIOLOGICAL MONITORING–POULTRY

The microbiological monitoring of gnotobiotic poultry for bacteria, bacteria-like forms, fungi, protozoa, and helminths does not differ substantially from procedures recommended for rodents.

The *Mycoplasma* species that affect poultry are different from those infecting rodents. Therefore, the serological testing for *Mycoplasma* antibodies should include antigens prepared from typical avian strains: *M. gallinarum*, *M. gallisepticum*, *M. synoviae*, *M. iners*, and *M. meleagridis*.

Viruses that commonly infect avian species and for which there is some evidence of vertical transmission are Newcastle disease virus (NDV), avian leucosis group, Rous sarcoma, and the RIF-RAV agents (Rous inhibiting factor and Rous associated virus). Methods for detection of antibodies or detection of the presence of these viruses are presented in *Methods of Virology* (47). Complement-fixation testing services for the avian leucosis group as well as Rous sarcoma antibody-testing services are available commercially. Serologic tests for infectious bronchitis virus and embryo resistance tests against infection by avian encephalomyelitis virus should also be run.

It is possible that other viral diseases could also be present, but since their transmission via the egg under natural conditions has not been proven, they are not considered here. It is expected that an investigator will run adequate controls to assure freedom from specific diseases under investigation.

Among the *Rickettsiales*, *Chlamydia* infect poultry but are not known to be transmitted through the egg. Complement-fixation tests may be used to detect antibody. Otherwise, the staining and cultivation procedures for rickettsiae outlined in Section V will detect these agents.

VI TRANSPORTATION AND CONSIGNEE (USER)/ CONSIGNOR (BREEDER) RESPONSIBILITIES

Premise: It is understood that both the consignor (breeder) and the consignee (user) of gnotobiotic animals are competent and knowledgeable in gnotobiotic technology. It behooves both parties, if possible and whenever practicable, to ascertain the other's competency and capabilities.

A. CONSIGNOR RESPONSIBILITY

Husbandry

Animals should be bred and reared according to Section II "Production, Care, and Maintenance of Germfree Rats and Mice."

Selection for shipment

Only animals conforming to normal growth rates for the strain, exhibiting good physical condition and clean coats, and demonstrating no gross abnormalities should be shipped.

1. Animals should be of the strain and species requested by the user.
2. Animals should be exactly the age, sex, and weight range specified by the user.
3. Animals should be reared on the diet specified by the user. If none is specified, details of diet and sterilization procedure used by breeder should be furnished.
4. When criteria such as strain, species, age, weight, and diet do not

conform with user's specifications, the user shall be so advised, and his reconfirmation should be received before shipment is made.

Microbiological procedures

The breeder should follow procedures as outlined in Section V, "Microbiological Monitoring."

1. Breeding or holding isolators contributing animals to a shipment should be checked 7 days prior to shipment. A second test should be performed on these animals 48 hours prior to shipment.

No animals should be added to any isolator during the 7-day holding period or until the animals for shipment have been removed and transferred into shipping units.

2. Wet fecal smears and routine cultures should be taken at the time of final transfer prior to shipment in accordance with Section V.

Stained fecal smears should accompany each shipment of animals. A duplicate slide should be held on file by the consignor for 30 days for comparative purposes and be made available to the consignee on request.

3. A statement of tests performed (date, time, and results) should accompany the shipment or be available on request.

4. Isolator(s) from which the shipment was obtained should be recorded, and the data should be kept on file for 30 days following shipment.

If a contaminant occurs in the donor isolator(s) within 7 days after shipment, the consignee shall be advised immediately.

If the donor isolator is placed under suspicion resulting from another user's report of contamination or from suspect cultures in the breeder's laboratory, the consignee shall be advised immediately. Such isolators should not be considered gnotobiotic until 2 consecutive negative tests are performed by the breeder's laboratory.

5. Each shipping unit should contain 2 sterile sealed specimen containers with NIH fluid thioglycolate medium or a transport medium such as Stuart's (48).

One specimen container with a fecal sample should be returned immediately to the consignor upon transfer of the animals from the shipping unit.

One specimen container with a duplicate fecal sample should be processed immediately at the consignee's laboratory upon transfer of the animals out of the shipping unit.

Routing of shipment

Since animals can be subject to many hazards in transit, the most expeditious routing should be arranged.

1. Advise the consignee of exact routing in advance of shipment so that local arrangements to pick up and deliver the animals to their final destination can be made.

2. Reserve space with the carrier (if required) and reconfirm prior to shipment. Verify that the animals can be carried on passenger equipment in the event an all-cargo plane cannot be utilized.

3. Weather conditions at the point of shipment and at the destination should be determined on the day of shipment, and the shipment should be postponed if extreme conditions exist.

Request the user to accept change in the prearranged shipping schedule or routing. If the user insists on shipment as originally planned, shipment shall be made under full responsibility of the user.

4. Label shipment and shipping container to provide essential information necessary to ensure the safe and rapid delivery of animals.

Detailed name and address as given by the consignee should be provided.

When available and agreeable to user, business and home phone numbers should appear.

Preplanned routing plus an alternate routing should be clearly marked on shipping container.

Special customer information, such as purchase or requisition numbers, contents of shipment, and date and time of shipment should appear on the shipping container.

Special instructions should appear on the exterior of shipping container to alert the commercial carrier not to open the container, to indicate the nature of contents, and to guard the shipment against exposure to extreme temperatures, direct sunlight, and rough handling.

B. SHIPPING CONTAINERS

Shipping containers fall into two general categories: disposable containers without mechanical air supply and nondisposable, returnable isolators with battery-operated mechanical blowers. In both instances they should be designed to ensure maintenance of the microbiological integrity of the animals and to withstand the stresses of transportation.

Disposable shipping units

These should be designed for economy, sturdiness, and ease of transferring animals to the receiving isolator with minimum risk.

1. Filters should be of sufficient area to provide adequate ventilation and heat transfer, and to ensure the sterility of the shipment.

2. The floor area should be designed to prevent overcrowding and overheating.

The number of animals should be reduced during warm or humid conditions.

The number of animals may be increased during extreme cold.

3. The external configuration of the shipping unit should preclude any restriction of ventilation while in transit, and it should be such that units cannot be stacked.

To prevent accidental opening, the shipping unit should be securely sealed in a manner that would make it apparent to the recipient that the unit should not be opened.

4. The following information should accompany units without mechanical ventilation:

Detailed instructions for animal removal and microbiological procedures.

Expiration time of food and water provisions.

Description of diet fed animals by consignor.

Date and time animals were placed in unit.

Battery-operated (mechanically ventilated) returnable shipping isolators

These units are employed when the consignee does not have a receiving isolator available or when he intends to conduct his experiment in the shipping isolator. Their design incorporates gloves, inlet filter, exhaust filter, and entry port. Since these units are costly to construct, ship, and maintain, it is recommended that lightweight disposable units be encouraged when the circumstances do not preclude their use.

1. Since rechargeable batteries that operate forced-ventilation blowers have a limited life, it is important to maintain the air supply to the isolator on an alternate electric source prior to shipment.

Routing is critical and must be consistent with battery life, allowing several hours' margin of safety.

Clearly state the expiration time of the battery and advise the consignee.

2. Provide detailed instruction for unpacking the unit and for switching from battery operation to local electric supply upon arrival.

3. The following information should accompany mechanically operated units.

Detailed instructions for animal removal and microbiological procedures.

Expiration time of food and water provisions

Description of diet fed animals by consignor

Date and time animals were placed in the unit

C. CONSIGNEE RESPONSIBILITY

Husbandry

Animals should be housed and maintained according to Section I.

Condition of shipment

Advise consignor of any apparent abnormality or irregularity immediately on arrival.

Since delays in transit may cause stress or death of animals, the time of arrival should be recorded.

Report deaths and delays to public carrier, since animals are usually insured in transit by the carrier. Filing a claim usually requires a joint inspection by the agent and the consignee.

Shipping units are sometimes damaged due to improper handling and should be carefully examined upon arrival for breaks or perforations before unpacking.

If an inner container is present, examine it carefully for breaks or perforations.

If animals are visible, check for dead or moribund animals.

Determine if food or water has been exhausted.

Examine the animals to determine whether they conform to specifications and number as requested.

Transfer of animals

Animals should be transferred as quickly as possible to isolators when received in a disposable unit.

When animals are received in a returnable, battery-operated unit, it should be returned according to consignor's instructions.

Animals become the consignee's responsibility 24 hours after transfer.

Microbiological tests

Collect fecal samples immediately upon arrival or upon transfer from shipping unit and place them in sterile containers provided in the unit for culture.

1. One properly identified tube should be sent to the consignor immediately.

2. A second tube should be retained for culture according to Section V.

If sterile containers are not provided, the consignee shall take fecal samples immediately upon transfer for culturing according to Section V.

A wet smear of fecal material should be prepared at the time of transfer.

Examine for motility and numbers of organisms per field.

Prepare a stained smear of fecal material.

If a slide is supplied by the consignor, it should be compared with the stained smear prepared on arrival of shipment.

Advise the consignor immediately if the animals are suspected of contamination on initial culture. Supply details as to motility, morphology, staining characteristics, and other available information regarding the organism. Damage to container or other pertinent information should be furnished.

Inform the consignor if contamination is discovered in the initial cultures within 21 days after receipt of shipment.

Slow-growing organisms, which are not common, may not be detected during the early stages of incubation but could become apparent at a later date.

Postponement of scheduled shipment at the request of the consignee

The consignee should advise the consignor, when possible at least 48 hours prior to the expected time of shipment, of conditions that will entail postponement of shipment.

Causes for postponement may be:

1. Sterilizer breakdown
2. Temporary loss of trained personnel
3. Suspected contaminations in existing isolators
4. Local bad weather conditions
5. Anticipated inability to accept delivery because of labor problems with public carrier and/or institutional personnel

GLOSSARY

A number of attempts have been made to systematize the terminology used in gnotobiotic technology, but no single system is universally accepted. In this publication the nomenclature has been limited to terms that, by general usage, are familiar to and understood by most workers in the field.

GNOTOBIOTIC A word derived from the Greek *gnotos* and *biota*, meaning known flora and fauna.

GNOTOBIOTE (GNOTOBIOTIC ANIMAL) One of an animal stock or strain derived by aseptic cesarean section or sterile hatching of eggs that is reared and continuously maintained with germfree technics under isolator conditions and in which the composition of any associated fauna and flora, if present, is fully defined by accepted current methodology.

GERMFREE ANIMAL (AXENIC ANIMAL) A gnotobiote that is free from all demonstrable associated forms of life, including bacteria, viruses, fungi, protozoa, and other saprophytic or parasitic forms. Since criteria for establishing "germfreeness" depend on currently available diagnostic tests, animals now designated as germfree may possibly in the future be found to harbor one or more viable agents.*

DEFINED FLORA ANIMAL A gnotobiote maintained under isolator conditions in intentional association with one or more known types of micro-organisms. Such terms as "monoinfected," "monocontaminated," "monoassociated," and "polycontaminated" have been employed to describe this type of gnotobiote. Of these, the terms "monoassociated" and "polyassociated" are preferable for describing intentional, rather than accidental, association with microbes.

CONVENTIONAL ANIMAL An animal with an uncontrolled flora, reared under open animal room conditions in association with other animals of the same type.

*So far all "germfree" mice tested harbor a leukemogenic virus (49). These animals may be considered gnotobiotes, but they can no longer be called germfree.

CONVENTIONALIZED ANIMAL An animal originally born or delivered under gnotobiotic conditions, which has subsequently been intentionally provided with the microflora of a conventional animal.

EX-GERMFREE ANIMAL An animal born or delivered under germfree conditions, which, accidentally or intentionally, acquired a microbial flora.

CESAREAN DERIVATION When used in connection with gnotobiotes, refers to an operation performed under rigidly sterile conditions to obtain gnotobiotic animals from the uterus of the mother.

HYSTERECTOMY A cesarean operation in which the intact gravid uterus is aseptically removed at term and introduced by sterile technics into a sterile isolator where the young animals are removed from the uterus.

HYSTEROTOMY A cesarean operation in which a surgical incision is made through the floor of a sterile isolator in contact with the surgically prepared abdomen of a gravid female at term. The young animals are removed from the uterus *in situ* and transferred to the sterile isolator.

ISOLATOR A sterilizable life-support chamber, usually constructed of stainless steel or plastic, in which gnotobiotes may be housed and maintained free from contamination. The term may be further qualified according to function, e.g., shipping isolator, surgical isolator, rearing isolator, or jacket isolator.

AIR-DIFFUSION ISOLATOR An isolator in which air exchange is effected by free diffusion through a relatively large sterilizing filter area, in contrast to the usual system of air filtration under positive pressure.

GERMICIDE TRAP An entry or exit device on an isolator in which the sterile barrier is maintained by a germicidal solution. Such devices are used in the introduction of eggs, or a gravid uterus obtained by hysterectomy, and for removal of samples or materials from the isolator.

LOCK A double-portal sterilizable entry device by which access to an isolator may be obtained or with which two isolators may be connected.

GERMICIDE LOCK A lock that is sterilized by a chemical agent, usually peracetic acid.

CLAVE A type of lock that can be sterilized by steam under pressure and is similar in construction and function to an autoclave.

SUPPLY CYLINDER A metal cylinder provided with a fiber glass filter, which, with its contents, may be steam sterilized in an autoclave. Its subsequent linkage to the entry port of an isolator is sterilized by peracetic acid spray.

TRANSFER SLEEVE A flexible plastic sleeve used to connect the entry ports of two isolators. It is sterilized by the introduction of peracetic acid spray.

FREON TEST A test for leaks in isolator assemblies that is based on the introduction of freon gas and the detection of leaks by an electronic monitoring device.

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