ABSTRACT

STUDIES ON GNOTOBIOTIC PIGS

PART ONE. A TECHNIQUE FOR REARING GNOTOBIOTIC PIGS

PART TWO. A COMPARISON OF BODY WEIGHTS, ORGAN WEIGHTS, AND SOME HISTOLOGICAL FEATURES OF GNOTOBIOTIC AND FARM-RAISED PIGS

by Glenn L. Waxler

Part One describes the technique used in 4 experiments to obtain and rear young pigs to the age of approximately 3 weeks in the absence of bacteria or in the presence of known species of bacteria. A hysterectomy was performed on the anesthetized sow, and the pigs were removed from the uterus inside a sterile, vinyl-film isolator.

The diet, sterilized with steam under pressure, consisted of pasteurized, homogenized milk with mineral and vitamin supplements.

The technique was found to be successful in that 2 pigs were raised in the absence of any demonstrable bacteria. Four animals were raised in the presence of Staphylococcus aureus, 3 in the presence of Bacillus sp., and 4 in the presence of Achromobacteriaceae and an unidentified organism.

Part Two describes the comparison of body weights, organ weights, and the histology of selected tissues of the 13 pigs from Part One, 5 pigs raised in open cages on the sterilized diet, and 11 animals raised by the sow to the age of 3 weeks under farm conditions.

The farm-raised pigs were significantly heavier at the end of the 3-week period than were the artificially-raised animals. When the organ weights were expressed as relative weights, the mandibular lymph nodes of the farm-raised pigs were significantly heavier than those of the germfree pigs. This did not hold true for the other groups of lymph nodes studied. The kidneys of all artificially-reared animals were relatively heavier than those of the farm-raised animals. The relative adrenal weights of the germfree pigs and those animals raised in the presence of Staphylococcus aureus were greater than those of the farm-raised pigs.

Light-centered nodules, corresponding to the usual germinal or reaction centers were found in the lymph nodes of the germfree animals, but these did not appear as large or numerous as in the farm-raised pigs. The germfree pigs usually displayed more solid-centered primary nodules than did those animals raised under farm conditions. Numerous immature eosinophils, neutrophils, and small, intensely-staining cells resembling hematopoietic cells were found in the lymph nodes of all groups of animals. The histological picture of the lymph nodes seemed to be influenced more by the diet and rearing conditions under which the animals were kept than by the presence or absence of bacteria in the environment.

No significant differences were found in the histology of the spleens of the various groups of animals, except for the fact that the artificially-reared animals appeared to have more iron-containing pigment in this organ.

The hepatic interlobular septa of the artificially-reared animals were less well-developed than in the farm-raised pigs, and in many cases the only indication of the borders of the lobules was a lining up of the hepatic cells and reticular fibers in this area.

Two germfree pigs left in the germfree environment 3 and 5 days, respectively, after death showed no gross evidence of post-mortem degeneration. However, dehydration was noted. Histological examination revealed less degenerative changes than would be expected under conditions in which the animals were in contact with bacteria. Of the tissues examined, the myocardium and erythrocytes showed the least degeneration, the lymphatic tissue and the adrenals the most.

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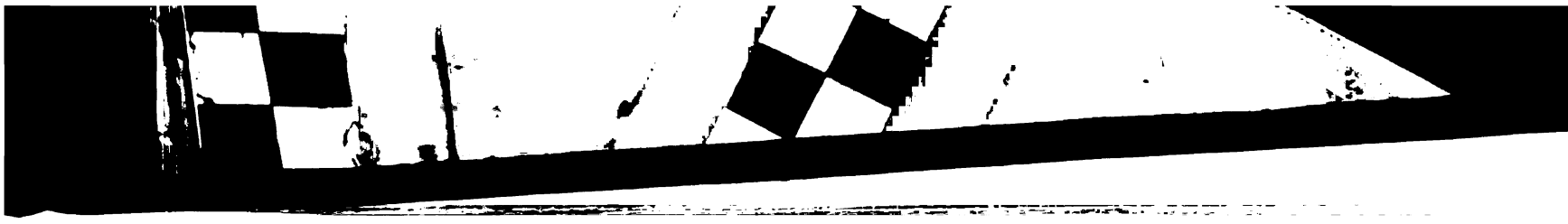

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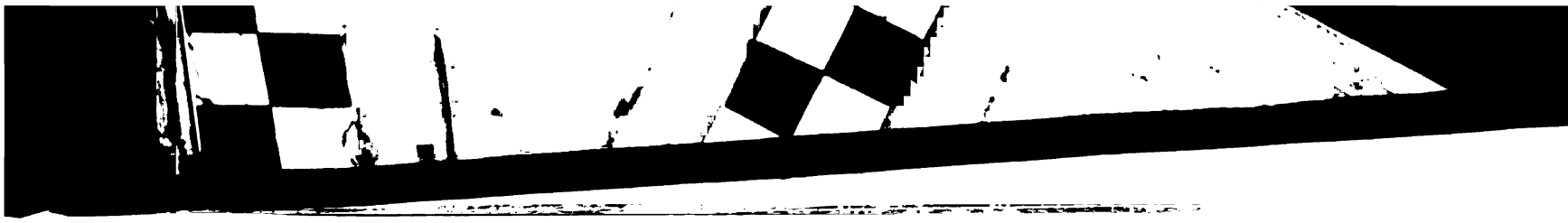

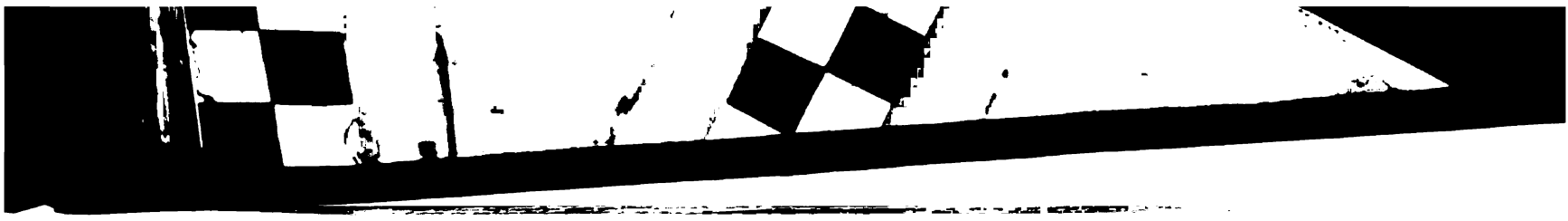

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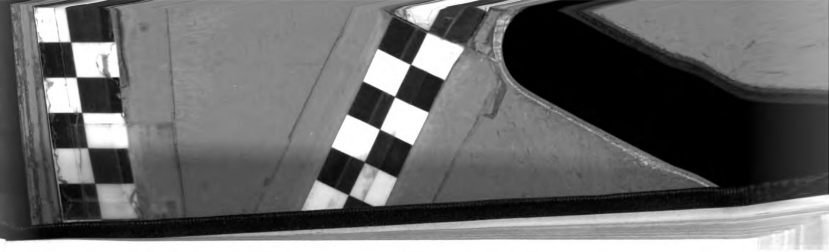


PART ONE

A TECHNIQUE FOR REARING GNOTOBIOTIC PIGS

I. INTRODUCTION

Gnotobiotics, the science of rearing organisms by themselves or in the presence of specifically known associates, has received much attention during the past several years. Most investigations have involved the rearing and use of laboratory animals. However, germfree chickens have been produced quite extensively, and a few attempts have been made to utilize larger domestic mammals in this type of research. The size of the equipment necessary to house the larger animals and the volume of diet required have been among the factors limiting the usefulness of these species in gnotobiotic research.

The specific fields of research in which germfree animals have been used include, among others, the areas of nutrition and infectious diseases. Investigative work in the area of nutrition is made difficult by the various interrelationships which exist among the numerous nutritional factors. In like manner, the infectious agents often exert an influence on one another, and it has been demonstrated that interrelationships exist between nutrition and infectious agents. By using germfree animals it is possible to control the environmental conditions of the experiment more rigidly and thus more thoroughly delineate some of the above-described interrelationships.

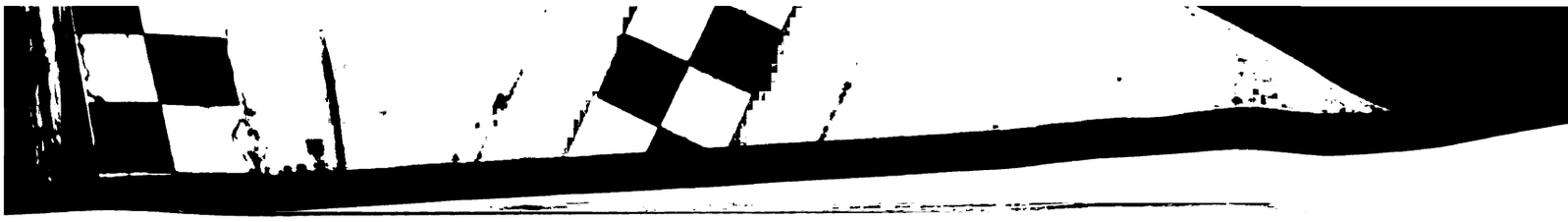
In the field of swine diseases it is recognized that there is a need for much basic research. Diagnosis of many diseases is made

quite difficult by the vast array of microorganisms which may be isolated from affected individuals. This is especially true in some of the enteric disturbances of young animals. It is felt that adequate prevention and control of these conditions in swine will be accomplished only when it has been found possible to clearly define the roles played by the various microorganisms involved.

The research on these problems will be greatly enhanced and the results made more reliable if a dependable, practical technique for obtaining and rearing young pigs free from microorganisms can be developed. It will then be possible to introduce known species of bacteria and study their ability to produce pathological changes in the animal.

In preliminary work at this institution, using pigs derived by Caesarean section as a source of experimental animals for various studies, it was found that certain factors often complicated the results. One of the most common of these was the appearance of bacterial infections, usually manifested by enteritis, in the young colostrum-deprived animals. In an attempt to control these complicating factors, work was begun in 1959 to investigate the possibilities of adapting gnotobiotic techniques to the rearing of young pigs. It was hoped that this would result in the obtaining of more reliable experimental data in the field of research utilizing young pigs. The attempts to obtain germfree pigs as reported in this manuscript were made from the period of May, 1960, to January, 1961.

Part One of this manuscript includes a description of the equipment, sterilization techniques, surgical procedures, diets, rearing methods, and bacteriological techniques used in a series of



4 experiments designed to investigate the feasibility of obtaining and raising gnotobiotic pigs to an age of approximately 3 weeks. Part Two includes some phases of a morphological characterization of the animals obtained in these 4 experiments. This includes a study of the body weights, weights of various organs, and the histology of selected lymph nodes, the spleen, and the liver of these animals as compared to the same observations made on pigs of comparable age raised under ordinary farm conditions.

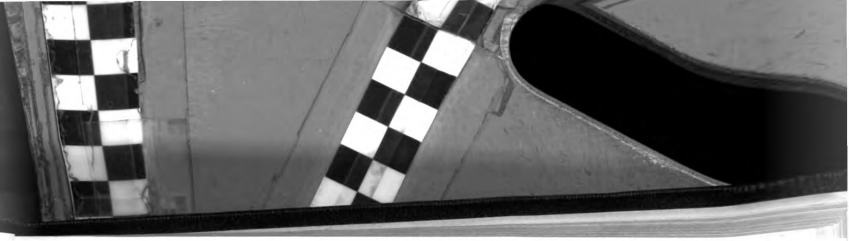
II. REVIEW OF LITERATURE

A. Gnotobiotic Techniques

1. Terminology

Reyniers et al. (1949b) have reviewed the various terms used to describe the technique of working with animals in an environment free from detectable microorganisms and the terms applied to these animals themselves. They point out that such designations as "pure", "germ-free", "sterile", "aseptic", and "bacteria-free" have appeared in the literature from time to time. More recently the term "germ-free" has by most authors been changed to one word and now appears as "germfree". Baker and Ferguson (1942) suggested the term "axenic" which is derived from the Greek and means "free from strangers". According to these workers, "axenic" organisms are individuals of a species free from any demonstrable life apart from that produced by their own protoplasm. Just (1959) suggested the term "bion" which refers to an organism that is morphologically and physiologically independent, a condition satisfactorily met by germfree animals. Under this system of nomenclature "biontology" refers to the field of research dealing with germfree forms. A "holobiont" is an organism free from microorganisms, a "monobiont" is raised in contact with a single species and a "dibiont" with 2 species of microorganisms.

Reyniers et al. (1949b) and Reyniers (1956) have suggested the term "gnotobiotics" to describe the conditions and concepts involved in this type research. This term is derived from 2 Greek words, one meaning "known" and the other "life", and it may be used to



indicate the field of investigation concerned with growing living things by themselves or in an association with other completely known kinds of organisms. The animal grown under these conditions may then be referred to as a "gnotobiote", and the environment may be referred to as a "gnotobiotope".

Trexler (1960b) has suggested the grouping of "gnotobiotes" upon the basis of the certitude with which their specific content can be defined. He described "alpha-gnotobiotes" as those maintained in a secure environment and subject to extensive examinations requiring reproduction to furnish the necessary specimens. The "beta gnotobiotes" include animals maintained under the same rigid security but unable to reproduce under germfree conditions. The "gamma gnotobiotes" are gnotobiotic animals as used at the present time in which the status of each isolator is determined by rather extensive analysis by competent microbiologists. He also described "delta gnotobiotes" as those requiring the least reliable control procedures, such as simple microscopic observations of fecal smears combined with a few culture media. Jenkins (1960) has suggested a similar classification of laboratory animals on the basis of associated organisms.

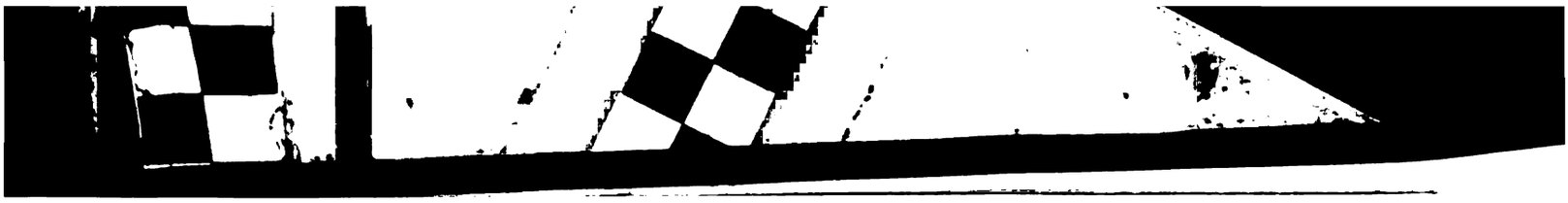
2. Chronological Survey

Credit for the first attempts to cultivate complex organisms in the absence of bacteria is generally given to Duclaux (1885) who was able to grow peas and beans in an environment free of bacteria. The seeds were sown in a sterile soil moistened with milk, and Duclaux observed that after one or 2 months the milk was still intact with no coagulation. Pasteur (1885) in a preface to Duclaux's publications,

expressed an interest in the question of whether or not it would be possible to raise animals in an environment and on a diet both free of the ordinary microbes. It was Pasteur's firm belief, however, that life under these conditions would become impossible.

Schottelius (1899), who studied under Pasteur, expressed the opinion that it is inconceivable that the relationship between the intestinal mucosa and the intestinal flora should not have developed into some sort of symbiosis. If the normal flora is not allowed to establish itself at birth, as would be the case in the germfree experiment, he concluded that life would not be possible. The interest of Schottelius in the possibility of germfree life was followed by several attempts to obtain germfree chickens (Schottelius, 1899, 1902, 1908, 1913), and the failure of these birds to grow seemed to strengthen the contentions of both Pasteur and his student. These chickens were below their initial weight at the end of the 30-day experimental period. Control animals on the sterilized diet developed normally. When the germfree chickens were infected with pure cultures of Bacterium coli from hen feces or milk, they developed almost as well as the control chickens. A study of the diet used by Schottelius suggests that it was probably poor in accessory nutritive substances as compared to diets used for chickens in later successful trials (Gustafsson, 1948).

Nencki (1886) from Switzerland was opposed to the theories presented by Pasteur and Schottelius and belonged to the group of those believing that the bacterial flora has the ability to produce more or less poisonous substances which would impair the health of the animal host. A few years later the German workers Nuttal and



Thierfelder (1895-1896, 1896-1897) demonstrated that guinea pigs could live in the absence of bacteria.

Metchnikoff (1903), who, like Pasteur, never actually performed a germfree experiment, clearly lined up with the Nencki-Nuttal-Thierfelder forces in believing that the bacterial flora can assume the role of the fighting antagonist against the host. Cohendy (1912a, 1912b), a student of Metchnikoff, was quite successful in rearing germfree chickens and raised 17 such birds, some of them to the age of 40 days. In some cases the physical condition and growth of these germfree chickens compared favorably to control animals. Cohendy concluded that (1) life is possible for vertebrates (chickens) in the germfree state and (2) germfree life does not cause any breakdown in the organism. Cohendy and Wollman (1914) reared germfree guinea pigs to ages varying from 16 to 29 days and also used these animals in studying cholera infection (Cohendy and Wollman, 1922).

Küster (1912, 1913, 1915a, 1915b) a student of Schottelius, was the first to work with larger mammals under germfree conditions. He raised 2 germfree goats, and one of these lived to the age of 35 days.

Excellent reviews of these early efforts in the field of germfree research have been presented by Gustafsson (1948), Reyniers et al. (1949a), Reyniers (1956), and Gordon (1960).

Balzam (1937) conducted 3 experiments, using germfree chickens, to study the correlation between the digestibility of food and vitamin requirements of normal chickens. From the results of these experiments, Balzam concluded that (1) the intestinal flora of the chicken does not exert an appreciable influence on the

digestibility of food and (2) the lack of an intestinal flora has no effect on the need for vitamins by germfree animals.

Much of the recent research in gnotobiotic techniques has been conducted at the Lobund Institute, University of Notre Dame, Notre Dame, Indiana. No attempt will be made at this time to review extensively the many reports published by this group. However, the following references may be cited as covering, in a general way, the research work conducted: Gordon (1959), Phillips et al. (1959), Reyniers (1959a, 1959b), Reyniers and Trexler (1943), Reyniers et al. (1946, 1949a), Trexler (1959), and Trexler and Reynolds (1957). The Lobund group has successfully reared germfree rats, mice, guinea pigs, chickens, rabbits, monkeys, turkeys, dogs, and other animals (Reyniers, 1959b).

The Swedish workers (Glimstedt, 1936, 1959; Gustafsson, 1946-1947, 1948, 1959a, 1959b) have utilized germfree rats and guinea pigs in their research. In Japan, Miyakawa (1959a, 1959b) and Miyakawa et al. (1957) have worked with the germfree guinea pig. In addition numerous other groups have conducted research involving germfree laboratory animals (Lev and Forbes, 1959; Horowitz et al., 1960; Phillips et al., 1960; Hickey, 1960; Snow and Hickey, 1960).

The interest in obtaining and rearing germfree farm animals, first evidenced by Küster (1912, 1913, 1915a, 1915b) has been dormant until quite recently. Smith and Trexler (1960) have obtained a germfree lamb and 2 germfree goats. The lamb was reared under germfree conditions for 4 months. Luckey (1960) also reported the successful rearing of germfree lambs. In addition to the present work, at least one attempt has been made to rear germfree pigs (Trexler, 1960d).

3. Techniques and Equipment

According to Reyniers (1956) the ideal germfree animal would be one resulting from the removal of all contaminants from a conventional animal. This animal, left with the "experience but not the cause of this experience", would then be comparable to the conventional. This goal of complete decontamination has never been accomplished, although much work has been done in this direction. As a result, it has been necessary to rely on techniques designed to obtain young animals before they become associated with microorganisms.

The probability of obtaining germfree mammals and birds is closely associated with the contention that these animals are free from microorganisms during their embryonic development. Reyniers and Trexler (1943) have expressed it in this manner: "That a placental animal may be obtained germ-free is based on the theory of its being free from contaminating microorganisms while *in utero*. This, of course, is the case with normal parent animals. The reasons for this theory are partially based on anatomical and membrane protection, germicidal activity of the amniotic fluid, self-sterilization of the developing embryo, and chance factors." Reyniers *et al.* (1949a) have also shown that only a small percentage of the eggs available to them were contaminated before incubation, and they suggested that the defensive mechanism of the developing chick is effective in preventing the growth of the few microorganisms which find their way through the shell. In the case of both mammals and birds advantage is taken of this freedom from microorganisms during embryonal development to procure the germfree young.

The equipment used by Nuttal and Thierfelder (1895-1896, 1896-1897, 1897) for rearing germfree guinea pigs and chickens consisted of a cylindrical glass jar with a volume of 6 liters. This jar was supplied with air filtered through cotton at both the intake and outlet. A rubber glove for handling the animals was attached to an opening in the side of the jar, and a rubber nipple was attached to a second opening. The lower portion of this apparatus was placed in a water bath to help maintain the proper environmental temperature. This equipment was sterilized with a combination of sublimate solution, alcohol, ether, flaming, and autoclaving. Using a protective tent, surgery was performed on the pregnant guinea pig with the onset of milk secretion. The young were transferred into the rearing apparatus by opening the cylindrical jar quickly at its ground-glass surface.

Schottelius (1899, 1902, 1908, 1913) used a sterile room in his efforts to rear germfree chickens. This room, constructed of metal and glass, was entered through an anteroom provided with a germicide tray containing sublimate solution. The caretaker, wearing steam-sterilized cap, mask, gown, rubber gloves, and shoes, entered through this anteroom. A small hatching compartment, built into the room, was separated from the room by a sliding door and gauze curtain. The temperature of this hatching compartment was regulated by a water bath. Air circulation to the sterile room took place by gravity through cotton filters. The room was prepared by washing with sublimate and lysol solution. This was followed by treatment with formaldehyde gas. All utensils used were sterilized by autoclaving. Eggs which had been incubated for 20 days were cleaned with mercuric

chloride solution, rinsed, and transferred into the hatching compartment, where they were left to complete incubation and hatching.

Cohendy (1912a, 1912b), in his efforts to rear germfree chickens, used a glass cylinder with metal end plates. Air entering and leaving the cylinder was forced through cotton filters. A coil of cold water was used for drying and cooling the air in the rearing unit, and at the same time the water condensing on this cold coil served as a source of drinking water for the birds. A hatching compartment was constructed in one end of the glass cylinder. The entire apparatus was sterilized with steam under pressure. The surface of the egg shell was sterilized with mercuric chloride before it was placed in the hatching compartment.

The apparatus used by Küster (1912, 1913, 1915a, 1915b) in rearing germfree goats consisted of a rearing room and a sterile lock. Air from a compressor was passed through a cotton filter and was then dried by being passed successively through concentrated sulfuric acid and potassium hydroxide. An electrical heating unit was inserted in the air flow before it entered the unit. The rearing room was supplied with rubber gloves for handling the animals. The sterile lock was equipped with 2 heavy doors. Before being sterilized, the rearing room was painted with a "germicidal" paint. It was then washed with lysol solution and treated with formaldehyde gas. The lock was sterilized with steam under pressure or with formaldehyde gas. Pregnant goats near term were subjected to surgery inside a protective tent. The young goat was then transferred by way of the sterile lock into the rearing room.

The equipment used by Balzam (1937) in rearing germfree chickens consisted of a double box equipped with cotton filters for sterilizing the air. The apparatus was sterilized with formaldehyde vapor for 3 days, with the exception of the filters which were sterilized separately by autoclaving. Food and other materials were sterilized in a metal box which was then placed in the outer compartment of the unit, and the surface of the box was sterilized with a combination of condensing steam and mercuric chloride solution. The fertile eggs from which chickens were to be hatched within the isolator were introduced after the shells were sterilized with mercuric chloride.

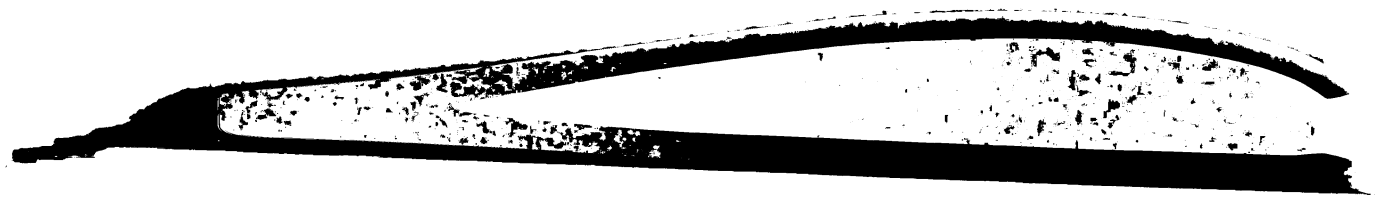
The equipment used by Reyniers et al. (1949a) in their early work on rearing germfree chickens consisted of a simple glass churn jar fitted with a cotton filter and attached to a flask of sterile water. This unit was sterilized with dry heat at 180° C. for one hour. The fertile egg, whose surface had been previously sterilized with mercuric chloride, was placed in the glass jar inside a sterile hood. At the same time, a supply of steam-sterilized food was added.

The Notre Dame group also described a germfree apparatus of the bell-jar type with the bottom of the glass jar being supported in a germicidal trap. A rubber glove attached to this trap allowed manipulation of the animals and materials within the unit. Air entering the unit was sterilized by passing through glass wool or cotton filters. The entire unit was sterilized before use by completely filling it with germicide. A modification of this unit was also constructed of metal.

The more elaborate isolation equipment designed by the Notre Dame group was first described by Reyniers (1943) and Reyniers and

Trexler (1943). Since these reports, numerous descriptions of this equipment and its use in germfree studies have appeared in the literature. Among these the following may be listed: Reyniers (1956, 1957, 1959b), Reyniers et al. (1946, 1949a), Horowitz et al. (1960), and Hickey (1960). The basic Reyniers germfree isolator has been described as a heavy metal cylinder, usually constructed of stainless steel and varying from 28 to 36 in. in diameter and up to 12 ft. in length. To this isolator were attached a window, one or more pairs of shoulder-length rubber gloves, a supply lock (which was essentially an autoclave opening into the isolator), inlet and outlet filters containing glass fibers, and outlets for attaching a steam supply. The units were sterilized by steam under pressure, each isolator simply acting as its own autoclave.

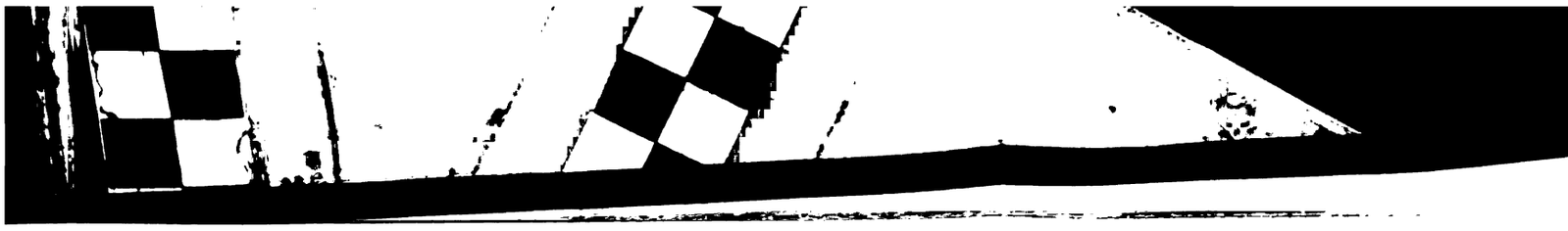
Four specialized isolators were described by Reyniers (1959b). These were rearing isolator, examining isolator, transport unit, and surgical isolator. These units could be attached to each other for transferring animals, food, etc. The surgical unit was separated into upper and lower units by a metal shelf with an opening in it. This opening was covered with sterile cellophane. At the time of surgery, the abdomen of the anesthetized full-term mammal (rat, mouse, guinea pig, or rabbit) was brought into contact with this cellophane from below. Using cautery, the operator was then able to cut through both the cellophane and abdominal skin, bring the uterus inside the isolator, and remove the young. The animals were then passed to the rearing unit. A special attachment has been described for bringing fertile eggs into the rearing isolator through a germicidal trap (Teah, 1960).



Reyniers (1956, 1957) has also described a large, colony-type isolator suitable for rearing large numbers of animals. The attendant, wearing a plastic suit, entered this unit through a germicidal trap.

The Japanese workers (Miyakawa, 1959a) have also utilized the heavy steel cylinder in designing an isolator patterned somewhat after the Reyniers unit. However, the animals, diet, cages, etc., inside the isolator were manipulated with a remote control device. This allowed the isolator to be made larger since the arm's reach of an individual was not the limiting factor. The air entering the isolator was sterilized by filtration and was also heated to 400° C. and cooled. The humidity of the air was also carefully controlled.

Gustafsson (1946-1947) has described an isolator constructed of a metal cylinder on end with a large petri dish on top as the lid. A sluice tank containing iodine was used to transfer the young rats taken by Caesarean section from the surgical isolator to the rearing unit. In a later publication (Gustafsson, 1959a) he described a larger unit constructed of thin stainless steel in a square, angled design. The top of the isolator was covered with thick glass, and heavy duty surgical gloves were fastened to rubber sleeves which were, in turn, secured to openings in the side of the isolator. The young animals were passed into this unit through the sterile food autoclave or through a germicide trap. Both these isolators were sterilized within an autoclave which was evacuated to 0.05 atmospheres before steam was introduced. Air to be passed into the isolator was first cooled and dehumidified. It was then preheated and passed through a carborundum column heated by an electrical element to 300° C., after which it was



filtered through glass-wool filter material. A heat exchanger was used to cool the air after sterilization. The outgoing air was also sterilized by heat.

The construction of an isolator from rigid transparent plastic has been described by workers at Syracuse University (Phillips et al., 1960). The walls were made of 3/8-in. Plexiglas type R (Rohm and Hass Company, Philadelphia, Pennsylvania), and were cemented together. The air entering and leaving the isolator was filtered through commercially-available filters attached to the unit. Neoprene gloves were attached to openings in the walls of the isolator. Fertile eggs, sterilized diet, water, etc., were passed into the unit through a germicidal lock containing mercuric chloride. A series of chemicals was used in the sterilization of this equipment. Included were a detergent, an iodine compound, a quaternary ammonium solution, formaldehyde, and ethylene oxide.

Trexler (1959, 1960a) and Trexler and Reynolds (1957) have described isolators constructed of flexible plastic film. The air for these isolators was sterilized by passing through glass-wool mat, and the flow of air leaving the unit was controlled by an outlet trap which was a variable orifice relief valve with a liquid seal. This outlet trap maintained a back pressure equivalent to 1/4 to 3/8 in. of water in the isolator. Shoulder-length rubber gloves, attached to the wall of the unit by means of aluminum rings, allowed access to the interior. The flexible plastic isolators were ordinarily sterilized with an aerosol of peracetic acid. Materials could be passed into the isolator by connecting it directly to an autoclave. Steam-sterilized materials in containers could also be passed through a lock in which

the outside of the container was sterilized with an aerosol of peracetic acid. It was also possible to use a germicidal dip bath in introducing materials into the isolator. Work has been conducted (Trexler, 1959, 1960c) on the use of a large plastic-lined room for rearing germfree animals. This room was chemically sterilized and was serviced by an attendant wearing a plastic garment which was also chemically sterilized.

Hickey (1960) has made a comparison of the apparatus currently in use for obtaining and earing germfree animals.

4. Diets

According to Wostmann (1959a) the main difficulty facing the nutritionist when working with germfree animals is that, while much may be known about the requirements of the host-flora complex, virtually no quantitative knowledge is available about the nutritional requirements of the host per se. It is therefore necessary to use the known requirements of the normal stock animal as a base line. In using these, correction must be made for the absence of possible intestinal synthesis (mostly of vitamins) and for the effects of the necessary sterilization, which results in loss of vitamins and of nutritional value of protein.

Nuttal and Thierfelder (1895-1896, 1896-1897) fed their germfree guinea pigs a diet consisting of undiluted milk which had been obtained by milking cows under precautions of surgical sterility. This milk was boiled and bottled in a steam bath for 1/2 hour on 3 consecutive days.

Schottelius (1899, 1902, 1908, 1913) fed his chickens a diet consisting of millet seed, chopped egg white, and egg shells. This diet was sterilized by autoclaving.

Cohendy (1912a) used different combinations of a number of ingredients in preparing diets for germfree chickens. These ingredients included egg albumin, bread crumbs, cracked corn, potatoes, rice, barley, lettuce, milk, dead flies, millet, chicory, sand, and Sprat's flour. These ingredients were sterilized with steam under pressure.

Küster (1912, 1913, 1915a, 1915b), after repairing the mother following the Caesarean section, used the animal as a source of milk for his germfree goats. The milk was stored in bottles and sterilized by steam under pressure within the sterile lock attached to the rearing unit. In addition, sterilized oatmeal was fed.

The diet fed to chickens by Balzam (1937) consisted of corn meal, corn, meat and bone flour, fish meal, cod-liver oil, brewer's yeast, sodium chloride, and calcium carbonate. This diet was also steam sterilized. This is the first recorded investigation in which vitamins were purposely added as part of the diet.

In the early work on rearing germfree chickens at Lobund Institute (Reyniers et al., 1949a), commercial starting rations, supplemented in some cases with cod-liver oil, brewer's yeast, and milk, were used. Somewhat later this same group used a number of synthetic-type diets. These diets varied quite widely in their component parts. Steam sterilization at 120° C. for 25 minutes was used for liquid and solid diets, dry heat at 170° C. for one hour was used for oils, and heat-labile substances were passed through a Seitz bacteriological

filter. These workers also investigated the destruction of vitamins by steam sterilization and reported that the loss of thiamin could be held to 30 per cent if the proper pH was maintained. About 25 to 30 per cent of the pantothenic acid was destroyed by autoclaving.

Luckey (1959) has reported further work on the preparation of diets for germfree chickens and described a number of practical and synthetic-type diets. He also investigated the nutrient loss in synthetic-type diets and found an 11 per cent loss of both fat and protein due to steam sterilization at 17 lbs. per sq. in. for 15 minutes and storage at room temperature for one week. Under the same conditions, the loss of vitamins ranged from 3 per cent for choline up to 92 per cent for thiamin. Luckey also found considerable loss of some nutrients due to the mixing procedure. In addition to sterilization by steam under pressure, Luckey stated that filtration, intermittent heating, gamma rays, and ethylene oxide may all be used for sterilization of various diets.

A description of some of the early diets used by the Lobund group in attempting to rear germfree rats was given by Reyniers et al. (1946). The liquid diets included such raw materials as rat milk, stomach contents of suckling rats, and milk from cows, dogs, rabbits, and sows. Various synthetic-type diets were also formulated. The most successful liquid diet contained casein, lactalbumin, a salt mixture, raw cream, sodium hydroxide, and distilled water. This was sterilized with steam at 15 lbs. per sq. in. for 20 minutes. A vitamin supplement was also fed to the animals. The solid diet consisted of a commercial rat diet or a synthetic-type diet, both supplemented with vitamins. These workers described the steam sterilization of dry food. Their

procedure consisted of evacuating the supply lock to 29 in. of mercury, allowing steam to flow freely for 10 minutes, building up the steam pressure to 15 to 20 lbs. per sq. in., holding this pressure for 20 to 45 minutes, and finally evacuating the lock to 29 in. of mercury for about 45 minutes to help dry the contents. The initial evacuation before the introduction of steam helped to insure the circulation and penetration of steam into the solid food.

Pleasants (1959) has recently described various synthetic-type diets used at Lobund Institute for rearing germfree rats, mice, and rabbits through weaning. He also described the rubber nipples and forced-feeding techniques used for the young animals. Wostmann(1959a) has listed both liquid and solid diets successfully used in rearing germfree laboratory mammals. He stated that steam sterilization (123° C., 17 lbs. per sq. in. for 25 minutes) is the most satisfactory method for sterilizing diets. Wostmann also stated that steam sterilization may cause loss of dietary thiamin of 80 to 90 per cent. Losses of other vitamins do not usually exceed 40 to 50 per cent. The nutritional value of protein is also affected by steam sterilization, with the availability of lysine especially being affected. Phillips et al. (1959) have investigated a number of liquid, dry, and semisolid diets suitable for rearing germfree guinea pigs.

Miyakawa (1959b) reported the use of a liquid synthetic-type diet for rearing guinea pigs for the first few days of life. The diet was then gradually changed to a synthetic-type solid diet so that only the solid diet was fed after the first month.

The liquid diet used by Glimstedt (1936) for his germfree guinea pigs consisted of cows' milk to which was added either blood

plasma or cream and plasma. This diet was sterilized by steam at 110° C. for 3/4 of an hour on 3 successive days. The various solid diets used were sterilized by steam at 110° C. for 2 hours. The vitamin supplements used were sterilized by filtration.

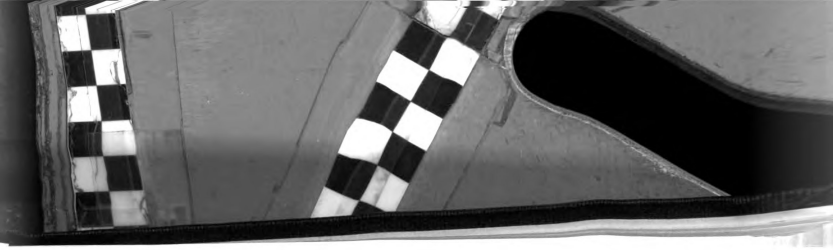
Gustafsson (1948) reported the use of a milk mixture made with enzymatically digested casein as a source of protein. Gustafsson (1959b) used a stock diet composed of casein, wheat starch, arachis oil, a salt mixture, and a vitamin supplement. This diet was sterilized by steam at 121° C. for 30 minutes.

Phillips et al. (1960) sterilized solid diets for chickens by electron beams, using radiation doses of 4 to 5.5 megarads without visible alterations in the diet.

Luckey (1960) used a diet containing casein, glucose, corn oil, salts, roughage, acetate, and vitamins in his attempts to rear germfree lambs.

5. Use of Germfree Animals

Much of the early work with germfree animals was concerned with the development of techniques suitable for rearing the various species of animals free from contamination. Küster (1912), however, realized the promising scientific field of research opened by these germfree rearing attempts and suggested that animals raised under such conditions might be suitable for studying such things as digestion and absorption, development of natural and artificial immunity, wound healing, and destruction of pharmaceutical and therapeutic preparations.



One of the areas of particular interest in germfree research has been the anatomical comparison of germfree and conventional animals. This has involved primarily the intestinal tract (Gordon, 1959; Gordon and Bruckner-Kardoss, 1958-1959) and the lymphatic system (Glimstedt, 1936; Thorbecke, 1959; Thorbecke and Benacerraf, 1959; Thorbecke et al. 1957; Miyakawa, 1959b; Miyakawa et al., 1957).

Germfree animals have also been used quite extensively in nutritional studies, especially in attempts to define the role of the intestinal synthesis of certain vitamins. The following may be mentioned as contributions of the germfree techniques in the study of the nutrition of monogastric animals: Gustafsson (1959b), Wostmann (1959a), Luckey (1959), Reyniers (1946, 1956), and Luckey et al. (1955a, 1955b). In addition, Smith and Trexler (1960) and Luckey (1960) have introduced the use of germfree animals into the study of the process of ruminant digestion.

The serologic aspects of germfree life have also been investigated. Wostmann (1959b) has studied the serum proteins in germfree vertebrates as have Wostmann and Gordon (1958). The formation of antibodies in germfree animals has been investigated by Springer et al. (1959), Wostmann (1959b), and Wagner (1955, 1959).

Germfree and gnotobiotic animals have proved to be of value in a study of the mode of action of antibiotics in growth promotion. Most evidence (Luckey et al., 1956; Wagner and Wostmann, 1958-1959; Forbes and Park, 1959; Lev and Forbes, 1959; Forbes et al., 1959; Gordon et al., 1957-1958) points to the fact that conventional chickens fed antibiotics show an increased growth rate because of the inhibiting action of the antibiotics on certain undesirable bacterial

species. These same workers in general found no growth promoting action when antibiotics were fed to germfree chickens. Luckey et al. (1956), however, found that low levels of certain antibiotics produced growth increments in germfree chickens.

Phillips and Wolfe (1959) and Phillips et al. (1955) have studied the relationship between bacteria and infection with Entamoeba histolytica. They were unable to produce lesions in the germfree guinea pig when the parasite alone was given. When certain species of bacteria were also given, lesions were produced. Newton et al. (1959) and Phillips (1960) have also studied the development of helminths in germfree animals.

Germfree animals have also been used in studying diseases caused by bacteria. Orland et al. (1954) were unable to demonstrate dental caries in germfree rats fed a diet which consistently led to the production of caries in conventional rats. Later work (Orland et al., 1955; Orland, 1959) demonstrated that Enterococcus in pure culture, combined with the cariogenic diet produced caries in germfree rats. Taylor (1959) has discussed the use of germfree animals in virology.

Among the miscellaneous areas of research utilizing germfree animals may be listed studies involving tumors produced in chickens by the injection of methylcholanthracene (Taylor et al., 1959; Reyniers and Sacksteder, 1959), liver necrosis (Luckey et al., 1954), hemorrhagic shock (Zwiefach, 1959), and otolaryngic experimentation (Kelemen, 1960).

B. Artificial Rearing of Pigs

Early attempts to rear baby pigs without the aid of colostrum often met with failure. Bustad et al. (1948) reported that pigs removed from the mother at birth and placed on a synthetic milk containing all known vitamins and with plasma or serum as colostrum substitutes failed to survive longer than 22 days. When no colostrum substitute was fed, the pigs died shortly after birth. These workers concluded that colostrum and serum or plasma contain something which is necessary for the survival of the pig. A severe diarrhea developed in all animals fed the synthetic milk. The apparently critical need for colostrum in the diet of the young pig was further emphasized by the numerous reports of successful artificial rearing of pigs weaned at an early age. Wintrobe (1939) successfully reared pigs weaned at 2 to 23 days of age on a diet of casein, lard, sucrose, minerals, and vitamins. Becker et al. (1954), however, working with pigs allowed access to colostrum for 24 hours, concluded that the baby pig was unable to utilize sucrose as a source of carbohydrate. A synthetic-type diet with glucose as the carbohydrate did produce satisfactory results. Johnson et al. (1948) reported the rearing of pigs weaned at a minimum of 24 hours on a diet of casein, cerelose, lard, salts, and vitamins. Other successful attempts to rear pigs weaned at one to 2 days of age have been reported by Reber et al. (1953), Schendel and Johnson (1953), and McCrea and Tribe (1954, 1956).

It is now recognized that young pigs, along with the young of other farm mammals are incapable of producing antibodies themselves for some considerable time after birth and that they derive their immunity from the mother through the colostrum (Brambell et al., 1951).

Rutqvist (1958) was unable to detect gamma globulin in the serum from pig fetuses and newborn pigs. Large amounts of gamma globulin appeared in the serum shortly after nursing. It has been shown that gamma globulins form the chief component of circulating antibodies (Wostmann, 1959b). This definite correlation between the ingestion of colostrum and the level of antibody in the young pig has also been noted by Hoerlein (1957), Straub and Boguth (1956), and Wellman and Heuner (1958). Work by Hoerlein (1957), Asplund (1960), and Speer (1957) indicated that the ability to absorb gamma globulins from the colostrum is lost in the young pig after approximately the first 24 hours.

Young and Underdahl (1951), in discussing the rearing of colostrum-deprived pigs, stated that it is of vital importance to protect the young and vulnerable pig from infections that may be enzootic within the herd, even though such infections are not apparent in older swine or in pigs that have had colostrum. These workers caught pigs in sterile bags at the time of birth and raised them in an isolated room heated to 80 to 90° F., using a diet based on milk. This same group has developed a technique for removing the pigs from the sow by means of hysterectomy (Young et al., 1955). The full-term sow was anesthetized with carbon dioxide, and the entire gravid uterus was removed and passed into a hood previously treated with formaldehyde (Underdahl and Young, 1957a). Here the pigs were removed from the uterus, the umbilicus was ligated and cut, and the stump was treated with tincture of iodine. The young animals were then transported in a previously sterilized wooden box to the rearing room. Here the pigs were placed in individual isolation units in a room previously treated with formaldehyde (Young and Underdahl, 1953; Young et al., 1955).

The isolation units were kept at 90 to 100° F. for the first few days, and then the temperature was gradually lowered.

The diet described by Underdahl and Young (1957b) for starting the young pigs consisted of one quart of homogenized milk, one whole egg, and 5 ml. of a mineral mixture containing ferrous sulfate, copper sulfate, manganese chloride, and potassium iodide.

The pigs were transferred at one week of age to an isolation brooder (Underdahl and Young, 1957b) large enough to hold 12 pigs. They were continued on the same diet and gradually changed to a commercial pig starter feed. The pigs at 4 weeks of age were transferred to a clean area. The use of these "disease-free" pigs to repopulate herds affected with atrophic rhinitis and virus pneumonia of pigs has been described by Young et al. (1959).

Whitehair and Thompson (1956) reported the rearing of Caesarean-derived pigs in individual metabolism cages on a diet of casein, fat, lactose, minerals, and vitamins. Others successfully rearing colostrum-deprived pigs have included Haelterman (1956), Hoerlein et al. (1956), and Shuman et al. (1956). Catron et al. (1953) described a practical synthetic milk formula for baby pigs receiving no colostrum.

In attempts to improve the diets for rearing young pigs, various workers have studied the composition of sows' milk. Work has been reported by Heidebrecht et al. (1951) and Davis et al. (1951). The British workers (Braude et al., 1947) have found that sows' milk contains a higher percentage of total solids, solids-not-fat, protein, ash, calcium, and phosphorus than the milk of cows and goats and about the same percentage of lactose. Bellis (1957) also compared the composition of the milk of various mammals.

III. EXPERIMENTAL

A. Procedures

1. Equipment

The equipment used in 4 experiments on collecting and rearing gnotobiotic pigs was similar to that described by Trexler (1959, 1960a) and Trexler and Reynolds (1957) for rearing laboratory animals. Two principal pieces of equipment were used, one being the surgical isolator and the other the rearing unit. Both these isolators were constructed of vinyl film which was 8 mils (0.008 in.) in thickness.

The surgical isolator (Figure 1) measured 36 x 36 x 72 in. and was mounted on a sheet of plywood slightly wider and longer than the unit. The top of the plywood was covered with one layer of paper-packing material to help cushion and protect the bottom of the isolator. At one end of the sheet of plywood was fastened a fiber-glass ring 16 in. in diameter and approximately 12 in. in length. This ring served as an opening into the isolator, and it extended about 2 in. above the top of the plywood. A circular piece of the vinyl film about 12 in. in diameter was cut out of the bottom of the isolator, and the resulting opening was stretched over the top of the fiber-glass ring. The film and fiber glass were fastened together by 3 complete turns of Scotch Brand Tape No. 471 (Minnesota Mining and Manufacturing Company, St. Paul, Minnesota). At the opposite end of the isolator were attached the air filter and air-outlet trap to be described in more detail later.

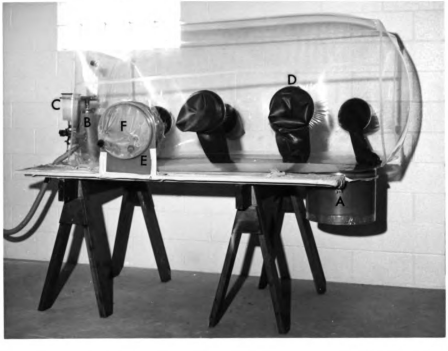


Figure 1. Surgical isolator

A. 16-in. fiber-glass ring

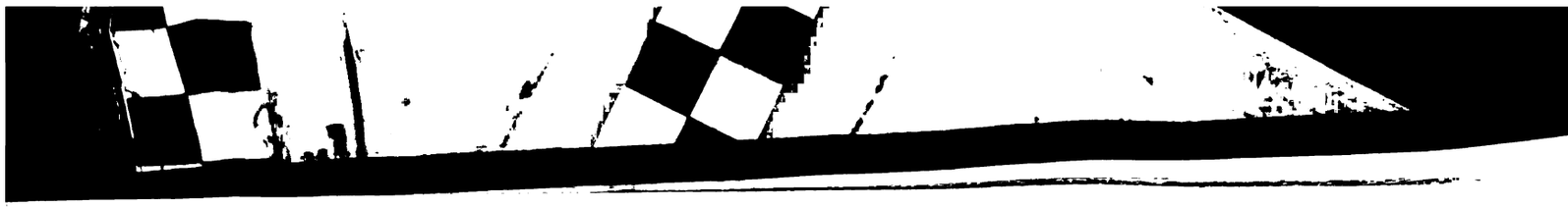
B. Air filter

C. Air-outlet trap

D. 30-in. dry-box gloves

E. 12-in. fiber-glass ring

F. Outer door



Three pairs of 30-in. dry-box gloves (Charleston Rubber Company, Charleston, South Carolina) were attached to the sides of this surgical unit. Two pairs, both 25 mils thick, were attached opposite each other on the end nearest the fiber-glass ring in the bottom of the isolator. The third pair, 15 mils in thickness, was attached on one side near the opposite end. The gloves were attached to the isolator by a method differing somewhat from that described by Trexler and Reynolds (1957). A circular opening approximately 7 in. in diameter was cut in the vinyl film at the place where the glove was to be attached. The cuff of the glove was cemented to the film and both were held in place in the outer, concave surface of a spun-aluminum ring approximately 9 in. in diameter.

Directly opposite the third pair of gloves was attached another fiber glass ring which was 12 in. in diameter and 6 in. in length. This ring was placed vertically. Both this and the larger ring were provided with flanges for securing the rings to the plywood base. The vinyl film was attached to the smaller ring in the previously described manner. Inner and outer doors or covers for this ring were constructed of 20-mil vinyl film with the door being made slightly smaller in diameter than the fiber-glass ring. The inner door was stretched over the interior opening of the ring and held in place with a large rubber band cut from a truck inner tube. The outer door was held in place with 3 turns of Scotch Brand Tape No. 471. This outer door was provided with 2 openings constructed of Tygon flexible tubing (U. S. Stoneware Company, Akron, Ohio) 1 in. in diameter and 3 1/2 in. long. These were either sealed to the vinyl film by use of a Callanan Dielectric Sealer (J. A. Callanan Company, Chicago,

Illinois) or were taped in place in much the same manner as the fiber-glass rings. Three such openings were provided in the isolator itself, one each for receiving the air filter and air-outlet trap, and the third for use during sterilization of the isolator. These openings were closed with rubber stoppers when not in use.

The rearing isolator (Figures 2, 3) measured 24 x 24 x 72 in., and it was attached to a plywood base with the ends and part of the sides built up to give added support. An air filter (Figure 4) and air-outlet trap (Figure 5) were attached to one side of the unit, along with a 12-in. fiber-glass ring fitted with inner and outer doors as previously described. On the opposite side one pair of gloves was attached. The floor of both the surgical and rearing units was covered with a sheet of ribbed polyethylene to prevent cages and sharp objects from perforating the vinyl film.

In the first 2 experiments no satisfactory method of weighing the pigs inside the isolator was found. In Experiments III and IV a hook was installed in the top of the rearing unit by cutting a small hole in the vinyl film. A piece of Tygon tubing 1/2 in. in diameter and approximately 3 in. long was taped into this hole as has been previously described. A length of small stainless steel rod was then forced through a hole drilled in a small rubber stopper. A hook was bent on either end of the rod, and the stopper was taped securely into the upper end of the tubing. This allowed the upper end of the hook to be attached to a spring-type milk scale. This scale, in turn, was suspended from the ceiling of the room. The pig was suspended from the lower hook in a small cloth bag or by means of a loop of string around the hock joint.

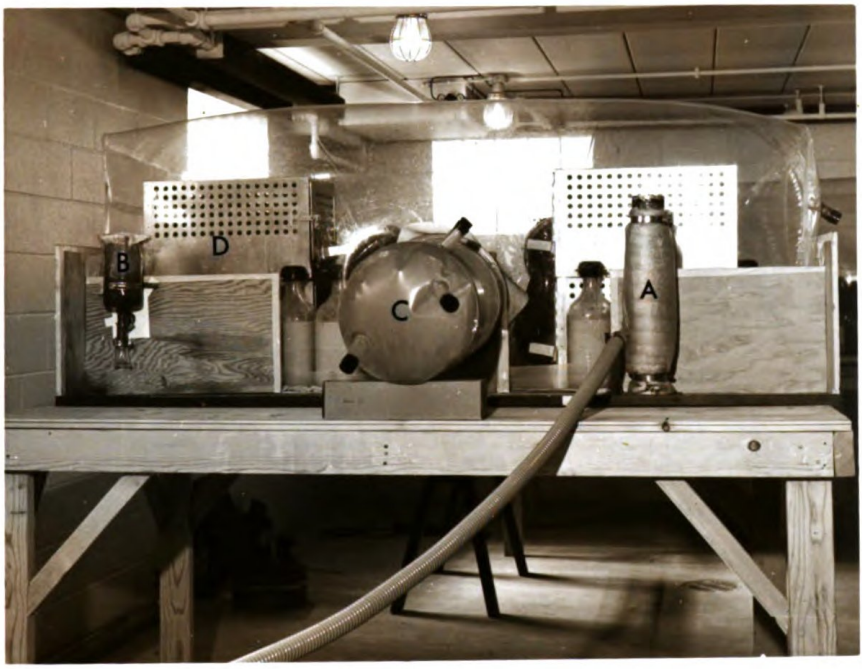


Figure 2. Rearing isolator viewed from filter side

A. Air filter

B. Air-outlet trap

C. Sterile lock

D. Rearing cage

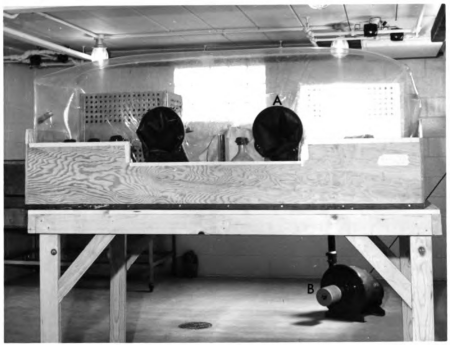


Figure 3. Rearing isolator viewed from glove side

A. 30-in. dry-box gloves

B. Spencer Turbo Compressor

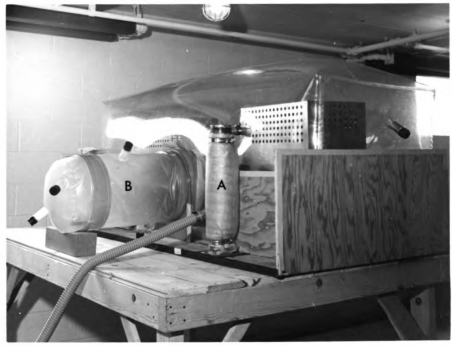


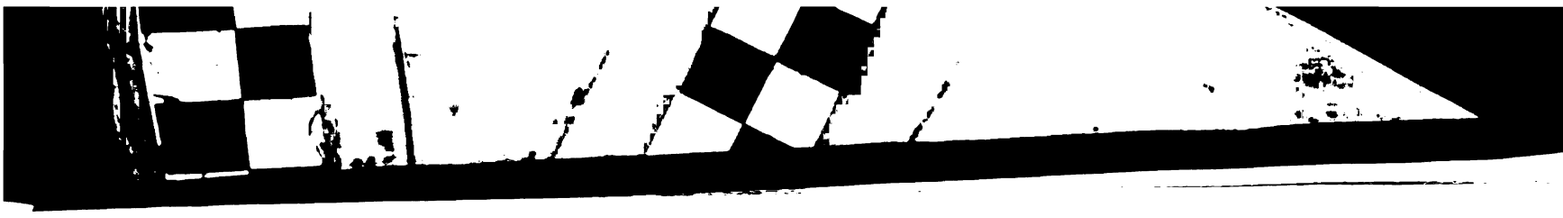
Figure 4. Air filter attached to rearing isolator

A. Air filter

B. Sterile lock

Figure 5. Air-outlet trap attached to rearing isolator

A. Air-outlet trap



The sterile lock (Figure 4) for the introduction of liquid diet, vitamins, minerals, etc., into the isolator consisted of a tubular piece of 20-mil vinyl film 12 in. in diameter and 24 in. in length. This lock had 2 Tygon-tubing openings, one near either end. The outer door was removed from the rearing isolator and one end of the lock was fastened to the fiber glass ring with 3 turns of Scotch Brand Tape No. 471. A fiber-glass ring 12 in. in diameter and 2 3/4 in. in length was taped on the other end of the lock, and the outer door was also taped to this ring. This formed a chamber which could be loaded from the outside and whose contents could be brought into the rearing isolator through the inner door.

The air filters were constructed with a core of wire mesh or expanded metal having solid ends (Figure 6). On the top end, a metal tube, 1 in. in outside diameter, was welded. This core was wrapped with four 1/2-in. layers of FG 50 glass-wool mat (American Air Filter Company, Louisville, Kentucky)(Figure 7) which were held in place by several turns of small stainless-steel wire. This amount of filter material has been found sufficient to retain a dried aerosol of washed bacterial spores at velocities encountered during use (Trexler, 1959). A cover of 8-mil vinyl film was held in place over the filter material by 2 radiator hose clamps (Figure 8). In actual use the air entered through a short piece of Tygon tubing attached to the cover, went through the 4 thicknesses of filter material, and left through the metal tubing attached to the filter core. This tubing was secured inside one of the Tygon-tubing outlets of the isolator by means of a small radiator hose clamp.

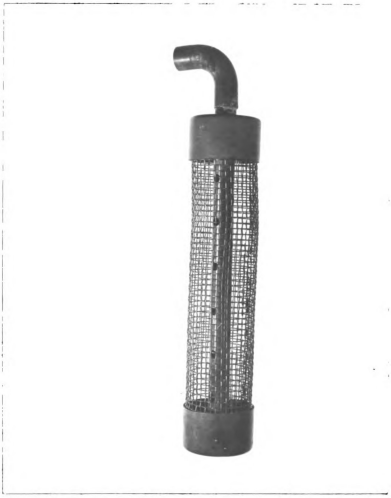


Figure 6. Air-filter core

Figure 7. Wrapped air-filter core

A. Air-filter core

B. Glass-wool mat

Figure 8. Assembled air filter

A. Vinyl cover

B. Radiator-hose clamp

C. Adjustable clamp

D. Tygon tubing for attachment to air source

E. Metal tube for connecting to isolator

Air filters of similar construction have been shown by Trexler and Reynolds (1957) to pass 2 to 3 cu. ft. of air per minute at an air pressure of 5 lbs. per sq. in.

The air-outlet trap (Figures 9, 10) was made of fiber glass. The trap was attached to the isolator by slipping the small side arm inside one of the short pieces of Tygon tubing forming one opening into the isolator and attaching it with a small radiator hose clamp. The lower end of the small fiber-glass tubing, covered with a short length of Tygon tubing sealed on one end, acted as a reservoir to prevent fluid spilled from the trap from running into the isolator. The upper end of this tube rose above the level of the liquid in the trap. The end of this tube was covered by an inverted plastic tumbler with 2 openings in its wall. These openings were at such a level that they were approximately 1/4 in. below the surface of the liquid when the tumbler was at its lowest position. A wire ring was mounted inside the large opening of the tumbler, and a short piece of wire was cemented to the upper surface. These both acted as guides. A wire screen covered the top of the trap to shut out insects. The air-outlet trap was filled to the proper level with Cellulube (Celanese Corporation of America, New York, N. Y.).

The air leaving the isolator entered the side arm of the trap, traveled into the upper end of the fiber-glass tube, raised the inverted tumbler, and escaped through the holes in the wall of the latter. Any decrease in pressure within the isolator, caused, for example, by withdrawing one's arms from the gloves, caused the tumbler to drop so that the outlet openings were below the surface of the

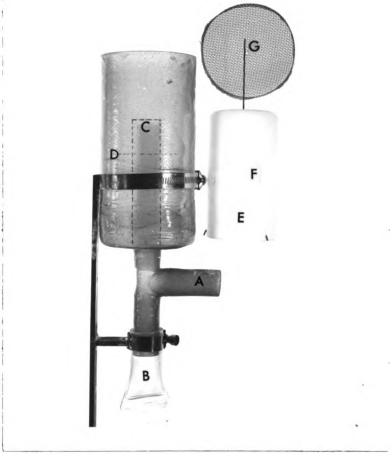


Figure 9. Air-outlet trap, disassembled

A. Side arm

B. Reservoir

C. Upper end of fiber-glass tubing

D. Fluid level

E. Plastic tumbler

F. Opening in wall of tumbler

G. Wire-screen cover

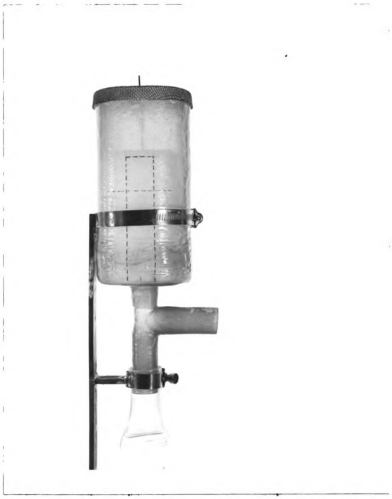
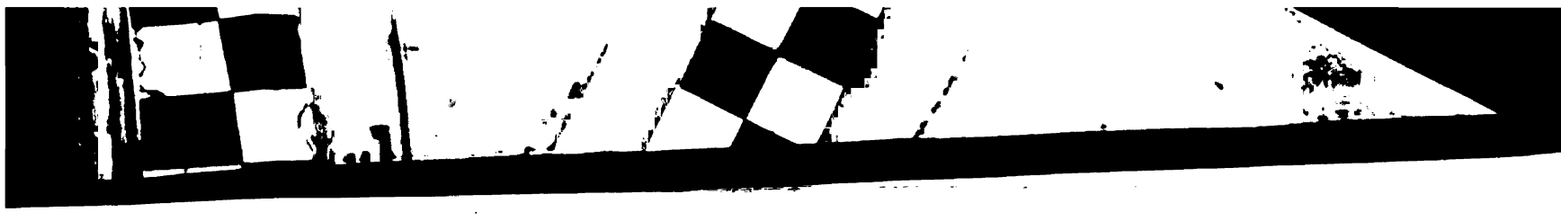


Figure 10. Air-outlet trap, assembled



liquid. This prevented a back-flow of contaminated air into the isolator.

In Experiment I the air-outlet trap of the rearing isolator consisted of a 6-in. length of vinyl film tubing 1 in. in diameter. One end of this tubing was attached to the Tygon tubing opening of the isolator. The other end, with a small metal weight attached, floated on the surface of a 1-liter beaker 3/4 filled with mineral oil. The air flow caused this tubing to float on the surface of the liquid. A decrease in pressure caused the end of the tubing to sink, and the back-flow was prevented.

Outlet traps of this design have been shown by Trexler (1959) to maintain a pressure equivalent to 1/4 to 3/8 in. of water within the isolator.

Air flow was provided either by small individual electric blowers of the squirrel-cage type (Universal Electric Company, Owosso, Michigan) or by a Spencer Turbo Compressor (Spencer Turbine Company, Hartford, Connecticut). The latter provided enough air flow for several isolators at one time and was fitted with a "rough" filter for removing dust and other extraneous material from the air. The air source and the filter on the isolators were connected by lengths of vacuum-sweeper hose. The flow of air was regulated by an adjustable clamp on the Tygon tubing of the air filter so that the inverted plastic tumbler of the outlet trap rode at the proper level.

The cages (Figures 11, 12) were constructed of stainless steel and measured 11 x 11 x 18 in. They were provided with a door, a removable floor of heavy hardware cloth, and a removable tray for collecting urine, feces, and spilled food material. The door contained

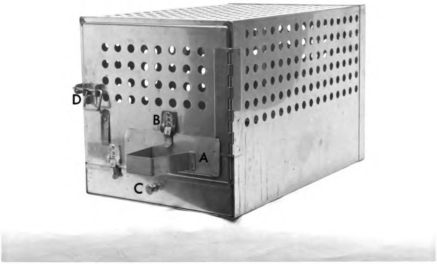


Figure 11. Stainless-steel rearing cage

A. Feeding pan

B. Metal clip

C. Removable tray

D. Door catch

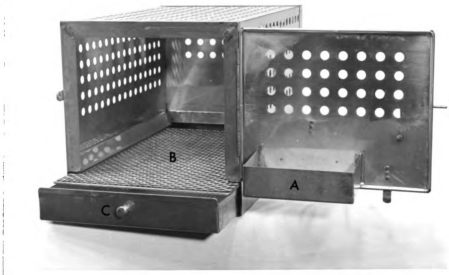


Figure 12. Interior of rearing cage

A. Feeding pan

B. Removable floor

C. Removable tray

an opening to receive the feeding pan which measured 5 3/4 x 4 in. and was 1 5/8 in. deep. Metal clips held the feed pan and the floor in place.

The containers for liquid diet (Figures 13, 14) were 2-liter Square-Pak Flasks (American Sterilizer Company, Erie, Pennsylvania). These containers were sealed by 2-piece lids. A rubber collar slipped over the neck of the container, and a hard plastic cap covered this collar. The collar and cap were self-venting so that liquid diet could be autoclaved within them. When the containers cooled, the collar and cap formed an air-tight seal.

2. Sterilization of Equipment

All equipment to be sterilized was first washed with water and a detergent, rinsed, and dried. The lower end of the 16-in. fiber-glass ring on the surgical unit was covered with a sheet of Mylar (E. I. duPont de Nemours and Company, Inc., Wilmington, Delaware) 1.5 mils in thickness. This was held in place by 3 complete turns of Scotch Brand Tape No. 853. The air filter was detached from the isolator, and the vinyl cover was removed. A small piece of Mylar was placed over the metal tube carrying air from the filter and was taped in place with Scotch Brand Tape No. 853. The filter was then wrapped in paper and sterilized in an oven at 150° C. for 1 1/2 hours. The filter was then attached to the isolator, and the cover was replaced.

The surgical isolator was next inflated with a mixture of Freon (10 per cent) and air (90 per cent). A Halogen Leak Detector Type H-2 (General Electric Company, Schenectady, New York) was used

Figure 13. Square-Pak Flask, disassembled

A. Rubber collar

B. Plastic cap

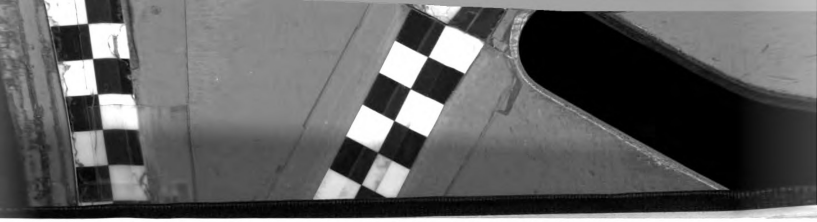
Figure 14. Square-Pak Flask, assembled

to check the unit for leaks, covering the entire surface of the vinyl film and gloves. Any leaks found were patched with 8-mil vinyl film using the Callanan Dielectric Sealer.

The interior of the surgical unit was sterilized with 2 per cent peracetic acid. Trexler (1959, 1960a), Trexler and Reynolds (1957), and Kline and Hull (1960) have found this material to be a very effective sterilization agent when it is properly used. Trexler and Reynolds (1957) have emphasized that peracetic acid is very corrosive, and many substances such as heavy metals catalyze its decomposition. They therefore recommended plastics, stainless steel, and glass as the construction materials of choice in cases where sterilization was to be accomplished with peracetic acid. Nacconal NRSF (National Aniline Division, Allied Chemical Corp., New York, N. Y.) in the amount of approximately 0.1 per cent was added as a detergent and wetting agent. This solution was made with distilled water. The interior surfaces of the gloves, isolator, and floor mat were thoroughly wet with this solution which was atomized with a small paint spray unit modified by inserting a small plastic tube inside the spray nozzle.

After the surfaces were thoroughly wet, the isolator was inflated with air and all openings sealed with rubber stoppers. The interior surface of the outer door was sprayed. The door was taped in place, and the area between the inner and outer doors was fogged through the 2 openings in the outer door.

The surgical unit was allowed to remain closed for at least 2 hours. Then the Mylar film covering the opening of the air filter was punctured from inside the isolator with a small length of



stainless steel wire, the isolator was connected to the air supply, and the interior of the isolator was allowed to dry.

Before the interior of the rearing isolator was sterilized, the cages were wrapped in paper and sterilized with steam at 250° F. for 30 minutes. Since the cages were too large to go through the 12-in. fiber-glass ring, it was necessary to cut an opening in the vinyl film, insert the wrapped cages, and reseal the opening with the Callanan Dielectric Sealer. The cages were then unwrapped and sprayed and the rearing unit was sterilized in the same manner as was described for the surgical isolator.

After the rearing unit was dry, the remaining supplies were sterilized and transferred into it. These supplies included surgical instruments, towels, cloth gloves, a length of cotton cord, vials of minerals and vitamins, screw-capped test tubes, a 4-oz. rubber bulb, a glass graduate, and the initial supply of milk in 2-liter flasks. This material was placed in a modified Reyniers unit, and a vacuum of approximately 25 in. of mercury was drawn in the unit to remove air pockets and insure better penetration of steam. The vacuum was then broken with steam, and the contents were sterilized at 252° F. for 28 minutes. A sterile lock attached to the rearing unit then permitted these supplies to be transferred from the Reyniers unit to the vinyl rearing unit.

3. Surgical Procedure

A small, round-bottomed, galvanized tank of approximately 25-gal. capacity was placed at the end of the surgical unit so that the 16-in. fiber-glass tubing extended into one end of the tank

(Figure 15). A piece of large-size polyethylene tubing, sealed at either end, was used to line this tank, and an opening was cut in this tubing to receive the 16-in. fiber-glass ring. Two Tygon openings were also attached to the polyethylene, and 20 gal. of water were added to the lined tank through one of these openings. The water covered approximately 4 in. of the outside of the fiber-glass ring. Peracetic acid was added to provide 50 parts per million, and the interior of the polyethylene was fogged with 2 per cent peracetic acid solution. Shortly before the unit was to be used, 1 gal. of 5.25 per cent sodium hypochlorite bleach was added to the solution in the lined tank.

The outer doors were removed from both isolators, and the 2 fiber-glass tubes were connected by a short length of 20-mil vinyl tubing 12 in. in diameter with 2 Tygon openings in the vinyl (Figure 16). This connecting tubing was taped in place with 3 turns of Scotch Brand Tape No. 471 on each end. The interior surface of this connection was then fogged with peracetic acid solution, and the Tygon openings were closed with rubber stoppers. After 30 minutes both inner doors on the isolators were opened, and the necessary towels, surgical instruments, and cotton cord were passed into the surgical unit.

The pregnant Yorkshire sow on the 112th day of gestation was thoroughly scrubbed with a detergent solution. She was then suspended by the rear limbs, head down, using a chain hoist and cables attached above the fetlock joints, and lowered into a large tile, 24 in. in diameter and 36 in. deep, sunken in the floor with the upper surface level with the floor. Seven pounds of dry ice had previously been pulverized and placed in this tile. After one minute surgical

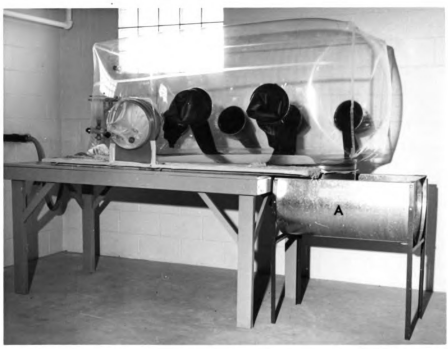


Figure 15. Surgical isolator with tank in place

A. Galvanized tank

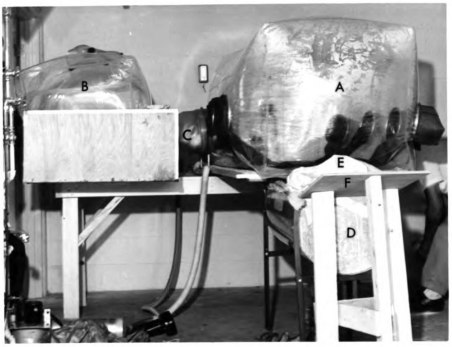


Figure 16. Surgical and rearing isolators connected

A. Surgical isolator

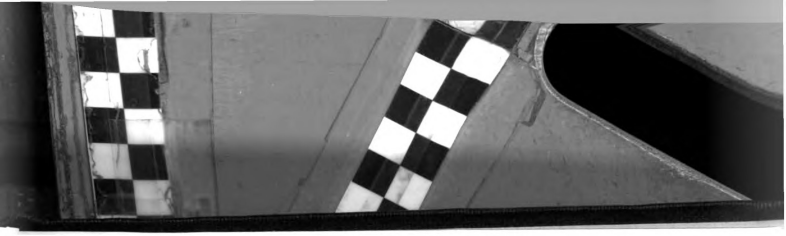
B. Rearing isolator

C. Connecting tube

D. Galvanized tank

E. Polyethylene lining tank

F. Plywood incline

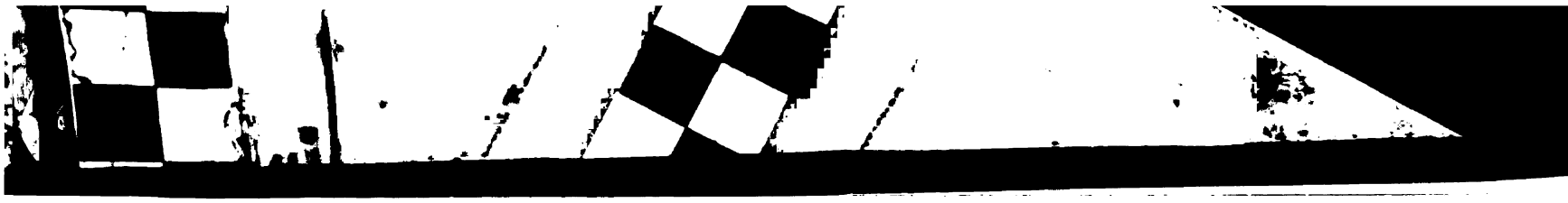


anesthesia was complete, and the sow was raised from the pit. The abdomen was then thoroughly scrubbed with Weladol Shampoo (Allied Laboratories, Inc., Indianapolis, Indiana) and dried. In Experiments II, III, and IV the ventral abdominal wall was then sprayed with Vi-Hesive Surgical Adherant (Aeroplast Corporation, Dayton, Ohio). A sterile piece of Mylar approximately 2 ft. by 3 ft. was then cemented to the ventral abdominal wall.

The abdominal cavity was then quickly opened in the ventral midline, cutting through the Mylar when it was used. The incision extended from just anterior to the brim of the pelvis to a point just posterior to the xiphoid cartilage. A heavy cotton ligature was then tied around the uterus just anterior to the cervix. In Experiment III, 2 heavy forceps of the intestinal type were applied instead of the ligature. The uterus was then removed by cutting just posterior to the ligature and also just anterior to the ovaries.

In Experiment I the uterus was passed directly into the germicidal tank after the top of the polyethylene bag lining the tank had been incised. In the other 3 experiments a piece of plywood approximately 15 in. square was set at the end of the tank at a slight incline (Figure 16). This was covered with sterile Mylar and the uterus was allowed to slide down this surface into the germicidal tank. The uterus was guided into the tank by an assistant wearing sterile, shoulder-length gloves. This assistant then kept the uterus in continuous motion in the germicide for 30 seconds.

In the meantime, the Mylar covering the bottom of the 16-in. fiber-glass ring was punctured with scissors by the operators working through the gloves of the isolator. The uterus was then pulled into



the surgical isolator and torn open as rapidly as possible. Blunt scissors or forceps were used to help puncture the uterine wall. The fetal membranes were removed from each pig, and a small alligator-type electrical clip or a Kelly forceps was placed on the umbilical cord approximately 1 in. from the body. The pigs were then given artificial respiration by alternately pressing and releasing the chest until all were breathing.

The umbilical cords were then ligated close to the body wall with heavy cotton cord, and the cords were cut between the ligature and the forceps. The pigs were dried with cotton towels and were then swabbed with a towel saturated with a solution of Hyamine (Rohm and Hass Company, Philadelphia, Pennsylvania) in Experiment I. In Experiment IV they were swabbed with a solution of Quan-Sept (Fort Dodge Laboratories, Fort Dodge, Iowa). The operator using the gloves opposite the 12-in. fiber-glass ring then passed the animals into the rearing unit.

The inner doors were replaced on both isolators, and the connecting tube was removed. The inner surfaces of the outer doors were sprayed with 2 per cent peracetic acid, the doors were taped in place, and the space between the doors was fogged with the solution through the Tygon openings. The rearing unit was then removed to the room in which the animals were to be kept.

4. Diets and Rearing Procedures

a. Experiment I

The diet used in Experiment I consisted of pasteurized, homogenized milk containing 4.5 per cent butterfat and fortified with

400 International Units of irradiated ergosterol per quart. Two quarts of this milk were placed in each 2-liter Square-Pak Flask, and the initial supply was sterilized with steam as described previously. The initial supply of milk was passed directly into the rearing unit from the autoclave.

During the course of the experiment, milk was sterilized by steam at 250° F. for 30 minutes. The flasks were then removed from the autoclave, and the exterior surfaces of the flasks were cleaned and dried. The sterile lock (Figure 4) was then attached to the rearing unit, and the outer door was removed. The interior surface of the lock was sprayed with peracetic acid solution, and the exterior surfaces of the Square-Pak Flasks were likewise sprayed before being placed in the lock. The exterior door was then replaced, and the space within the lock was fogged with the solution. After a waiting period of at least 30 minutes, the inner door of the lock was removed, the flasks were taken into the isolator, and the inner door was replaced.

The vitamin mixture used in this experiment to compensate for vitamin loss in sterilization was essentially B vitamin mixture 103 described by Pleasants (1959), except that it was twice as concentrated. The vitamins used are listed in Table I. This solution was made up and dispensed in 15-ml. quantities into 20-ml. glass ampules. The ampules were then sealed and sterilized with the initial milk supply, being passed directly into the rearing unit from the autoclave.

The mineral supplement used was salt solution 15 also described by Pleasants (1959). It consisted of solution A and solution B which were made up and sterilized separately in 15-ml. quantities

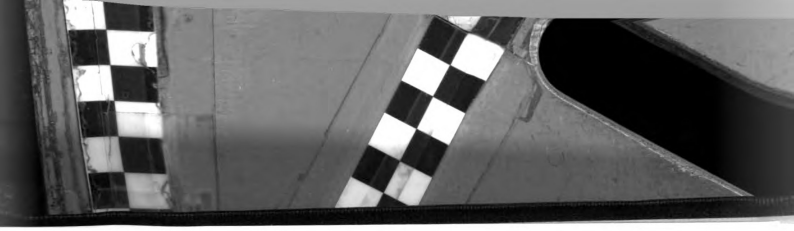


TABLE I. B vitamin mixture 103, concentrated

Vitamin	Mg. per 1.5 ml. water
Thiamine hydrochloride	3
Riboflavin	1
Pyridoxine hydrochloride	1
Niacinamide	2.5
Calcium pantothenate	12.5
Choline dihydrogen citrate	100
Biotin	0.025
Folic acid	0.25
Vitamin B_{12}	0.025
Inositol	100

as described for the vitamin mixture. The composition of the solutions are listed in Tables II and III.

TABLE II. Salt mixture 15, solution A

Ingredient	Mg. per ml. water
KH_2PO_4	176
Na_2HPO_4	180
KI	0.4

Before the milk was fed to the pigs within the isolator, 30 ml. of B vitamin mixture 103, concentrated, and 15 ml. each of mineral solutions A and B were added to each Square-Pak Flask

TABLE III. Salt mixture 15, solution B

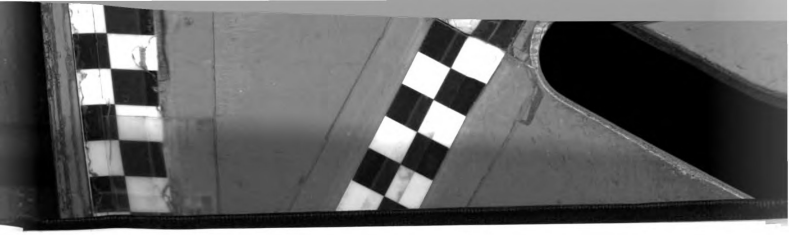
Ingredient	Mg. per ml. water
$MgSO_4$	14
$MnCl_2 \cdot 4H_2O$	4
Ferric ammonium citrate	24
$CuCl_2$	2.4
$ZnSO_4 \cdot H_2O$	5.2
$CoCl_2 \cdot 6H_2O$	0.8

containing 2 qts. of milk. The ampules were opened by filing a small notch in the neck of the ampule with a small 3-cornered file and then breaking the ampule at this notch. Cloth gloves were usually worn over the rubber gloves during manipulations within the unit to prevent the gloves being punctured by slivers of glass and other sharp objects.

The pigs were fed 3 times each day, at approximately 8 a.m., 1 p.m., and 8 p.m. The milk, vitamin, and mineral diet was placed in the feed pans, and the pans were left in the cages until the next feeding. The volume of diet fed each day is shown in Table IV.

TABLE IV. Volume of diet fed in Experiment I

Age of pigs (days)	Volume per pig per day (ml.)
0 - 4	300
5 - 6	360
7 - 17	450



Waste material in the cages was aspirated from the removable trays by means of the 4-oz. rubber bulb and placed in the empty Square-Pak Flasks from which the diet had been fed. This material was then passed outside the isolator as new supplies of milk were passed in.

The temperature in the room in which the pigs were maintained was kept at 90° F. during the course of the experiment.

A total of 7 pigs was obtained in Experiment I. Since only 4 cages were available in the rearing unit, the remaining 3 pigs were removed from the unit at 2 days of age and placed in open metabolism-type cages. These cages were kept in the same room as the rearing unit, and these animals were fed the same diet as the gnotobiotic pigs.

b. Experiment II

The diet used in Experiment II was the same as that used in Experiment I except that the vitamin and mineral supplements were changed. The vitamin supplement used (Table V) was a modification of that described by Reber et al. (1953). As modified, this supplement was calculated to allow for destruction of 90 per cent of thiamine and 50 per cent of the other vitamins as suggested by Wostmann (1959a) and still meet the minimum requirements. The fat-soluble vitamins were dissolved in absolute ethyl alcohol, the remaining vitamins were dissolved in distilled water, and the volume of the final solution was adjusted to contain 20 per cent alcohol. This solution was dispensed in 10-ml. ampules, sealed, sterilized at 250° F. for 15 minutes, and passed into the rearing unit. It was added to the milk at the rate of 5 ml. per quart.

The mineral solution added in this experiment consisted of solution B used in Experiment I with the addition of potassium iodide

TABLE V. Vitamin solution used in Experiments II, III, and IV

Vitamin	Amount per 5 ml.
A[a]	1600 I.U.
D[b]	240 I.U.
E[c]	2.0 mg.
K	0.5 mg.
Thiamine hydrochloride	3.0 mg.
Riboflavin	1.2 mg.
Pyridoxine	4.0 mg.
Calcium pantothenate	4.0 mg.
Inositol	20.0 mg.
p-Aminobenzoic acid	5.2 mg.
Biotin	47.2 mcg.
Nicotinic acid	5.2 mg.
Choline	260.0 mg.
Folic acid	0.1 mcg.
B_{12}	5.2 mcg.

[a] As PGB-250 dry vitamin A (Distillation Products Industries)
[b] As irradiated ergosterol
[c] As alpha tocopherol acetate

in the same amount as it has been in solution A. The resulting modified solution B was added to the milk before feeding at the rate of 15 ml. for each 2 qts. of milk.

The pigs in this experiment were fed 4 times each day for the first 11 days. The feedings were made at approximately 8 a.m.,

1 p.m., 5 p.m., and 10 p.m. During the remaining time the 10 p.m. feeding was eliminated. The volume of diet fed per day is indicated in Table VI.

TABLE VI. Volume of diet fed in Experiment II

Age of pigs (days)	Volume per pig per day (ml.)
0 - 6	480
7 - 17	600

The temperature in the rearing room was held at 90° F. for the first 4 days, after which it was reduced to 85° F. for the remainder of the experiment.

Of the 9 animals in the litter used in this experiment, 3 died while in the surgical isolator. Four of the remaining animals were transferred to the rearing unit, and 2 were transferred to individual isolation units, similar to those described by Young et al. (1955). These units were designed to operate under positive pressure. At the end of 7 days these 2 animals were removed from the individual isolation units and placed in open metabolism-type cages in the same room where the vinyl rearing unit was maintained. These pigs were fed the same diet as the gnotobiotic animals.

c. Experiment III

The diet fed in this experiment was identical to that used in Experiment II. The pigs were fed 4 times a day for the first 6 days and 3 times a day thereafter. The approximate amounts of diet fed are indicated in Table VII.

TABLE VII. Volume of diet fed in Experiment III

Age of pigs (days)	Volume per pig per day (ml.)
0 - 2	300
3 - 7	400
8 - 9	500
10 - 11	600
12 - 16	450
17 - 21	525

For the first 9 days of this experiment the room temperature was maintained at 90° F. After this it was reduced to 85° F. for the remainder of the experiment.

Of the 13 pigs in this litter, only 4 lived. Since the number of animals in this group was so small, it was thought advisable to leave all the pigs in the plastic isolator and not place any in open cages.

d. Experiment IV

The diet fed in Experiment IV was identical to that used in Experiment III. The milk was sterilized in 1 1/2 liter amounts in the 2-liter Square-Pak Flasks to reduce the amount of boiling over during the sterilization process. After the flasks were transferred into the rearing unit, they were brought to the usual 2-qt. volume before addition of vitamins and minerals. The pigs were fed 6 times a day, with the feedings being made at 8 a.m., 11 a.m., 2 p.m., 5 p.m., 8 p.m., and 11 p.m. The amounts of diet fed are indicated in Table VIII.

TABLE VIII. Volume of diet fed in Experiment IV

Age of pigs (days)	Volume per pig per day (ml.)
0 - 6	360
7 - 9	450
10 - 12	540
13 - 16	630
17 - 21	720

Twelve pigs were obtained in this litter. Six of these were placed in the rearing unit, with 2 pigs being placed in each of 2 cages. Five of the remaining animals were allowed to nurse a sow which had farrowed the day these pigs were delivered. These pigs were given 2 ml. of Armidexan (Armour and Company, Kankakee, Illinois) at 2 to 3 days of age.

At the termination of these 4 experiments, the animals were removed from the rearing units, weighed, bled, and killed. Various organs were weighed and blocks of tissue were saved for use in Part Two. Experiments I and II were each terminated at the end of 17 days, and Experiments III and IV were terminated at the end of 21 days.

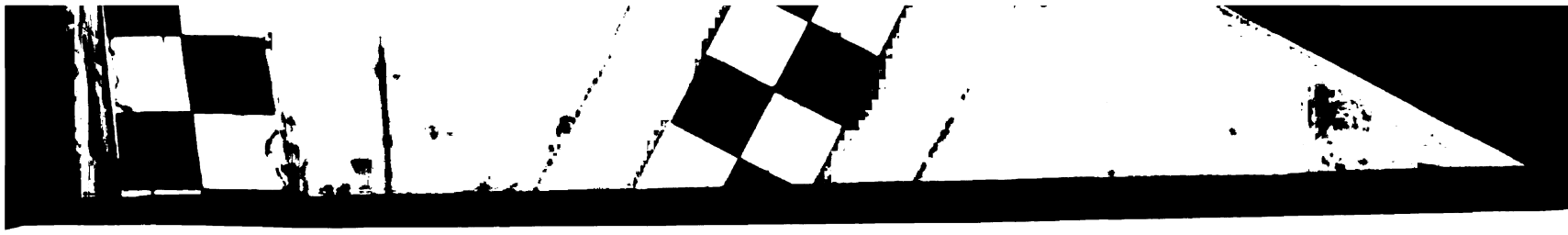
5. Bacteriological Procedures

Bacteriological determinations on the pigs in the rearing unit were made twice during each experiment, once at about 10 days of age, and again at the termination of the experiment. Samples for these determinations were obtained from 2 sources. Sterile cotton swabs in screw-capped test tubes, which had been placed in the rearing

unit at the time of initial sterilization, were used in collecting rectal swabs from the pigs. The second source of material for culture consisted of a mixture of urine, feces, and milk dipped from the bottom of the tray in each cage. This was placed in a sterile, screw-capped test tube within the isolator. These samples, one rectal swab from each pig and one liquid sample from each cage, were then passed out through the sterile lock.

In Experiment I wet mounts of the samples were made and examined, along with smears which were stained with Gram's stain. Eugonagar (Baltimore Biological Laboratories, Baltimore, Maryland) (with 20 per cent bovine blood) and thioglycollate broth were also inoculated.

In Experiments II, III, and IV, a method which was essentially that of Larson and Hill (1955) was used to determine the flora of the animals within the isolator. Wet mounts and Gram's stains were made as in Experiment I, and Eugonagar (with 20 per cent bovine blood) was inoculated and incubated both aerobically and anaerobically at 37° C. The liquid samples from the cages were used in inoculating the remaining media. Violet red bile agar (Difco Laboratories, Detroit, Michigan) was used for coliform determinations. "SF" medium (Difco) was inoculated for determination of fecal streptococci. Lactobacillus Selection Medium (B.B.L.) was used to detect the presence of lactobacilli. Clostridia were determined by the use of a modified Wilson-Blair medium, and potato dextrose agar (Difco) was utilized in checking for yeasts.



When growth was obtained on any of the media used, attempts were made to identify the bacteria involved, using standard bacteriological procedures.

A quantitative determination of the bacteria in the feces was made, utilizing a composite weighed sample of feces from the 2 animals raised in open cages in Experiment II and using the techniques of Larson and Hill (1955).

B. Results

1. Experiment I

Some difficulty was encountered with the surgical procedure in this experiment. An attempt was made to support the gravid uterus manually while it was being excised and removed. This resulted in a tear in the uterine wall, and one pig dropped from the uterus before it could be placed in the germicidal lock.

No problems were met in getting the young pigs to drink the liquid diet from a pan, and they soon were consuming the entire amount shortly after it was offered to them. Although the milk was slightly changed in color, becoming a very faint tan, and had a slight "cooked" odor, this did not appear to affect its palatability for the young pigs. The odor from the air-outlet trap at the beginning of the experiment was that of the autoclaved milk. Later, however, the exhaust air took on a rather "sour" odor. At no time did the residual milk in the feed pans of the gnotobiotic pigs appear coagulated.

Although it was not possible to obtain birth weights on these animals, they did not appear to grow very rapidly. The hair coat was not as smooth as is normally seen in 3-week-old pigs. It

was also noted that the animals did not appear to possess as much subcutaneous fat as do animals nursing the sow. The feces of the pigs in the isolator were normal for the first few days, except for the fact that they were dark in color. By the tenth day the fecal material had become rather soft and semi-fluid in consistency. This continued until the termination of the experiment, with the feces becoming slightly more fluid as time went by. This dark, semi-fluid fecal material covered the rear quarters of the animals in the isolator.

Bacteriological cultures revealed the presence of Staphylococcus aureus in the feces and the material from the cages. An occasional culture showed the presence of a mold, suggesting the possibility of a mold contamination.

The littermate pigs maintained in open cages also grew quite slowly, although they were somewhat smaller at the beginning of the trial than those left in the isolator. The fecal material was more nearly normal in consistency for 2 of the 3 animals (pigs B-1, B-2, Table IX. The third animal (B-3, Table IX) had diarrhea during almost all of the experiment.

The body weights of the pigs in this experiment are shown in Table IX. The gnotobiotic animals are represented by group A, their littermates raised in open cages by group B. Table X summarizes the sources of the various groups of pigs in these experiments.

2. Experiment II

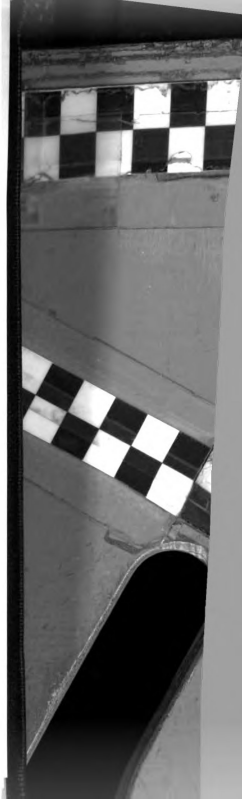
The use of the Mylar-covered incline leading to the germicidal trap provided more support for the uterus as it was excised and

TABLE IX. Total body weights (lbs.)

	Experiment I				Experiment II				Experiment III		Experiment IV			
	Group A		Group B		Group C		Group D		Group E		Group F		Group G	
Pig No.	Sex	Wt.	Sex	Wt.	Sex	Wt.	Sex	Wt.	Sex	Wt.	Sex	Wt.	Sex	Wt.
1	F	3.7	M	2.8	F	5.2	M	5.0	M	4.4	F	6.0	F	11.6
2	M	4.5	M	4.2	M	4.6	F	5.0	M	4.1	M	6.9	M	12.6
3	F	3.4	M	3.5	M	4.9					M	5.6	F	13.1
4	M	3.8									F	6.5	F	14.1
5													F	10.5
6													F	12.8
7													F	12.2
8													M	12.0
9													M	12.2
10													M	8.4
11													M	7.0
Average		4.08		3.50		4.90		5.00		4.25		6.25		11.50

TABLE X. Source of groups of pigs

Group	Source
A	Maintained in the presence of Staphylococcus aureus.
B	Littermates of group A, maintained in open cages.
C	Maintained in the presence of Bacillus sp.
D	Littermates of group C, maintained in open cages.
E	Maintained in the absence of any demonstrable microorganisms.
F	Maintained in the presence of Achromobacteriaceae and unidentified organism.
G	Maintained on the sow.



placed in the tank, and no problem was encountered with tearing of the uterus as in Experiment I.

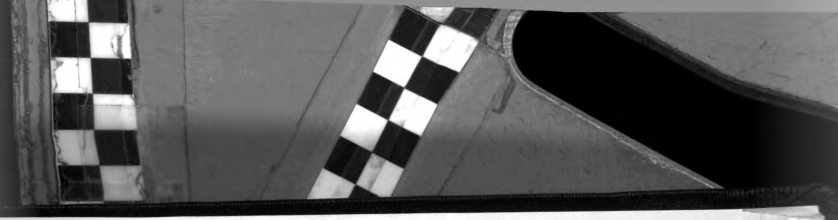
The air leaving the isolator developed a "sour" odor when the pigs were about 12 days of age, although there was no coagulation of the residual milk in the feeding pans.

One pig within the vinyl rearing unit died a few hours after delivery. Death was due to hemorrhage from the umbilical cord. It appeared that the stump of the cord had been torn in some way above the ligature. The pigs did not differ markedly in appearance from those in Experiment I. The consistency of the fecal matter was again semi-fluid, and it was of a dark color.

Bacteriological cultures revealed the presence of Bacillus sp. The organism was not further identified.

The littermate pigs in open cages grew at about the same rate as did the gnotobiotic animals. Pig D-1 developed some diarrhea toward the end of the experiment, but pig D-2 had well-formed feces. The quantitative determinations of the intestinal flora of this group of animals, using a composite sample from the 2 animals, revealed that the bacterial count was essentially that of animals raised on the sow. The total numbers of aerobic and anaerobic organisms, along with the numbers of coliforms, streptococci, and clostridia, were nearly identical to that of a series of pigs the same age nursing the sow. The lactobacilli were reduced by a factor of about 10,000 in the pigs raised in open cages on the sterilized diet.

The body weights of the animals in this experiment are shown in Table IX. The gnotobiotic animals are represented by group C, while group D represents the 2 littermate animals raised in open cages.



3. Experiment III

The viability of the litter used in this experiment was quite low, and only 4 of 13 pigs lived. Three of the remaining 9 were mummified, and attempts to initiate respiration in the remaining 6 animals were unsuccessful. The animals started eating quite slowly, and they were much weaker than the animals in previous experiments.

One of the 4 pigs placed in the rearing unit died on the third day and another on the fifth day after delivery. Both these animals were left in the rearing unit at 90° F. until the eighth day of the experiment. There was no evidence of bacterial decomposition as evidenced by bloating, discoloration of the skin in the abdominal region, or odor. The carcasses did become dehydrated, and the eyes were quite sunken at the time of removal from the rearing unit.

The odor from the air-outlet trap was that of the sterilized milk throughout the experiment, and the pigs themselves had no disagreeable odor at the time they were removed from the rearing unit.

The feces of the 2 remaining pigs were well-formed at the start of the experiment but began to be rather soft after about one week. At the end of the 21-day experiment the fecal material was dark in color and semi-fluid to pasty in consistency.

Bacteriological examination failed to reveal the presence of any demonstrable bacteria in rectal swabs or waste material from the cages. A Gram-stained smear of fecal material from one pig in this group is shown in Figure 17. For comparison, a smear of fecal material from a 3-week-old pig raised on a sow is shown in Figure 18.

Since facilities for weighing the pigs had been installed in the rearing unit before the start of this experiment, it was possible

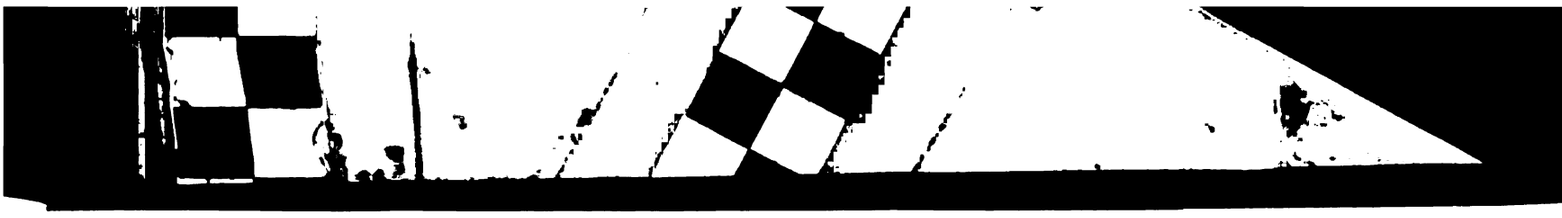


Figure 17. Gram-stained fecal smear from germfree pig

Note absence of bacteria. x 1600

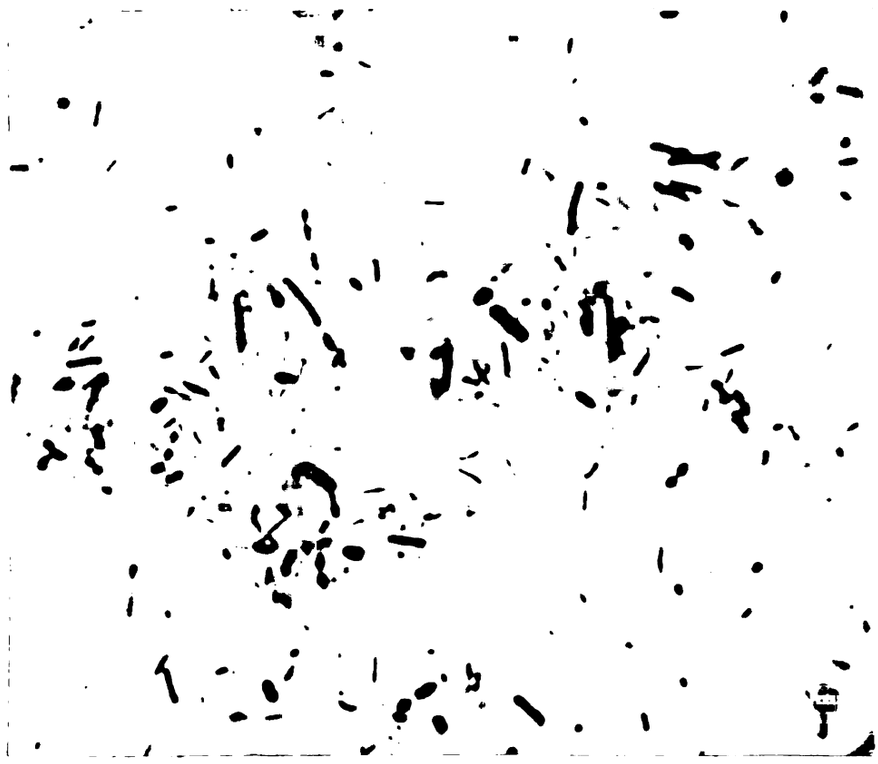
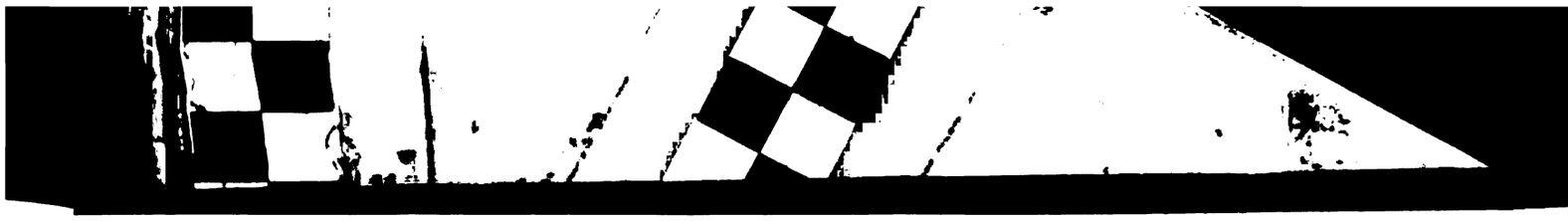


Figure 18. Gram-stained fecal smear from farm-raised pig

Note numerous bacteria of various shapes and sizes.

x 1600



to follow the growth rate of these pigs. The body weights at different ages are shown in Table XI, and the final body weights are shown as group E in Table IX.

TABLE XI. Body weight of pigs in Experiment III (lbs.)

Pig No.	Age (days) 0	7	14	21
E-1	1.4	2.0	3.0	4.4
E-2	1.7	1.8	2.7	4.1

4. Experiment IV

Shortly after the pigs were transferred to the rearing unit, the umbilical ligature on pig F-1 loosened, and the animal lost enough blood to become rather weak. It soon regained its strength, however. On the second day of the experiment, one of the pigs was lost because of hemorrhage from the umbilical cord. It had been placed in a cage with another pig, and apparently the ligature had been sucked or bitten from the umbilical cord. The remaining pigs did quite well in comparison to earlier groups. Two of the pigs (Figure 19) from this group are shown after their removal from the rearing unit.

Some difficulty was encountered in the one remaining cage containing 2 pigs. One of these was somewhat larger than the other, and it became necessary to hold each animal while the other was being fed to prevent the larger animal from getting more than its share of the milk.

The outlet air from the rearing unit did not develop a significant odor, although it was thought possible at times to detect

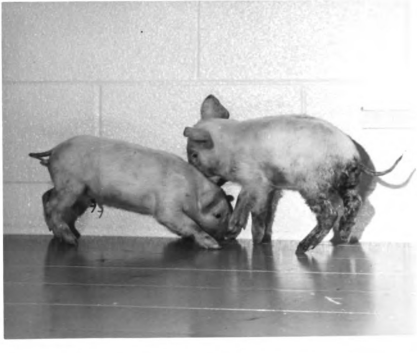


Figure 19. Gnotobiotic pigs from Experiment IV

somewhat the same "sour" odor that had been encountered in previous groups contaminated with bacteria.

The fecal matter was noted to be somewhat soft on day 9 of the experiment, and it became somewhat more liquid as time progressed. The 2 pigs in the same cage became covered with this dark, semi-fluid fecal material.

Bacteriological determinations revealed the presence of a bacterial strain belonging to the Achromobacteriaceae and a small Gram-negative, anaerobic rod which was lost before it could be further identified. A Gram-stained smear of fecal material from one of the pigs in this litter is shown in Figure 20. Examination of the isolator at the termination of the experiment revealed a small hole in the vinyl film which may have accounted for the contamination.

The body weights of the 4 gnotobiotic animals killed at the termination of this experiment and 3 of their littermates raised on a sow are shown in Table XII. The remaining animals of this litter were

TABLE XII. Body weights of pigs in Experiment IV (lbs.)

Pig No.	Source	Age (days) 0	7	14	21
F-1	Gnotobiotic	2.4	3.3	4.7	6.0
F-2	"	2.7	3.7	5.3	6.9
F-3	"	2.8	3.2	4.5	5.6
F-4	"	2.6	3.5	4.7	6.5
G-10	On sow	2.4	4.3	6.0	8.4
G-11	"	2.4	3.5	5.7	7.0
G-12	"	2.8	4.8	7.5	9.5

Figure 20. Gram-stained fecal smear from gnotobiotic pig in Experiment IV

Note several rod-shaped bacteria in center of field. x 1600.

lost. The final body weights of the gnotobiotic pigs are shown as group F in Table IX, and 2 of the sow-raised littermates are shown as pigs G-10 and G-11.

The remaining animal raised in the vinyl isolator was transferred to a sterile rearing unit when the other animals were killed. It was kept in this unit for an additional 2 weeks, and it was then removed from the isolator and placed in a room where pigs had been previously kept. It was gradually changed to a conventional pelleted diet and pasteurized milk. While the animal was in the isolator, it was noted that the tongue protruded from the mouth and could not be completely retracted. This condition gradually improved after changing to the conventional diet, and the feces also soon became normal in consistency.

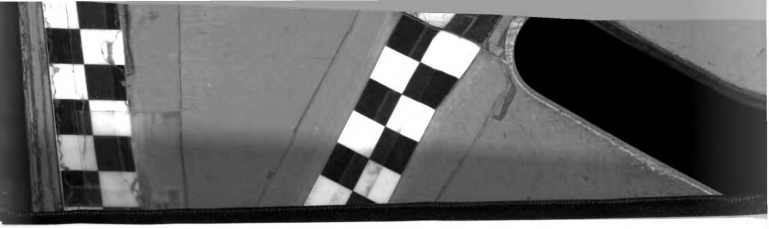
The final body weights of all pigs in these 4 experiments are shown in Table IX. Included also, as group G, are 11 pigs raised on sows to the age of 21 days. These data, along with various organ weights, will be compared in Part Two.

IV. DISCUSSION

The fact that young pigs were raised to the age of approximately 3 weeks in the presence of not more than 2 species of bacteria indicates that this technique and equipment can be successfully used in producing gnotobiotic pigs for use in research. It is thought that the bacteria encountered in Experiment I probably entered as a result of the tear in the uterine wall during the hysterectomy. The exact reason for the contamination in Experiment II is not known, but it is assumed that the most likely cause was improper sterilization of the diet since the contaminant was a spore former. The bacteria encountered in Experiment IV probably entered through the tear in the vinyl film of the isolator.

The percentage of live pigs obtained in Experiments II and III was rather disappointing. The small size of the animals obtained in the latter case suggests that the breeding date of the sow used may have been in error. Postponement of the hysterectomy for another 2 or 3 days or perhaps even longer might have resulted in heavier and stronger animals.

It is extremely important to be able to predict the expected farrowing date of the sow or gilt to be used in this procedure. An error of a few days in either direction can result in the failure of an attempt to obtain viable pigs by hysterectomy. Glimstedt (1936) used radiographs of the pelvis in determining the end of the gestation period in guinea pigs, depending on the amount of separation of the symphysis pubis. Phillips et al. (1959) also relied on the diastasis of the pubic



bones of the pregnant guinea pig in determining the proper time to perform surgery. They found a gradual increase in the separation of the pubic bones until the distance between the bones reached 17 to 18 mm. Following this they found a rapid increase during the next 24 to 36 hours, at which time the separation was 21 or 22 mm. This latter period of rapid diastasis appeared to be the most favorable time for Caesarean section, but germfree guinea pigs derived from dams with a pubic spread of 16 mm. or more could usually be reared successfully. These workers determined the amount of diastasis either radiologically or manually by palpation. Gustafsson (1946-1947) utilized 2 female rats bred on the same day to the same male. One female was allowed to give birth spontaneously. The other animal was then checked every half hour and surgery was started as soon as labor began. The Lobund group (Pleasants, 1959) has reported delaying surgery in rats until one of the young was delivered spontaneously. Since none of these procedures seem practical in the sow, emphasis should be placed on accurate breeding dates. If these are available, the best plan is to perform surgery on the 112th day of gestation. If no accurate breeding date is available, one must rely on the appearance of milk in the nipples as a sign of approaching parturition, but this is not highly accurate, as milk may be present for only a short time or for several days prior to farrowing.

Another factor that no doubt affected the viability of the young pigs was the fact that the time elapsing between the attainment of surgical anesthesia in the sow and the initiation of respiration in the pigs was longer than ordinarily encountered in the procurement of specific-pathogen-free pigs. This was due to the need for completely

aseptic procedures in the current work. It is felt that the development of equipment and techniques for the utilization of a Caesarean section in obtaining gnotobiotic pigs would result in a higher degree of viability. The placental attachment of each animal could be interrupted on an individual basis, each animal could be handled individually, and more time could be devoted to the initiation of respiration in each pig. It might also be possible to salvage the sow for market or perhaps for raising subsequent litters.

A need for larger cages was evidenced in these experiments. The present cages appeared adequate for the length of time the animals were kept in them, but it is possible that the animals might consume more food and grow more rapidly if more space for exercise were provided. If attempts to rear gnotobiotic pigs to an older age were to be attempted, larger cages would no doubt be required.

Another modification which would be of value would be the provision of larger fiber-glass rings for the introduction of material into the rearing unit. This would allow the cages to be placed within the isolator without cutting and resealing the vinyl film itself. It would also allow the construction of a larger sterile lock and permit the introduction of more diet at a given time.

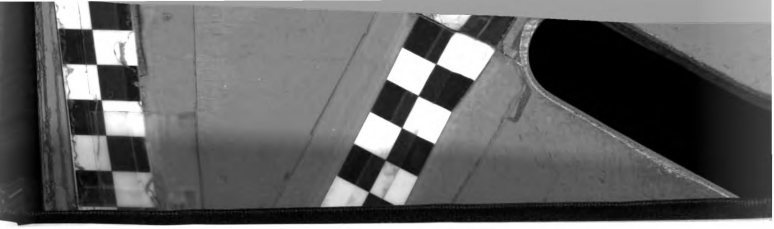
The diet itself seemed adequate to support life in the young pig, but it would be desirable to have a diet which would, if possible, produce growth rates and weight gains more nearly comparable to those seen in pigs raised on the sow. This suggests that much more work needs to be done on formulating such a diet. In order to study this problem thoroughly, the sterilized diet should be fed to both gnotobiotic animals and those possessing a normal flora. Additional information as to

the adequacy of the diet could also be gained by feeding the sterilized diet to one group of animals and the same diet before sterilization to a comparable group.

In formulating a ration more suitable for rearing young pigs, it may be necessary to resort to something other than cows' milk as a base. It is known (Bellis, 1957) that sows' milk contains more total solids, fat, protein, and ash than does cows' milk. It is also quite high in energy. Sows' colostrum is even higher in total solids and protein than is sows' milk. It may be necessary to formulate a new synthetic-type diet to provide optimum nutritional conditions for the young pigs.

The appearance of semi-fluid feces in the animals raised in the isolator suggests that a deficiency of some nutritional factor may have existed. Whitehair (1958) has listed diarrhea as a symptom seen in some of the B-vitamin deficiencies. The fact that some of the animals raised in open cages on the same diet had feces which were rather well-formed points to a possible influence of the bacterial flora on the nutritional adequacy of the diet. It is conceivable that one or more of the vitamins were destroyed during the sterilization process to such an extent that there was not sufficient quantity left in the diet to meet the minimum need. It is likewise conceivable that certain bacteria in the intestinal flora of the pigs raised in open cages were able to synthesize the vitamin or vitamins in question and thus counteract this deficiency and prevent the formation of semi-fluid feces.

It is possible that something in the diet, either as an ingredient in the original diet or as a result of the sterilization process, may have produced the fluid feces by interfering with absorption



from the intestinal tract or by increasing peristalsis. This seems unlikely, however, since the same thing was not seen in the pigs in open cages.

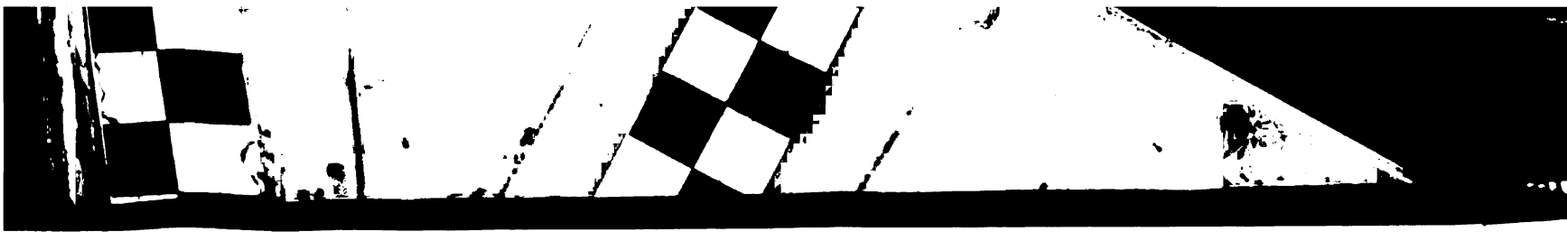
The possibility exists that the bacteria, by their bulk, influence the physical characteristics of the fecal material. It is hoped that further work will result in the clarification of this problem.

Glimstedt (1936) reported that the stools of his germfree guinea pigs were not formed but were rather viscous. He did not consider this as being truly diarrheal in nature.

The improved growth rate seen in the pigs in Experiment IV may be attributed to the greater amount of diet fed per day, the increased number of feedings per day, or a combination of both.

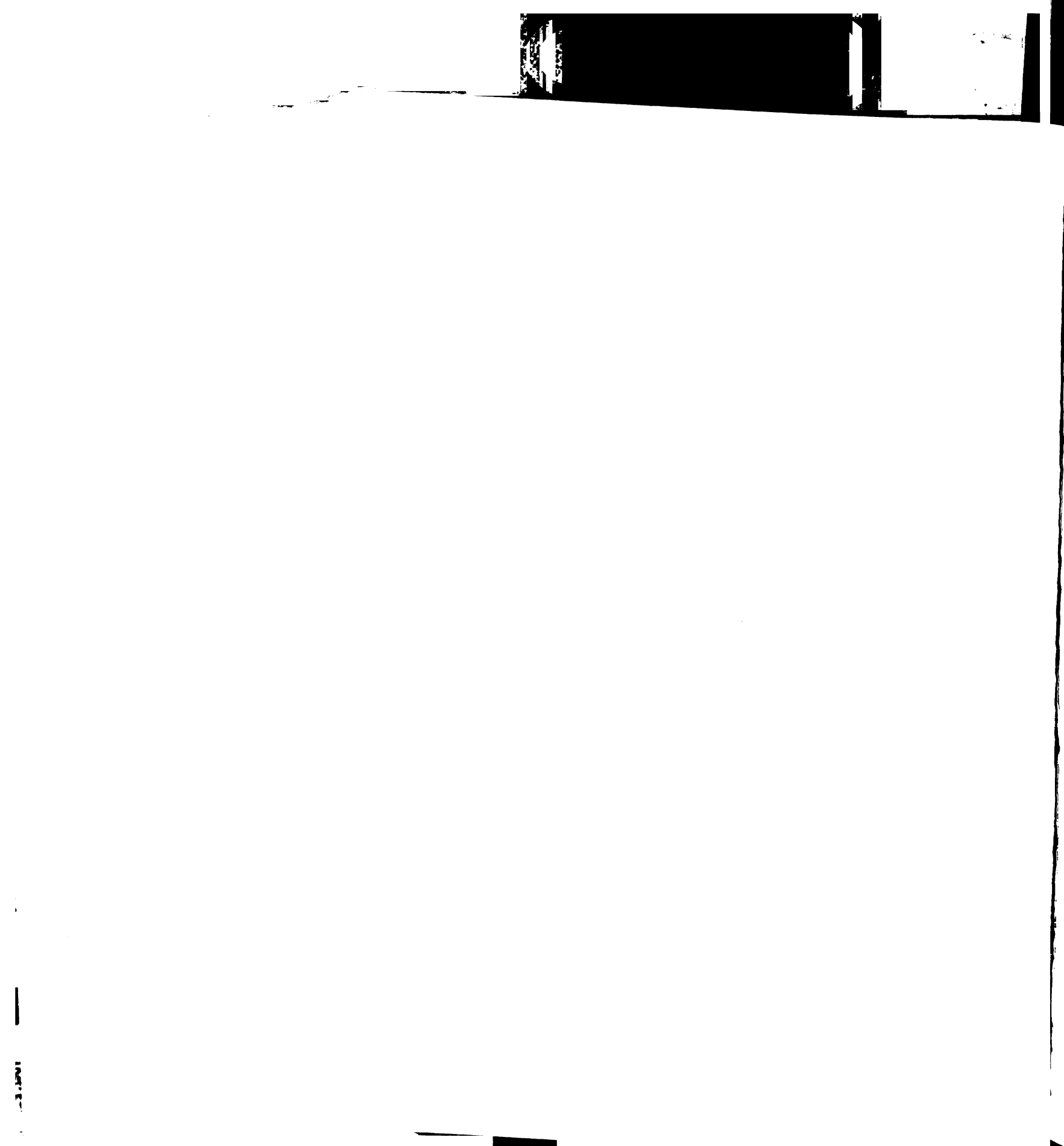
The bacteriological procedures were apparently adequate for picking up bacterial contamination, but they were quite time-consuming and could, no doubt, be simplified. For detecting bacteria during the course of the experiment, the use of wet mounts and Gram's stains of fecal smears along with the inoculation of thioglycollate broth would probably be adequate. It would be necessary, of course, to incubate this medium at different temperatures, for example, 25, 37, and 55° C. More elaborate cultural procedures could then be used, if desired, at the end of the experiment, or if examination of Gram-stained fecal smears indicated the presence of organisms in sufficient quantities to suggest that the thioglycollate broth was not supporting growth.

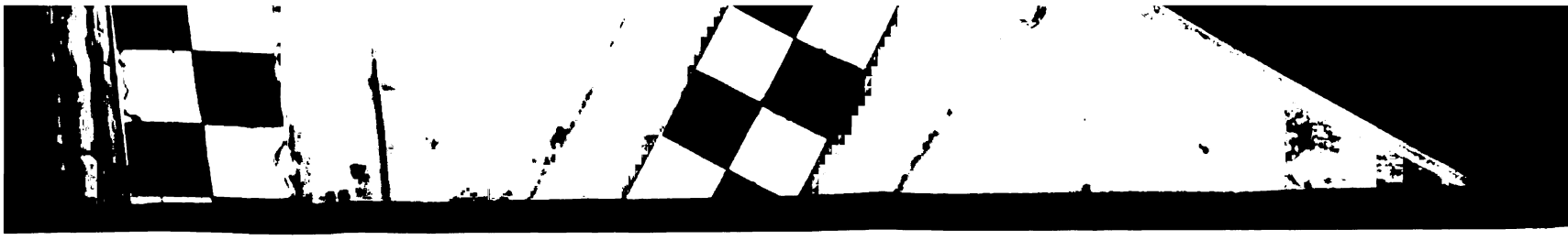
The lack of the usual post-mortem decomposition in the 2 pigs in Experiment III left in the isolator for 3 and 5 days, respectively, after death must be attributed to the absence of bacteria. This



suggests that autolytic changes are not, in themselves, responsible for a marked change in the external appearance of animals after death.

Very careful planning is required to collect young pigs by this technique, and the success of the procedure depends upon everything being in order and ready for use at the time the sow approaches the end of the gestation period. The synchronization of all the different factors involved in a procedure of this type, including the estimation of the farrowing date of the sow, the preparation and sterilization of equipment, the preparation and sterilization of the diet, and the provision of necessary media and equipment for bacteriological procedures, poses a very real problem. Each of these factors needs to be the subject of careful study, and much time and effort could be devoted to the improvement of each.





V. SUMMARY

A series of 4 experiments was conducted in an attempt to obtain and rear young pigs to the age of approximately 3 weeks in the absence of bacteria or in the presence of known species of bacteria. The pregnant sow was anesthetized with carbon dioxide, and the uterus was excised and passed into a sterile vinyl-film isolator by means of a germicidal trap. The pigs were removed from the uterus and passed into another vinyl-film isolator where they were kept for the remainder of the experiment. The isolators were sterilized with peracetic acid, and air entering the units was sterilized by passing it through 4 layers of glass-wool mat.

The diet used consisted of pasteurized, homogenized milk with mineral and vitamin supplements. This diet was sterilized with steam under pressure.

Two pigs were raised in the absence of any demonstrable bacteria, 4 were raised in the presence of Staphylococcus aureus, 3 in the presence of Bacillus sp., and 4 in the presence of Achromobacteriaceae and an unidentified organism. In addition, 5 animals were raised in open cages on the same diet, and 2 were raised on a sow.

The pigs raised on the experimental diet grew rather slowly, and those raised in the isolators developed semi-fluid feces during the latter part of the growing period. Pigs kept in open cages but maintained on the same diet generally had feces which more nearly approached normal.

Two pigs left in the sterile isolator for 3 and 5 days, respectively, after death showed no external post-mortem changes. They did, however, show evidence of dehydration.

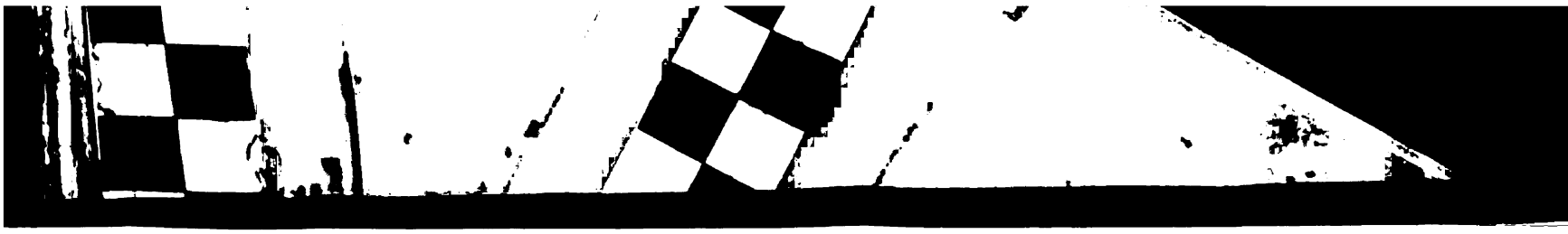
PART TWO

A COMPARISON OF BODY WEIGHTS, ORGAN WEIGHTS, AND SOME HISTOLOGICAL FEATURES OF GNOTOBIOTIC AND FARM-RAISED PIGS

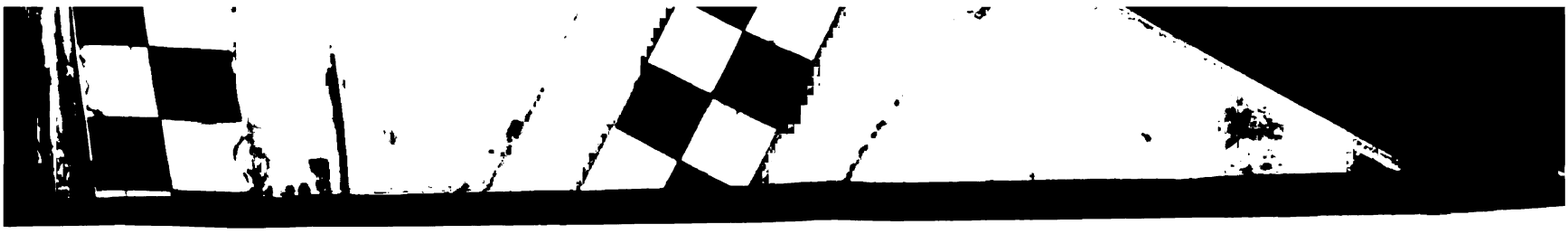
I. INTRODUCTION

The morphological and physiological characteristics possessed by gnotobiotic pigs must be studied sufficiently to determine whether or not they deviate significantly from those seen in animals raised on the sow. The possible influence of these characteristics on the outcome of investigations utilizing the gnotobiotic pig as an experimental animal should also be considered. Only after the results of these studies become known, can the gnotobiotic pig assume its greatest value in the field of research.

It is beyond the scope of the present investigation to attempt to explore all the possible areas in which gnotobiotic pigs differ from conventionally-raised pigs. It seemed important, however, to explore some of the areas most likely to show differences between the 2 groups of animals. In view of results reported in the literature on studies in germfree animals of other species, it seemed advisable to study total body weights and the weights (both absolute and relative) of certain body organs. The importance of the lymphatic system in the body's defensive mechanism suggested that this system should be a part of such a study. Interest has been expressed in the significance of the comparatively large amount of connective tissue seen in the pig's liver. Therefore, this organ seemed to merit investigation. The intestinal



tract in germfree animals of other species has been shown to differ from the conventional. The findings relative to the intestinal tract of the gnotobiotic pig are the subject of a separate report.



II. REVIEW OF LITERATURE

A. Growth Rates of Germfree Animals

Gustafsson (1946-1947), in discussing the comparison of germfree animals with other animals, stated that little value can be attached to investigations where conclusions have been drawn from comparisons between germfree animals and control animals reared by the mother. He suggested the use of 4 groups of Caesarean-derived animals for adequate comparisons. One group included the animals raised in a sterile environment on a sterilized diet. The second group consisted of animals raised in a non-sterile environment on a sterilized diet. The third group included those raised in a non-sterile environment on a non-sterilized diet. The fourth group consisted of animals taken by Caesarean section and raised by another female. In Gustafsson's work the 3 groups of rats fed the artificial diets were all about the same weight at the end of 28 days. The littermates raised by another female rat were somewhat heavier. Reyniers et al. (1946) found that their germfree rats and the animals maintained in a non-sterile environment but fed a sterilized diet grew more slowly than the normally suckled rats. Pleasants (1959) reported the successful weaning of hand-reared rats, mice, and rabbits. He found that, although growth and development were subnormal during the suckling period, few cases of permanent handicap resulted. Gordon (1959) noted that third- to eighth-generation germfree rats and mice showed a retardation of growth in comparison to normal animals also fed a sterilized diet.

Glimstedt (1936) found that his germfree guinea pigs grew more slowly than did the conventional animals. Later, however, the growth

rate became balanced with the conventional animals. Miyakawa (1959b) reported that for the first 20 days the weight gains of his germfree guinea pigs were below those of conventional animals. After 30 days the weight gains became parallel to those of conventional animals on the same diet. The body weight of Miyakawa's germfree guinea pigs was always less than that of conventional animals of the same age.

The growth of germfree chickens (Gordon, 1959) was found to compare favorably with the normal-stock controls fed a sterilized diet. Reyniers et al. (1960) found that the body weight of comparable germfree and conventional chickens generally showed no essential difference up to the age of one year.

B. Organ Weights of Germfree Animals

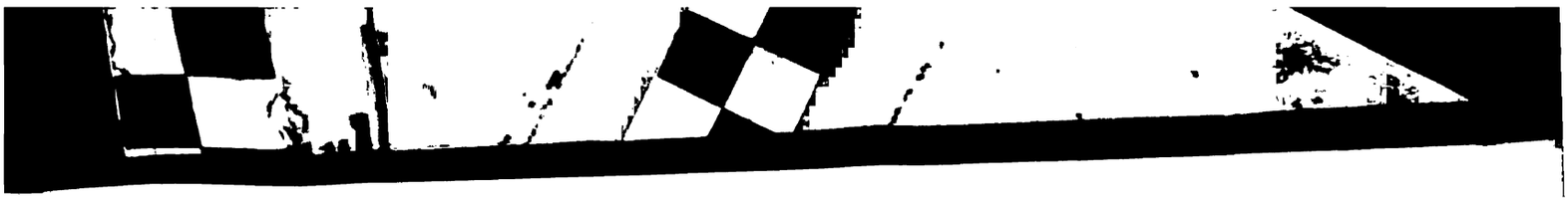
Gordon (1959) stated that, in terms of weight, the organs of his germfree animals fell into 2 distinct groups: one harboring, or in close association with, an abundant flora in normal life showed reduced weight in the germfree animal, and the other group of organs that is normally remote from bacteria showed practically no difference between the germfree and normal stock. As examples of the former group, Gordon listed various parts of the digestive tract and its associated lymph nodes and, in some instances, the liver. As examples of the latter group, Gordon listed the heart and brain. He also found that the adrenal glands of germfree rats were heavier than those of the normal-stock controls fed the sterilized diet. These conclusions were drawn from work with chickens, rats, and mice. Reyniers et al. (1960) found a lower weight in the ileo-cecal-colic junction (harboring the "cecal tonsils") in germfree chickens than in conventional birds. The heart,

brain, thymus, spleen, and endocrine glands displayed similar weights in the 2 groups.

In rearing germfree guinea pigs, Glimstedt (1936) found that the liver, kidney, adrenal, heart, and lungs showed a moderate inhibition of development. The development of the intestinal canal was inhibited to a greater degree in the germfree animal. The thymus in the 30-day-old germfree animals weighed 49 to 56 per cent of that found in the conventional animals. The corresponding figure for the 60-day-old animals was 39 to 46 per cent. The total lymphatic tissue amounted to 22 to 26 per cent in the 30-day-old animals and 25 to 34 per cent in the 60-day-old animals of that seen in the control guinea pigs. Corresponding values for the spleen were 49 to 65 per cent and 57 to 66 per cent, respectively. In 2 germfree animals, Glimstedt found that the amount of splenic white pulp was increased, while in the remaining animals it was reduced to 67 to 72 per cent of the mean value of the control animals.

From the above results Glimstedt (1936) concluded that the normal, nonpathogenic bacteria occurring in the guinea pig appeared to influence favorably the development of the animals. His investigation did not clarify the mechanism of this influence.

Thorbecke and Benacerraff (1959) found that the combined relative weight of liver and spleen was significantly lower in germfree than in control mice, chiefly because of a difference in relative liver weight. The liver weights per 20 gm. body weight in 9 control mice were 1180 $\pm$ 80 mg., and in 15 germfree mice they were 900 $\pm$ 130 mg.



C. Histology of the Lymphatic System

Since a study of the lymphatic system forms a part of the present investigation, and since this system has been the subject of many scientific papers in the past, it seemed appropriate to review the highlights of the histology of the lymphatic system. Flemming (1885) studied the microscopic structure of lymph nodes and described light secondary nodules or germinal centers which serve as the principal foci of proliferation of lymphocytes. He emphasized that these are not constant parts of the lymphatic nodules but show fluctuations. Flemming also described large, peculiarly-staining granular formations in cells of the germinal centers. These he called "tingible Körper". Maximow (1932) stated that these are phagocytosed inclusions in large macrophages and that they are mostly darkly-staining particles of chromatin originating from degenerated lymphocytes.

Hellman (1921, 1930) attached a different significance to the light centers found in lymphatic nodules. According to his theory, these centers are of no importance for the regeneration of lymphocytes, which occurs everywhere in the diffuse lymphoid tissue. They are, on the other hand, centers of reaction to bacterial and toxic stimuli acting upon the lymphoid tissue. If the stimulus is light, productive changes occur; if it is intense, the cells of the center display degenerative phenomena.

Maximow (1927, 1932) has described in detail the structure of lymph nodes and stated that the stroma of lymphoid tissue contains 2 types of cells. One of these is the reticular cell which is large and stellate or spindle shaped and has the ability to store vital dyes. The other is the cell of the reticular syncytium which has not undergone

differentiation and has no ability to store vital dyes. Maximow described the primary nodule or follicle of lymphocytes with its central area or secondary nodule which stains lighter than the periphery. This secondary nodule contains large pale nuclei with numerous mitoses. Maximow agreed with Flemming that these centers are the chief place of lymphocyte proliferation. He further stated that such centers are not found in embryos or newborn mammals and that they reach their height of development in the young animal.

According to Maximow (1932) the lymphoid tissue in the germinal centers under goes cyclic transformations and all the germinal centers of a lymph node, perhaps of the whole body, show similar conditions at a given time. He described the "active phase" of this cycle in which numerous mitotic divisions, mostly in medium-sized lymphocytes, occurs. The reticular syncytium may also show mitosis. During the "resting phase" no mitoses are present, and the arteriole in the center of the nodule is surrounded by a small pale area containing nuclei of the reticular syncytium and a few degenerated small lymphocytes. The rest of the nodule is made up of small lymphocytes and reticular nuclei. Maximow also recognized the "beginning of a new active phase" and the "transition from active into resting conditions". In the latter phase the medium-sized lymphocytes are transformed into small lymphocytes either by mitosis or without mitosis.

Maximow (1927) also described the transformation of the undifferentiated reticular syncytium of the lymphoid tissue into lymphocytes, myeloid cells, macrophages, and monocytes.

Conway (1937) described the voluminous literature on lymphatic tissue as ".....a maze of contradictory experimental and descriptive

data, opinions, and theories". She described a "bare" germinal center as one without a surrounding area of densely-packed, small lymphocytes. Conway demonstrated that, in an induced bacterial infection in rabbits, the lymphatic nodule is primarily a focus of rapidly-proliferating lymphocytes and only secondarily does it become a reaction center. She thus attempted to reconcile the 2 opposing views of Flemming and Hellman. She also stated that the reticular fibers of the lymphatic nodules are not arranged in a permanent pattern but are mechanically pushed apart by the growth pressure of the lymphocytes.

Bloom (1938) reviewed the literature on lymphatic tissue and attempted to clarify the confusion of terminology appearing in the literature.

Osogoe et al. (1960) stated that secondary nodules are formed as a reaction to bacterial infections and that the term "reaction center" is preferred to "germinal center". They counted the mitotic figures in sections of rat mesenteric lymph nodes and found more mitoses in the pale-staining centers than in the remainder of the lymphatic tissue.

Gyllensten (1950) found that the lymphatic system of newborn and young guinea pigs exhibits regional differences in regard to the degree of maturity. The maturity decreases in the following order: Peyer's patches and cervical lymph nodes, lymph nodes of the extremities, tracheal and mesenteric lymph nodes, and white splenic pulp. He stated that secondary nodules occur between the first and 23d day after birth in guinea pigs.

One of the aspects of lymphoid tissue which has received considerable attention has been the function of this tissue. Maximow and Bloom (1957) listed one of the functions of lymph nodes as the production

of lymphocytes. In some conditions, the lymph nodes also become the site of the formation of granular leukocytes. The lymph nodes also serve as filters in which various particles, arising locally or brought with the lymph from other parts of the body, are taken up and often destroyed. Maximow and Bloom (1957) stated that, just like all the other organs containing many macrophages, the lymph nodes probably elaborate antibodies. They further suggested that lymphocytes possibly contain antibodies. Ham (1957) described the chief function of lymphatic tissue as the filtering of lymph before it is returned to the blood. The lymph nodes also add lymphocytes to the lymph that flows through them.

Miller and Bale (1954) studied the synthesis of the plasma proteins and found that all fractions are synthesized by the liver with the exception of gamma globulin. They concluded that if antibody proteins are regarded as gamma globulins, antibodies are formed by extrahepatic tissue, most likely by radiosensitive cells of the hematopoietic system. McMaster and Hudack (1935) demonstrated the formation of agglutinins within the draining lymph nodes of mice following intradermal injections of killed cultures of microorganisms.

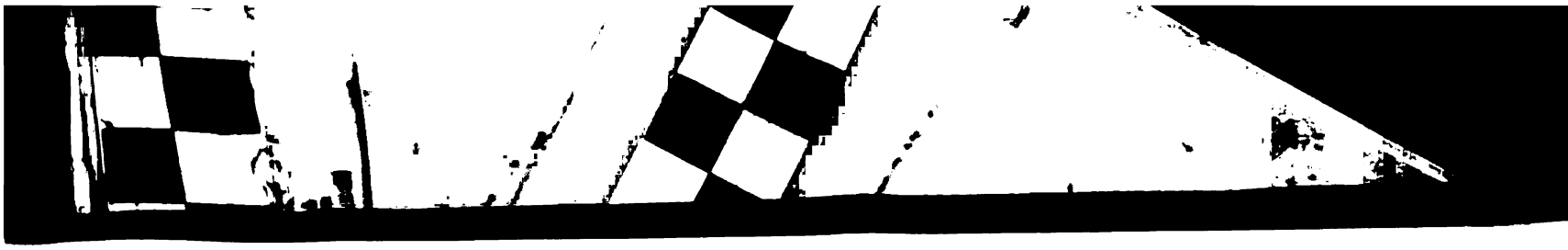
Numerous attempts have been made to determine which specific cells within the lymph nodes are responsible for the elaboration of antibodies. Fagraeus (1948) listed 3 groups of cells which have been suggested as the site of antibody formation. These were (1) different cells belonging to the reticulo-endothelial system, (2) lymphocytes, and (3) plasma cells. He further stated that, in human pathology, there is never an increase in the level of serum globulin associated with an increase in the number of lymphocytes alone. Fagraeus concluded that

antibody formation occurs in cells belonging to the reticulo-endothelial system. On the occasion of intense antibody formation, a development of reticulo-endothelial cells into plasma cells occurs, and, in the course of this process, antibodies are being formed.

Ortega and Mellors (1957) used fluorescent antibody techniques to study the cellular sites of the formation of gamma globulin in lymphatic tissue. They found that gamma globulin is formed in "intrinsic" cells of the germinal centers of lymphatic nodules. They distinguished these cells from medium and large lymphocytes and the primitive reticular cells that occur elsewhere and do not produce gamma globulin. They also found that gamma globulin is formed in the cytoplasm of mature and immature plasma cells.

Coons (1955), White et al. (1955a, 1955b), and Leduc et al. (1955) have studied antibody production by the injection of various antigenic materials into laboratory animals. Their conclusion was that the formation of antibody is closely associated with the proliferation of plasma cells in the lymph nodes, spleen, liver, and in case of some antigens, at the site of injection.

Carlson and Gyllensten (1958) studied the plasma cells in the growing lymphatic system of young guinea pigs. These cells were detected by their pyroninophilia. They found no mature plasma cells in newborn animals, but these cells began to form in the medullary cords of the lymph nodes and in the red pulp of the spleen immediately after birth. The number of plasma cells increased during the first and second months of life. In white splenic pulp, lymph node cortex, Peyer's patches, and thymus, mature plasma cells were found only exceptionally, and immature cells were very rare.



Speirs (1958) stated that eosinophils accumulate in lymph nodes draining an antigen-injected area or an area of infection and in the spleen following an intravenous injection of foreign material. He described 2 stages in antibody formation. In the first the antigen reacts with certain cells (possibly eosinophils), producing enzymatic changes or forming an enzymatic template. In the second step the specific enzymes are utilized for synthesis of the antibody. This takes place in the lymphoid and reticulo-endothelial tissues, and the eosinophil is phagocytized.

D. Histology of Germfree Animals

Numerous histological studies have been made on germfree animals, and most of these studies have involved either the lymphatic system or the intestinal tract. Glimstedt (1936) found that the cellular composition of the lymphatic tissues of germfree guinea pigs was the same as in control animals but that quantitative differences existed in the numbers of cells. The number of lymphocytes was reduced so that the cortex and medullary cords appeared narrow, the boundary between cortex and medulla was indistinct, and the intermediary sinus was wide. The number of large free cells in this sinus was also reduced. There was also a reduction in the number of lymphocytes in the white pulp of the spleen. Glimstedt stated that secondary nodules were not found in either the lymph nodes or spleen of germfree animals. Conway (1937) has disputed this claim on the basis of the various sizes of lymphocytes seen in some of Glimstedt's published photomicrographs.

Miyakawa (1959b) and Miyakawa et al. (1957) also studied the lymphatic tissue of germfree guinea pigs. They found an underdevelopment

of lymphatic tissue in these animals and were unable to demonstrate reaction centers or Flemming-type nodules. Peyer's patches were poorly developed. Miyakawa et al. (1957) stated that there was a smaller number of sinus reticulum cells in the lymph nodes of germfree animals and the phagocytic power of these cells was reduced when compared to those found in normal animals.

Gustafsson (1948) found that the number of free cells in the lymphatic organs of germfree rats was reduced and the reaction centers were missing.

Thorbecke (1959) found no such outstanding differences between germfree and conventional rats. He described secondary nodules in the spleen of germfree rats and stated that they were occasionally found in the lymphoid tissue of the appendixes and in the mesenteric lymph nodes. These nodules were not as numerous in germfree animals, nor were plasma cells as numerous.

Gordon (1959) and Gordon and Bruckner-Kardoss (1958-1959) have studied the scattered reticulo-endothelial elements in the mucosa and submucosa of the intestine of germfree chickens. They found a reduction in the number of globule leukocytes within the epithelium and in the lymphocytes, plasma cells, and possibly the macrophages of the lamina propria and submucosa. Gordon (1959) also found a lower percentage of connective tissue and a higher percentage of epithelium in the small intestine of germfree chickens and rats as compared to conventionally-raised animals.

Thorbecke (1959) found no plasma cells or secondary nodules in the intestinal tract of germfree chickens up to 6 weeks of age. No secondary nodules were seen in the spleen at this age, and the numbers

of plasma cells in the spleens of germfree chickens were always less than in the spleens of control chickens. No differences were observed in the size or histological features of the bursa of Fabricius in germfree and control chickens at the age of 6 weeks. Thorbecke et al. (1957) stated that secondary nodules could sometimes be found in the spleen and ileo-cecal-colic junction of germfree birds.

Reyniers et al. (1960) described a method for determining the lymphocyte concentration per unit volume of tissue. By using this method they found no differences in the lymphocyte concentration of the spleen of germfree and conventional animals. The ileo-cecal-colic junction of the germfree birds contained a lower concentration of lymphocytes than did the same tissue of the conventionally-raised birds.

E. Histology of the Lymphatic System and the Liver of the Pig

1. Histology of the Lymphatic System

Richter (1902) studied the lymph nodes of domestic animals and described the cell-rich cortical substance which contains germinal centers and occupies the central part of the lymph nodes, while the cell-poor substance occupies the peripheral part of the node. He stated that the medullary substance is very poorly developed in swine lymph nodes. Richter also described the fine-meshed reticulum of the medulla with its individual elements being more strongly built than those of the cortex.

Baum and Hille (1908) also found the cortex to be centrally located and to reach the capsule in only a few areas. They described the medulla as surrounding the cortex and sometimes extending in streaks between areas of the cortex. The germinal centers lie close to lymph sinuses surrounding the septa. Baum and Hille found no germinal centers

in very young pigs. In 3 to 4-week-old pigs these centers were described as being more distinct and numerous but not clear-cut or typical. They also described fatty degeneration of lymph nodes in older animals.

Trautmann (1926) described in detail the structure of the lymph nodes of swine. He noted the frequent fusion of small lymph nodes to form a node with a lobed appearance. His description of the cortex and medulla agreed with that of Richter (1902) and Baum and Hille (1908). Trautmann (1926) described the circulation of lymph through the lymph nodes of swine. The lymph enters through the afferent lymphatic vessels whose branches extend into the trabeculae, and the lymph then empties into the wide sinus system surrounding the septum or trabeculae. From here the lymph may go directly into the marginal sinus where the trabeculae reach the capsule, but usually it goes through the medullary substance, receiving "washings" from the germinal centers. The marginal sinuses empty into the efferent lymphatic vessels. Trautmann (1926) also described the coloring of swine lymph nodes caused by the collection of blood components in the sinuses.

Goldkuhl (1927) described the histology of swine lymph nodes and stated that the afferent lymphatic vessels enter through one or more pseudohili. The efferent vessels leave the node here and there on its convex surface without hilus formation or through one or more small hilus formations.

Hellman (1930) recognized the unusual arrangement of the cortex and medulla of swine lymph nodes and reviewed some of the earlier investigations of the lymph nodes of swine.

Trautmann and Fiebiger (1957) stated that the usual description of lymph nodes does not apply to the nodes of swine. In this animal the lymphatic tissue that contains germinal centers and corresponds to the cortex of the node in other species occupies a more central position. The peripheral zone is filled with a tissue that is comparable to that of the medulla of other species, although it has a somewhat different structure. They noted that the same type of nodular lymphatic tissue always lies closer to the afferent vessels, whether in swine or in other species.

Bouwman (1959) described the histology of various lymph nodes from pigs ranging in age from late fetal life to 3 years. He also studied the lymph flow in these nodes.

Trautmann and Fiebiger (1957) described the spleen of swine and ruminants as being intermediate between the "storage" spleen of the horse, dog, and cat, and the "defense" spleen of the rabbit and man. The reticulum of the red pulp is especially well-developed in the pig. These authors also stated that the destruction of red cells is particularly well-demonstrated in the swine spleen.

Calhoun and Smith (1958) stated that the spleen of swine contains considerable white pulp and relatively little red pulp.

Snook (1950) described the pig spleen as being of the non-sinusoidal type and having penicillar ellipsoids. He stated that the ellipsoid sheaths are quite large and measure 195 by 62 μ.

2. Histology of the Liver

Illing (1905) studied the histology of the pig's liver. He found that the lobules are 5, 6, or several-sided and are completely separated by heavy bands of connective tissue which are not as distinct

in the young animals. Illing found the swine liver lobule to be larger than that of other domestic animals, measuring an average of 0.872 mm. in suckling pigs and 1.573 mm. in adult animals.

Johnson (1916-1917, 1919) studied the development of the lobules of the pig's liver and found that the lobules are fused together until late fetal life. At this stage the pig's liver resembles closely the liver of most adult mammals. The start of segmentation becomes apparent just before birth and the formation of connective tissue septa is not completed until several months after birth. Johnson found that the liver cells themselves take part in the separation of the liver parenchyma. The cells along the boundaries of the lobules become granular and stain more deeply than the cells elsewhere. Soon these cells become arranged in parallel rows or sheets extending from one branch of a portal vein to another. The rows of cells become split apart by a thickening of the reticulum between them. Collagen fibers gradually spread into this thickened reticulum from around the portal veins. Johnson found that the lobules increase in size from 0.43 mm. at one day of age to 1.2 mm. in the adult.

Johnson (1918a, 1918b) described a method of separating the lobules in blocks of hepatic tissue so that the shapes of the lobules could be more readily studied. He found that the lobules are irregular polyhedrons with 4 to 15 or more surfaces. The surface lobules are irregularly prismatic in shape, but the deeper lobules seldom approach any geometrical solids. Compound lobules are formed due to incomplete connective tissue septa. New lobules are formed by bifurcation of the central veins and splitting of the lobules.

White (1938-1939) also described the development of a lobular pattern in the liver of young pigs and stated that the first indication of a lobular pattern was found at 5 to 7 days of age due to a columnar arrangement of the liver cells between branches of the portal vein. The first outgrowth of collagen fibers usually began at 20 to 24 days of age, but there were striking variations in the development of connective tissue, even in pigs of the same litter. The clearly marked lobulation seen in some animals before the outgrowth of collagen fibers is due to thick bundles of reticulum surrounding the lobules. White listed the diameter of the lobule as 0.37 mm. at 5 days of age and 1.55 mm. at 297 days of age.

Elias et al. (1954) have stated that the liver of the pig, characterized by sharply defined lobules separated from each other by connective tissue septa, exhibits a form of portal cirrhosis of unknown etiology. They noted the similarity between this condition and cirrhosis of the liver in man.

III. EXPERIMENTAL

A. Procedures

1. Animals Used

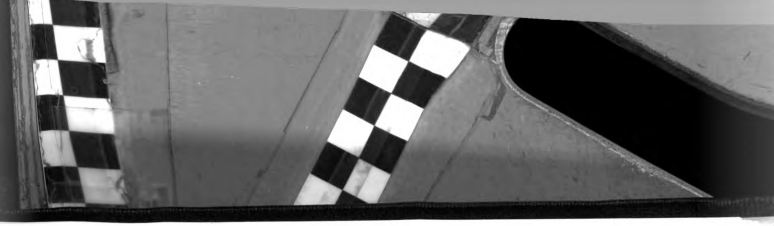
The source of the various groups of animals has been summarized in Table X. The procedures used in rearing the gnotobiotic pigs in groups A, C, E, and F has been discussed in detail in Part One, as has the rearing of the pigs in groups B and D.

The 11 pigs in group G were reared by the sow to the age of 3 weeks under conditions of management similar to those ordinarily seen on the farm. Five different litters were represented. Pigs G-1 and G-2 were from one litter, G-3 and G-4 from a second, G-5 from a third, G-6, G-7, G-8, and G-9 from another, and G-10 and G-11 from a fifth litter. The latter 2 animals were littermates of the 4 pigs in group F. After the animals were derived by hysterectomy, these 2 animals along with 3 others were placed on a sow which had farrowed on the same day.

The first 9 pigs in group G were given supplemental iron by a combination of methods. One method was to give each pig a tablet containing iron, copper, and cobalt (FeCuCo, Ft. Dodge Laboratories, Ft. Dodge, Iowa). Another method consisted of swabbing the sow's udder once a day with a solution containing $FeSO_4$, $CuSO_4$, and sugar in water. Iron was also provided by placing sod in the pen with the pigs. Pigs G-10 and G-11 were each injected with 2 ml. of iron dextran solution (Armidexan, Armour Laboratories, Kankakee, Illinois).

2. Body and Organ Weights

Pigs in groups A, B, C, and D were killed at 17 days of age, and pigs in groups E, F, and G were killed at 21 days of age. When



necropsies were to be performed, the animals were weighed with a spring-type milk scale, using a loop of cotton cord around the leg above the hock joint to suspend each animal from the hook of the scale. Blood samples were then drawn for hematological examinations, using either heparin or a combination of ammonium and potassium oxalates as the anticoagulant. The animals were then anesthetized with sodium pentobarbital. After anesthesia was accomplished, the pigs were exanguinated, and the necropsies were performed.

The weights of the various organs were determined by weighing them on a laboratory-type balance or a small torsion balance. The lymph nodes were dissected out, using the description of swine lymph nodes by St. Clair (1958) as a guide. The nodes were stripped of as much extraneous fat and fascia as possible before they were weighed.

The pericardial sac was opened, and the parietal layer was reflected back. The large vessels were then cut off just above the heart. The atria and ventricles were then opened and washed before the heart was weighed.

The trachea was removed at its bifurcation, and the mediastinum and its lymph nodes were removed before the lungs were weighed.

The gall bladder was removed from the liver before this organ was weighed.

The other organs were dissected out, freed of extraneous fat and fascia, and weighed in their entirety.

After the total body weights and organ weights were determined, the data were analyzed statistically, using an analysis of variance and the "F" test (Snedecor, 1948). The data were then checked

for significant differences between the various group averages, using the multiple range test as described by Duncan (1955).

In order to compensate for the relatively slow growth rate of the gnotobiotic animals, 2 methods of expressing organ weights were used. In the first method, the weights were expressed as grams. In the second method, they were expressed as relative weights (milligrams of tissue per 100 gm. of total body weight).

3. Histological Procedures

Blocks of tissue from the organs to be studied in this investigation were saved in Zenker's fixative (Armed Forces Institute of Pathology, 1957) except that acetic acid was not added before the fixative was used. In some cases blocks were also saved in 10 per cent buffered formalin and Carnoy's fixative (Armed Forces Institute of Pathology, 1957). The Zenker-fixed tissues were left in the fixative for 12 to 24 hours and were then washed for 12 to 24 hours in running tap water. The tissues were then stored in 80 per cent ethyl alcohol until the time of processing.

After the blocks were trimmed to size, they were dehydrated in a graded series of alcohols, cleared in xylene, and embedded in paraffin. Triplicate sections were cut at 6 micra with a rotary microtome, placed on slides, and stained with Delafield's hematoxylin and eosin, Heidenhain's aniline blue, and Foot's modification of Bielschowsky's method for reticulum. Some extra sections were also cut and stained for iron by Gomori's method, and Giemsa's stain was applied to a few sections. Formalin-fixed and Carnoy-fixed blocks of liver tissue were sectioned and stained for fat and glycogen, respectively,

using the Sudan IV technique for the former and the Best's carmine technique for the latter. All staining techniques were carried out according to the Manual of Histologic and Special Staining Technics (Armed Forces Institute of Pathology, 1957) with the exception of the Sudan IV stain which was carried out according to the description by Mallory (1938) with the substitution of Harris's hematoxylin for alum hematoxylin.

B. Results

1. Body and Organ Weights

The total body weights are shown in Table XIII. At the bottom of the table is shown the range of the group averages, in ascending order. The method of expressing significance between the averages is that described by Duncan (1955). Any 2 averages not underscored by the same line are significantly different, while any 2 averages underscored by the same line are not significantly different. The significance at the 5 per cent level is shown first, followed by the 1 per cent level. If the latter is not shown, it is an indication that there were no significant differences between the group averages at that level.

The various organ weights, both absolute and relative, are shown in Tables XIV through XXXV, and the same method of indicating significant differences was used.

2. Gross Observations

It was noted that, at the time of necropsy, there was very little odor associated with the pigs raised in the isolator. The odor which was present resembled more closely that of the sterilized milk than the somewhat offensive odor sometimes associated with young pigs.

TABLE XIII. Total body weights (lbs.)

Pig No.	Experiment I		Experiment II		Experiment III	Experiment IV	
	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1	3.7	2.8	5.2	5.0	4.4	6.0	11.6
2	4.5	4.2	4.6	5.0	4.1	6.9	12.6
3	4.3	3.5	4.9			5.6	13.1
4	3.8					6.5	14.1
5							10.5
6							12.8
7							12.2
8							12.0
9							12.2
10							8.4
11							7.0
Average	4.08	3.50	4.90	5.00	4.25	6.25	11.50

Significance of group averages at:

	B	A	E	C	D	F	G
5% level -	3.50	4.08	4.25	4.90	5.00	6.25	11.50
1% level -	3.50	4.08	4.25	4.90	5.00	6.25	11.50

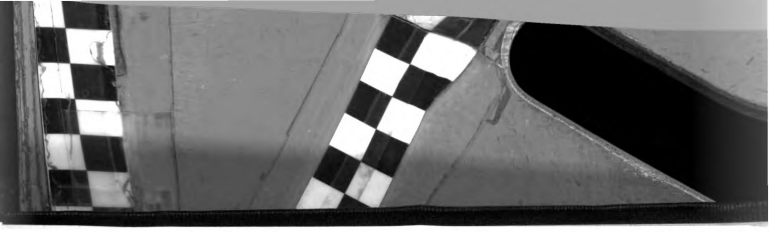


TABLE XIV. Weights of mandibular lymph nodes (gm.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1			0.11[a]	0.26	0.14	0.15	0.69
			0.12	0.26	0.14	0.21	0.52
2			0.20	0.17	0.17	0.40	0.78
			0.19	0.17	0.19	0.27	0.81
3			0.17			0.28	0.80
			0.22			0.35	0.88
4						0.36	0.85
						0.32	0.96
5							0.34
							0.41
6							0.60
							0.71
7							0.58
							0.71
8							0.76
							0.79
9							0.66
							0.82
10							0.70
							0.59
11							0.42
							0.40
Average			0.168	0.215	0.160	0.292	0.672

Significance of group average at:

	E	C	D	F	G
5% level -	0.160	0.168	0.215	0.292	0.672
1% level -	0.160	0.168	0.215	0.292	0.672

[a] In case of paired organs, first weight is right side, second weight is left side.

TABLE XV. Relative weights of mandibular lymph nodes (mg. per 100 gm. body wt.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1			4.7 5.1	11.5 11.5	7.0 7.0	5.5 7.7	13.1 9.9
2			9.6 9.1	7.5 7.5	9.1 10.2	12.8 8.6	13.6 14.2
3			7.6 9.9			11.0 13.8	13.5 14.8
4						12.2 10.8	13.3 15.0
5							7.1 8.6
6							10.3 12.2
7							10.5 12.8
8							14.0 14.5
9							11.9 14.8
10							18.3 15.5
11							13.2 12.6
Average			7.67	9.50	8.32	10.30	12.90

Significance of group averages at:

	C	E	D	F	G
5% level -	7.67	8.32	9.50	10.30	12.90

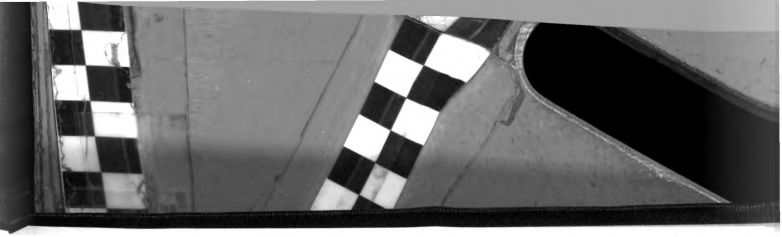


TABLE XVI. Weights of external inguinal lymph nodes (gm.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1			0.32 0.29	0.28 0.29	0.38 0.37	0.32 0.40	0.82 0.86
2			0.20 0.19	0.22 0.18	0.33 0.32	0.37 0.41	1.06 1.23
3			0.16 0.13			0.36 0.40	0.86 0.89
4						0.47 0.51	0.88 0.88
5							0.55 0.45
6							0.91 1.01
7							0.62 0.69
8							0.80 0.76
9							0.75 0.82
10							0.61 0.65
11							0.70 0.65
Average			0.215	0.242	0.350	0.405	0.793

Significance of group averages at:

	C	D	E	F	G
5% level -	0.215	0.242	0.350	0.405	0.793
1% level -	0.215	0.242	0.350	0.405	0.793

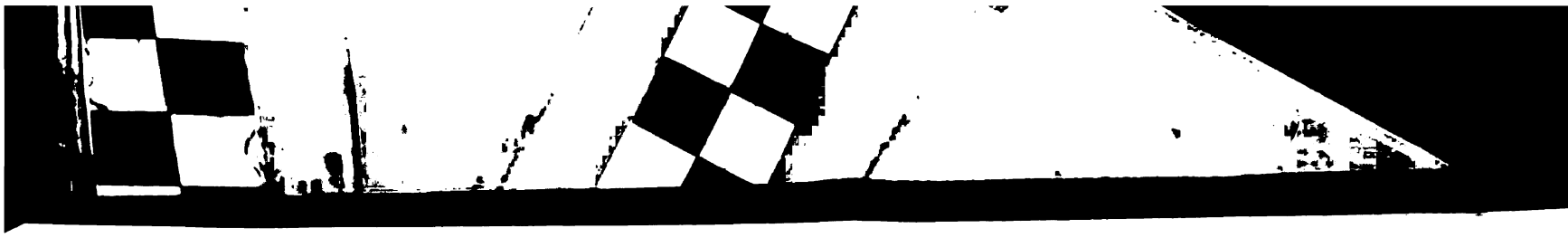


TABLE XVII. Relative weights of external inguinal lymph nodes (mg. per 100 gm. body wt.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1			13.6	12.3	19.0	11.7	15.6
			12.3	12.8	18.5	14.7	16.3
2			9.6	9.7	17.7	11.8	18.5
			9.1	7.9	17.2	13.1	21.5
3			7.2			14.2	14.5
			5.8			15.7	15.0
4						15.9	13.7
						17.3	13.7
5							11.5
							9.4
6							15.7
							17.4
7							11.2
							12.5
8							14.7
							13.9
9							13.5
							14.8
10							16.0
							17.0
11							22.0
							20.5
Average			9.60	10.68	18.10	14.30	15.40

Significance of group averages at:

	C	D	F	G	E
5% level -	9.60	10.68	14.30	15.40	18.10

TABLE XVIII. Weights of prefemoral lymph nodes (gm.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1	0.18		0.14	0.21	0.15	0.15	0.36
	0.17		0.15	0.20	0.18	0.13	0.38
2	0.18		0.15	0.10	0.20	0.25	0.56
	0.20		0.14	0.10	0.18	0.23	0.54
3	0.12		0.18			0.16	0.35
	0.08		0.18			0.16	0.31
4	0.18					0.15	0.66
	0.14					0.15	0.68
5							0.25
							0.24
6							0.48
							0.47
7							0.30
							0.34
8							0.41
							0.45
9							0.36
							0.28
10							0.34
							0.35
11							0.18
							0.16
Average	0.156		0.157	0.152	0.178	0.172	0.384

Significance of group averages at:

	D	A	C	F	E	G
5% level -	0.152	0.156	0.157	0.172	0.178	0.384
1% level -	0.152	0.156	0.157	0.172	0.178	0.384

TABLE XIX. Relative weights of prefemoral lymph nodes (mg. per 100 gm. body wt.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1	10.7		5.9	9.2	7.5	5.5	6.8
	10.1		6.4	8.8	9.0	4.8	7.2
2	8.8		7.2	4.4	10.7	8.0	9.8
	9.8		6.7	4.4	9.7	7.3	9.4
3	6.1		8.1			6.3	5.9
	4.1		8.1			6.3	5.2
4	10.4					5.1	10.3
	8.1					5.1	10.6
5							5.2
							5.0
6							8.3
							8.1
7							5.4
							6.1
8							7.5
							8.3
9							6.5
							5.1
10							8.9
							9.2
11							5.7
							5.0
Average	6.01		7.07	6.70	9.22	6.05	7.25

Significance of group averages at:

	A	F	D	C	G	E
5% level -	6.01	6.05	6.70	7.07	7.25	9.22

TABLE XX. Weights of hearts (gm.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1			12.0	12.4	13.0	20.3	27.0
2			13.5	12.1	12.8	21.7	31.1
3			14.5			17.1	32.0
4						19.1	35.2
5							24.6
6							28.9
7							29.9
8							—
9							31.9
10							22.8
11							21.7
Average			13.33	12.25	12.90	19.55	28.51

Significance of group averages at:

	D	E	C	F	G
5% level -	12.25	12.90	13.33	19.55	28.51
1% level -	12.25	12.90	13.33	19.55	28.51

TABLE XXI. Relative weights of hearts
(mg. per 100 gm. body wt.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1			508.3	546.3	650.8	745.2	512.7
2			646.4	533.0	687.7	692.7	543.7
3			651.8			672.6	538.0
4						647.2	549.9
5							516.0
6							497.3
7							539.8
8							---
9							575.9
10							597.9
11							682.8
Average			602.17	539.65	669.25	689.42	555.40

Significance of group averages at:

	D	G	C	E	F
5% level	539.65	555.40	602.17	669.25	689.42

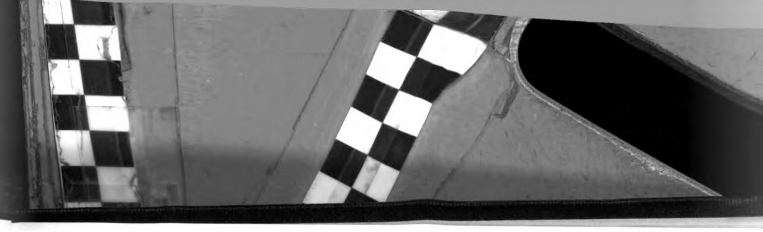


TABLE XXII. Weights of lungs (gm.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1			34.5	28.7	20.9	53.8	74.3
2			33.5	31.1	21.3	42.8	77.5
3			33.6			34.7	78.8
4						38.7	88.8
5							65.4
6							79.1
7							73.0
8							78.5
9							82.4
10							54.8
11							38.1
Average			33.87	29.90	21.10	42.50	71.88

Significance of group averages at:

	E	D	C	F	G
5% level -	21.10	29.90	33.87	42.50	71.88
1% level -	21.10	29.90	33.87	42.50	71.88

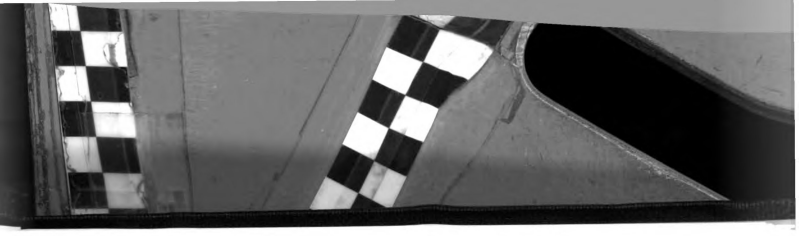


TABLE XXIII. Relative weights of lungs
(mg. per 100 gm. body wt.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1			1461.4	1264.3	1046.3	1975.0	1410.8
2			1604.1	1370.0	1144.3	1366.3	1354.8
3			1510.4			1364.9	1324.9
4						1311.4	1387.2
5							1371.9
6							1361.2
7							1318.0
8							1440.9
9							1487.7
10							1437.0
11							1198.9
Average			1525.30	1317.15	1095.30	1504.40	1372.12

Significance of group averages at:

	E	D	G	F	C
5% level -	1095.30	1317.15	1372.12	1504.40	1525.30

TABLE XXIV. Weights of livers
(gm.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1	47.0		59.5	57.5	54.4	71.0	193.7
2	47.5		66.1	55.1	54.7	95.3	216.3
3	44.5		59.9			82.6	154.8
4	34.2					83.7	193.3
5							141.1
6							140.3
7							127.0
8							156.4
9							152.3
10							102.8
11							82.8
Average	43.30		61.83	56.30	54.55	83.15	150.98

Significance of group averages at:

	A	E	D	C	F	G
5% level -	43.30	54.55	56.30	61.83	83.15	150.98
1% level -	43.30	54.55	56.30	61.83	83.15	150.98

TABLE XXV. Relative weights of livers (mg. per 100 gm. body wt.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1	2798.0		2520.3	2533.0	2723.3	2606.5	3678.0
2	2325.0		3165.1	2427.3	2938.6	3042.2	3781.2
3	2279.5		2692.6			3248.9	2602.8
4	1982.4					2836.3	3019.7
5							2959.9
6							2414.3
7							2293.0
8							2870.8
9							2747.9
10							2695.6
11							2605.4
Average	2346.22		2792.67	2480.15	2830.95	2933.48	2878.96

Significance of group averages at:

	A	D	C	E	G	F
5% level -	2346.22	2480.15	2792.67	2830.95	2878.96	2933.48

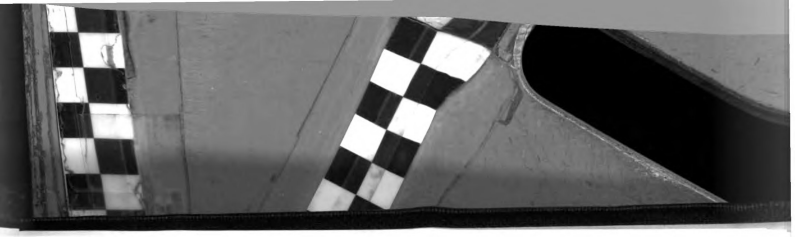


TABLE XXVI. Weights of spleens (gm.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1	2.1		2.7	2.6	3.8	4.5	8.6
2	2.1		2.5	2.8	3.6	6.7	15.3
3	2.8		2.2			4.4	8.1
4	2.0					5.8	13.6
5							7.0
6							7.6
7							7.0
8							7.2
9							7.6
10							5.6
11							6.1
Average	2.25		2.47	2.70	3.70	5.35	8.52

Significance of group averages at:

	A	C	D	E	F	G
5% level -	2.25	2.47	2.70	3.70	5.35	8.52
1% level -	2.25	2.47	2.70	3.70	5.35	8.52

TABLE XXVII. Relative weights of spleens
(mg. per 100 gm. body wt.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1	125.0		114.4	114.5	190.2	165.2	163.3
2	102.8		119.7	123.3	193.4	213.9	267.5
3	143.4		98.9			173.1	136.2
4	115.9					196.5	212.5
5							146.8
6							130.8
7							126.4
8							132.2
9							137.2
10							146.8
11							191.9
Average	121.78		111.00	118.90	191.80	187.18	162.87

Significance of group averages at:

	C	D	A	G	F	E
5% level -	111.00	118.90	121.78	162.87	187.18	191.80

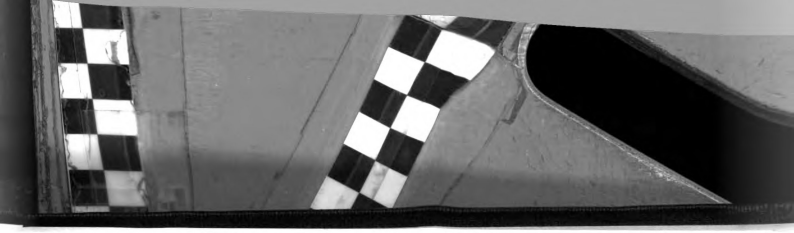


TABLE XXVIII. Weights of kidneys (gm.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1	7.5		9.9	9.1	7.5	9.5	18.0
	7.5		9.2	8.8	7.6	9.8	16.2
2	8.5		8.4	8.6	7.0	10.7	20.7
	8.5		9.4	7.8	7.3	10.5	21.6
3	8.0		5.7			9.6	17.0
	8.0		6.0			9.4	16.8
4	6.2					10.3	20.3
	6.3					11.2	20.3
5							13.1
							12.9
6							13.9
							15.6
7							13.2
							13.2
8							13.5
							14.5
9							13.9
							13.3
10							8.6
							9.5
11							7.7
							8.5
Average	7.56		8.10	8.58	7.35	10.12	14.65

Significance of group averages at:

	E	A	C	D	F	G
5% level -	7.35	7.56	8.10	8.58	10.12	14.65
1% level -	7.35	7.56	8.10	8.58	10.12	14.65

TABLE XXIX. Relative weights of kidneys (mg. per 100 gm. body wt.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1	446.5		419.3	400.9	375.4	348.8	341.8
	446.5		389.7	387.7	380.5	359.8	307.6
2	416.1		421.4	378.9	376.1	341.6	361.9
	416.1		450.1	343.6	392.2	335.2	377.6
3	409.8		256.2			377.6	285.8
	409.8		269.7			369.7	282.5
4	359.4					349.0	317.1
	365.2					379.5	317.1
5							274.8
							270.6
6							239.2
							268.4
7							238.3
							238.3
8							247.8
							266.2
9							251.0
							240.1
10							225.5
							249.1
11							242.3
							267.5
Average	408.66		367.73	377.78	381.05	357.65	277.75

Significance of group averages at:

	G	F	C	D	E	A
5% level -	277.75	357.65	367.73	377.78	381.05	408.66
1% level -	277.75	357.65	367.73	377.78	381.05	408.66

TABLE XXX. Weights of pancreases (gm.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1			3.3	3.6	3.2	5.2	11.6
2			3.0	3.9	3.2	6.5	16.5
3			3.1			6.2	7.7
4						5.0	11.4
5							7.6
6							7.0
7							5.6
8							7.3
9							6.7
10							5.2
11							3.9
Average			3.13	3.75	3.20	5.72	8.23

Significance of group averages at:

	C	E	D	F	G
5% level -	3.13	3.20	3.75	5.72	8.23

TABLE XXXI. Relative weights of pancreases
(mg. per 100 gm. body wt.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1			134.0	158.6	160.2	190.9	220.3
2			143.7	171.8	171.9	207.5	288.4
3			139.4			243.9	129.5
4						169.4	178.1
5							159.4
6							120.5
7							101.1
8							134.0
9							121.0
10							136.4
11							122.7
Average			139.03	165.20	166.05	202.92	155.58

Significance of group averages at:

	C	G	D	E	F
5% level -	139.03	155.58	165.20	166.05	202.92

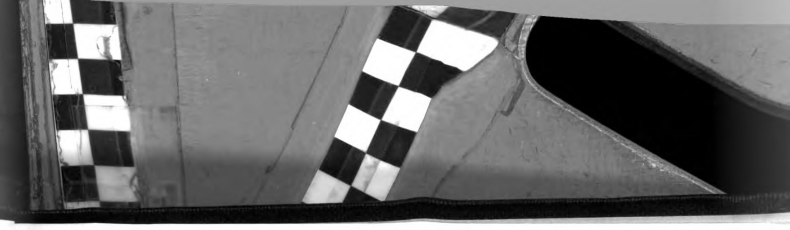


TABLE XXXII. Weights of thyroids (gm.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1	0.40		0.30	0.19	0.30	0.22	0.78
2	0.40		0.26	0.28	0.29	0.30	0.71
3	0.30		0.45			0.32	0.61
4	0.27					0.20	0.64
5							0.37
6							0.48
7							0.47
8							0.94
9							0.55
10							0.30
11							0.25
Average	0.342		0.337	0.235	0.295	0.260	0.555

Significance of group averages at:

	D	F	E	C	A	G
5% level -	0.235	0.260	0.295	0.337	0.342	0.555

TABLE XXXIII. Relative weights of thyroids (mg. per 100 gm. body wt.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1	23.8		12.7	8.4	15.0	8.1	14.8
2	19.6		12.4	12.3	15.6	9.6	12.4
3	15.4		20.2			12.6	10.3
4	15.7					6.8	10.0
5							7.8
6							8.3
7							8.5
8							17.3
9							9.9
10							7.9
11							7.9
Average	18.62		15.10	10.35	15.30	9.28	10.46

Significance of group averages at:

	F	D	G	C	E	A
5% level -	9.28	10.35	10.46	15.10	15.30	18.62
1% level -	9.28	10.35	10.46	15.10	15.30	18.62

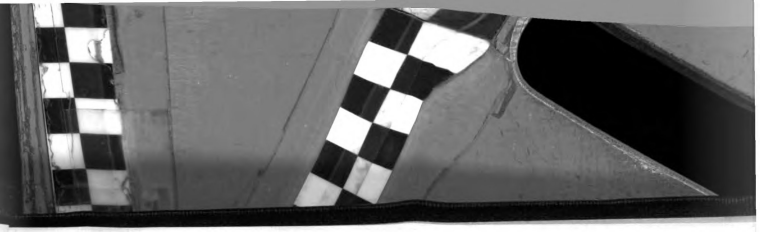


TABLE XXXIV. Weights of adrenals (gm.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1	0.18		0.13	0.21	0.16	0.25	0.35
	0.18		0.17	0.21	0.15	0.23	0.33
2	0.20		0.16	0.14	0.24	0.25	0.38
	0.20		0.16	0.15	0.23	0.26	0.37
3	0.22		0.19			0.22	0.22
	0.22		0.20			0.20	0.26
4	0.14[a]					0.22	0.32
	0.14					0.23	0.44
5							0.36
							0.33
6							0.28
							0.29
7							0.29
							0.25
8							0.27
							0.25
9							0.28
							0.28
10							0.35
							0.32
11							0.32
							0.30
Average	0.185		0.168	0.178	0.195	0.232	0.311

Significance of group averages at:

	C	D	A	E	F	G
5% level -	0.168	0.178	0.185	0.195	0.232	0.311
1% level -	0.168	0.178	0.185	0.195	0.232	0.311

[a] Missing weight. It was assumed that this adrenal had the same weight as the left one from the same animal.

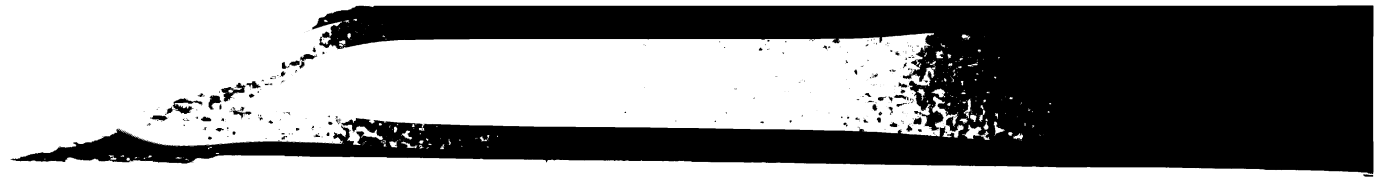


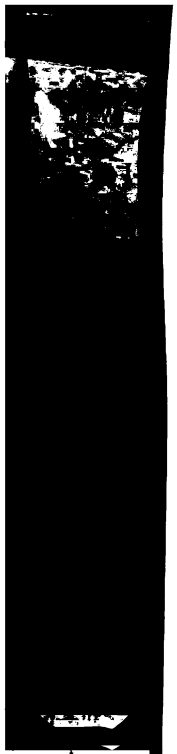
TABLE XXXV. Relative weights of adrenals
(mg. per 100 gm. body wt.)

Pig No.	Group A	Group B	Group C	Group D	Group E	Group F	Group G
1	10.7		5.5	9.2	8.0	9.2	6.6
	10.7		7.2	9.2	7.5	8.4	6.3
2	9.8		7.7	6.2	12.9	8.0	6.6
	9.8		7.7	6.6	12.4	8.3	6.5
3	11.3		8.5			8.6	3.7
	11.3		9.0			7.9	4.4
4	8.1[a]					7.4	5.0
	8.1					7.8	6.9
5							7.6
							6.9
6							4.8
							5.0
7							5.2
							4.5
8							5.0
							4.6
9							5.1
							5.1
10							9.2
							8.4
11							10.1
							9.4
Average	9.98		7.60	7.80	10.20	8.20	6.22

Significance of group averages at:

	G	C	D	F	A	E
5% level -	6.22	7.60	7.80	8.20	9.98	10.20

[a] Missing weight. It was assumed that this adrenal had the same weight as the left one from the same animal.



Since the findings on the intestinal tract are to be reported elsewhere, they will not be extensively described here. It may be mentioned, however, that the wall of the intestinal tract appeared thinner than normal, and the posterior portion of the small intestine contained a dark material which somewhat resembled a hemorrhagic exudate.

In the remaining tissues, the most constant gross lesion consisted of slight to moderate congestion or hemorrhage in the peripheral parts of the lymph nodes, especially those of the iliac group. Other nodes were sometimes involved also. These lesions appeared to be unrelated to the conditions under which the animals were raised. Pigs G-3 and G-4 showed marked enlargement of one small area of the mesenteric lymph nodes, and 2 nodes of the iliac group of pig G-4 were enlarged. Tissue was not saved from these enlarged areas. The peripheral lymph nodes of pigs G-10 and G-11 showed slight to marked peripheral staining with a brown-colored pigment.

Pig G-4 exhibited interstitial emphysema in the diaphragmatic lobes of the lungs, and there was one small area of consolidation on the dorsal surface of the lung above the right cardiac lobe.

Pigs C-1 and C-2 each had small cysts on the surface of the liver containing what appeared to be bile.

The umbilicus of pig G-10 was found to contain a moderate amount of purulent material.

Gross examination of the germfree animal left in the isolator 3 days after death revealed that the liver was quite friable, the intestines were somewhat soft, and the lungs showed areas of atelectasis and some emphysema. In the animal dead 5 days, it was noted that the

kidneys were quite soft, the intestines were soft, and the liver was rather friable. No objectionable odors were noted from either pig.

Hematological data obtained from blood samples drawn shortly before each animal was killed are shown in Table XXXVI to indicate that these data are, for the most part, within the normal range for animals of this age group.

3. Histology of the Lymph Nodes and Spleen

The histological picture of the lymph nodes was characterized by extreme variability. This variability existed not only between pigs but also between different lymph nodes in the same animal, and it was sometimes noted that different regions of the same lymph node presented somewhat different pictures.

One point of variability concerned the amount of fusion seen in the individual lymph nodes. Of the 4 groups examined the external inguinal nodes exhibited the most fusion, consisting of several small nodes separated in some cases by small bands of fatty tissue. In other instances the capsules were fused, and in still other instances the capsules were absent at the area of fusion and the medullary regions of the smaller nodes were in contact. The same type fusion was quite apparent in the mandibular and prefemoral nodes, although it seemed to occur to a somewhat lesser degree. The mesenteric nodes exhibited a small amount of such fusion.

Another point in which the lymph nodes varied was in the relative amounts and location of the cortex and medulla. In most nodes the tissue corresponding to the cortex of other mammals was located centrally, with the medulla surrounding it. It was occasionally found,

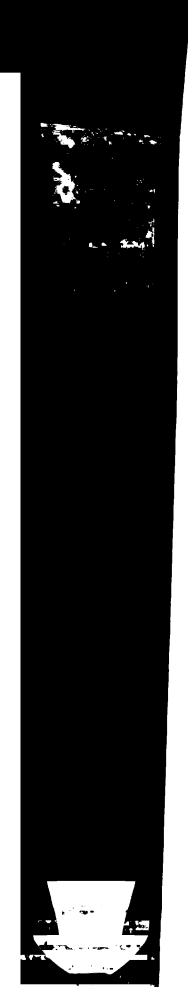


TABLE XXXVI. Hematological data

Group	Pig No.	Hgb. gm./100 ml.	Hct. vol.%	R.B.C. millions/cmm.	W.B.C. per cmm.	Neutrophils seg.,%	Neutrophils non-seg.,%	Lymph. %	Mono. %	Bas. %	Eos. %	Nuc. R.B.C.
A	1	Clotted										
A	2	10.9	34	6.05	7,600	29	10	60	0	1	0	
A	3	11.3	38	5.72	4,800	19	8	72	1	0	0	
A	4					45	11	43	1	0	0	
B	1	10.3	27	3.55	9,700	50		49	1	0	0	
B	2	12.5	37	5.08	10,050	52		49	0	0	0	
B	3	7.2	24	2.84	29,600	67		32	1	0	0	
C	1	8.4	29	5.83	6,050	33	0	64	2	1	0	
C	2	8.8	32	5.85	3,800	30	0	70	0	0	0	
C	3	8.8	30	5.71	4,750	31	2	66	1	0	0	
D	1	7.5	30	5.98	18,650	74	4	19	3	0	0	
D	2	8.1	29	6.16	4,800	30	1	67	1	0	0	
E	1	10.6	38	4.05	9,350	15	1	81	3	0	0	3
E	2	12.8	37	5.61	6,000	45	2	50	3	0	0	9
F	1	11.6	37	6.46	3,950	13	7	79	1	0	0	1
F	2	10.9	34	5.34	5,550	26	2	69	2	1	0	3
F	3	10.6	34	6.50	6,950	30	0	68	0	1	1	0
F	4	12.8	40	7.16	3,600	21	2	73	2	1	1	0
G	1	8.8	36	5.56	8,000	32	0	63	0	1	4	1
G	2	7.5	29	5.13	12,900	34	2	61	1	0	2	3
G	3	10.0	34	5.67	7,300	34	5	57	3	0	1	0
G	4	8.4	33	6.92	14,950	35	14	36	4	0	2	5
G	5	10.9	30	5.00	11,200	60	7	25	8	0	0	3
G	6	9.4	31	5.51	6,100	22	2	69	3	1	3	4
G	7	10.0	32	5.84	6,850	54	3	37	5	0	1	5
G	8	10.0	31	5.11	7,400	46	1	46	5	0	2	1
G	9	10.6	35	4.43	7,800	36	5	54	3	2	0	5
G	10	13.4	38	6.36	8,750	51	4	45	0	0	0	3
G	11	12.2	37	5.67	8,500	42	1	56	1	0	0	1

however, that the cortex was on the outside or perhaps along one edge. The relative quantities of the 2 types of tissue showed little uniformity, one node being predominantly cortex, another predominantly medulla.

The above mentioned variations made it extremely difficult to determine, quantitatively and qualitatively, whether or not differences existed between the various groups of animals.

Light-centered nodules corresponding to the germinal centers of Flemming (1885) or the reaction centers of Hellman (1921, 1930) were seen in all groups of animals. In the farm-raised animals, the mandibular nodes exhibited a greater degree of activity, as evidenced by the number of light centers and the amount of mitosis within these centers, than did the nodes of the external inguinal, prefemoral, and mesenteric groups. In the germfree animals, however, the mesenteric lymph nodes appeared to show more activity than did the other nodes. In the remaining groups of animals either the mandibular or the mesenteric nodes of the 4 groups examined, seemed to show the greater activity.

There was a definite tendency for the lymph nodes of the germfree animals to show more solid primary nodules consisting of dense collections of small lymphocytes, as in Figures 21 and 22. However, this type nodule was also found in the pigs raised on the artificial diet in open cages and occasionally in the farm-raised animals.

The lymphatic nodules found in the nodes of farm-raised animals exhibited numerous light-centered nodules as shown in Figure 23. However, such nodules were not absent from the gnotobiotic animals, as is evidenced by Figure 24 from a germfree animal and Figure 25 from an animal of group C (raised in the presence of Bacillus sp.). The

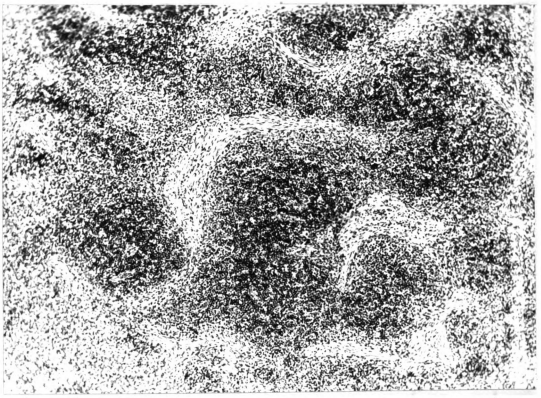


Figure 21. Solid primary nodules in prefemoral lymph node of germfree pig E-1

Hematoxylin and eosin. x 105

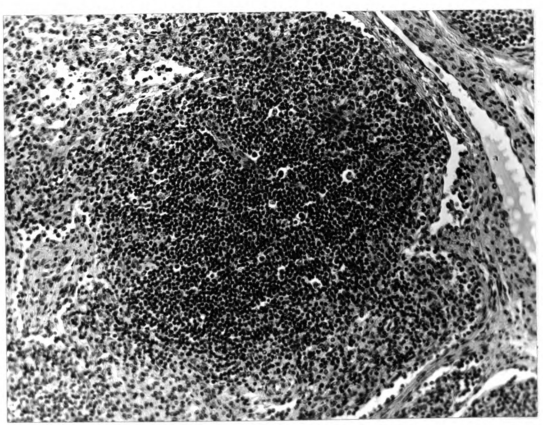


Figure 22. Solid primary nodule in mandibular lymph node of germfree pig E-2

Hematoxylin and eosin. x 215

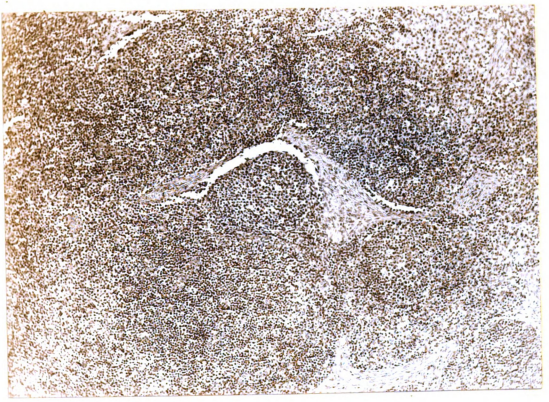


Figure 23. Light-centered nodules in prefemoral lymph node of farm-raised pig G-1

Hematoxylin and eosin. x 105

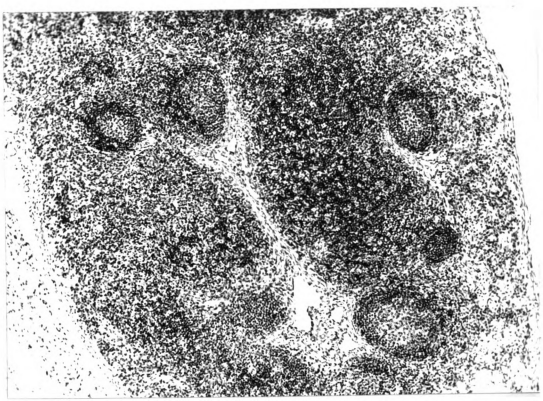


Figure 24. Light-centered nodules in mesenteric lymph node of germfree pig E-1

Hematoxylin and eosin. x 105

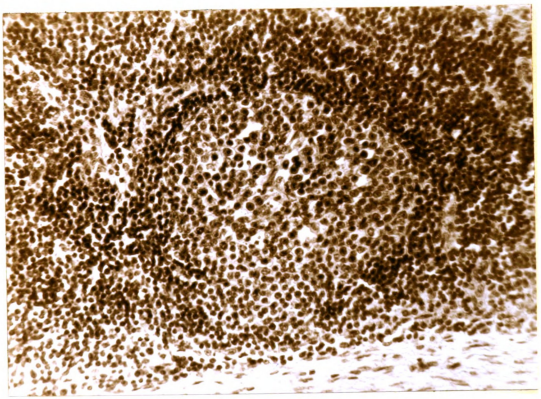


Figure 25. Light-centered nodule in mandibular lymph node of pig C-3

Hematoxylin and eosin. x 360

Note dark cells in center of nodule. One or 2 of these represent mitotic figures, the remainder are pyknotic nuclei.

opposite end of the lymph node pictures in Figure 24 consisted primarily of solid nodules as in Figures 21 and 22.

Another type of nodule seen rather frequently consisted of a collection of reticular cells, macrophages, and some large lymphocytes. These cells were often surrounded by a capsule-like reticulum as seen in Figure 26. These nodules were sometimes circled by small lymphocytes, but in many instances they were not. Mitotic activity was ordinarily negligible in these centers.

Figure 25 represents the more typical germinal or reaction center. These centers also contained numerous reticular cells, macrophages, and large lymphocytes. The mitotic activity in these centers was quite variable. In most instances at least a few mitotic figures could be seen, and sometimes several mitoses were present. When such centers were observed under low magnification, it appeared that considerable numbers of prophase mitotic figures were present. Examination under the oil-immersion objective, however, revealed that only part of these structures were mitotic figures. The rest were dark, apparently degenerating, nuclei. From the size and location of these nuclei, it appeared that some of them were from larger lymphocytes and some from the reticular cells or macrophages. Figure 27 shows at least 2 mitotic figures, as well as several degenerating nuclei. Also included in the figure are several small, round to irregularly-shaped objects which stain quite darkly and appear to correspond to the "tingible Körper" described by Flemming (1885). These are apparently composed of chromatin material from degenerated cells which have been engulfed by macrophages.

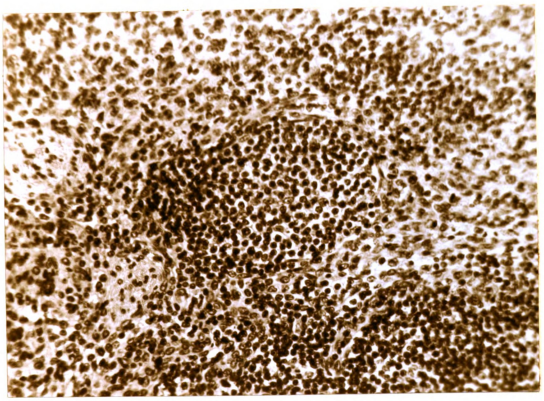


Figure 26. Light-centered nodule in mandibular lymph node of germfree pig E-1

Hematoxylin and eosin. x 360

Note connective tissue fibers surrounding nodule, scarcity of mature lymphocytes, and lack of mitotic activity in nodule.

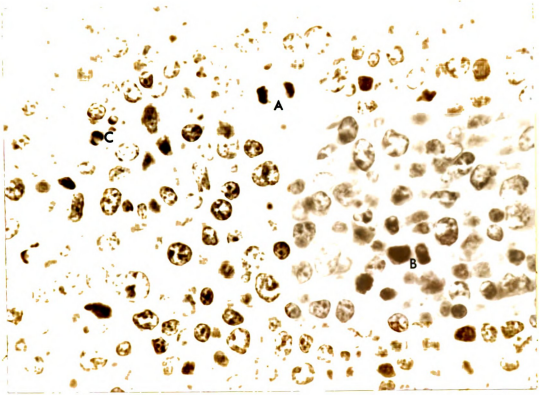


Figure 27. Cells in light-centered nodule from mesenteric lymph node of farm-raised pig G-10

Hematoxylin and eosin. x 960

A. Mitotic figure

B. Degenerating nuclei

C. "Tingible Körper"

Most of these germinal or reaction centers were surrounded by at least a small zone of mature lymphocytes. However, this zone was not usually as large or densely staining as that shown in Figure 25. This is probably due to the fact that some of the cells in this figure were hematopoietic cells (to be discussed later). Sometimes this zone of mature lymphocytes was absent, or present on one side and absent on the other. When the latter was the case, the border containing no small lymphocytes was usually adjacent to a trabecular sinus.

Although it was difficult to quantitate, it appeared that these typical germinal centers were larger and more abundant in the farm-raised pigs. No recognizable differences were found between the lymph nodes of animals in groups A and C (each raised in the presence of one species of bacteria) and their littermates (groups B and D, respectively) fed the sterilized diet in open cages.

Numerous cells of the granulocytic series were seen in the lymph nodes of these pigs. The most common cells were immature eosinophils, most of which appeared to be metamyelocytes or band cells. These cells were scattered in the medulla and cortex but were very seldom seen in the lymph nodules themselves. They ranged from a very few to many in a section, and no correlation could be found between the numbers of these cells and the conditions under which the animals were reared. The granules in these eosinophilic cells were in most sections very distinct. Also noted were some neutrophils. These granulocytes appeared to be more mature than the eosinophils and were primarily band forms with some segmented nuclei present. The granules were not distinct. In most sections these cells occurred in smaller numbers than did the eosinophils, and they had a tendency to be more in groups than

did the eosinophils. Here again, there was no correlation between the method of rearing the animals and the numbers of neutrophils present. Figure 28 illustrates these 2 types of cells in the cortex of a lymph node.

In most sections small, darkly-staining cells, resembling mature lymphocytes under low magnification, were found. Examination of these cells under the oil-immersion objective, however, revealed that they possessed nuclei which stained so intensely that no detail could be made out. Some of them appeared to contain no cytoplasm, whereas others had a small rim of eosinophilic cytoplasm. Figures 29 and 30 show these cells, which could not be distinguished from the cells of the small hematopoietic centers in the livers of these animals. The cortices of a majority of the nodes contained at least a few of these dark cells. Sometimes they were accumulated in groups in the sinuses of the medulla, and collections of these cells were commonly found in the trabecular sinuses as in Figures 29 and 30. In several of the lymph nodes these cells were so abundant that they could easily be seen under the scanning objective as dense black masses. In areas where these dark cells were quite densely packed, it was occasionally noted that spindle-shaped cells resembling degenerated fibroblasts accompanied these smaller cells.

An amorphous, brown-staining material was seen in the peripheral parts of all the lymph nodes of pigs G-10 and G-11, although the amount in the mesenteric nodes was much less than in the other 3 groups. Some of this material was free within the reticular sinuses, and part of it was intracellular. Gomori's iron stain showed this to be an iron-containing pigment (Figure 31).

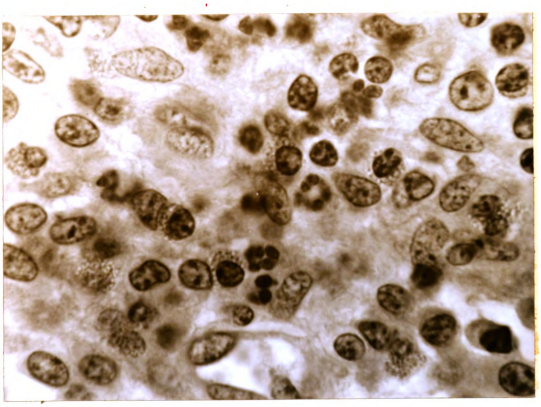


Figure 28. Eosinophils and neutrophils in medulla of mandibular lymph node of farm-raised pig G-2

Giemsa. x 1600

Figure 29. Hematopoietic cells in medulla of superficial inguinal lymph node of farm-raised pig G-1

Hematoxylin and eosin. x 175

A. Trabecula with cells in surrounding sinus.

Note dense collections of these cells in lower portion of figure.

Figure 30. Hematopoietic cells in trabecular sinus of superficial inguinal lymph node of farm-raised pig G-1

Hematoxylin and eosin. x 960

Note dark staining of these cells in comparison to surrounding cells.

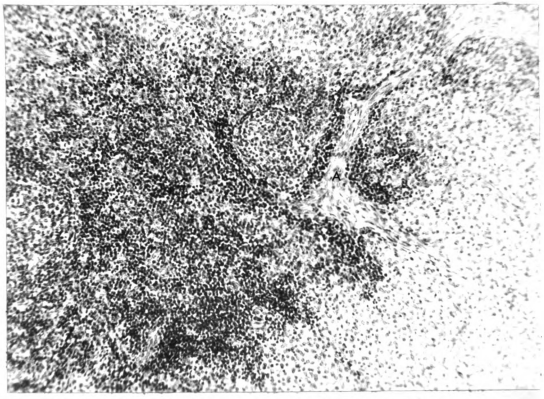


Figure 29

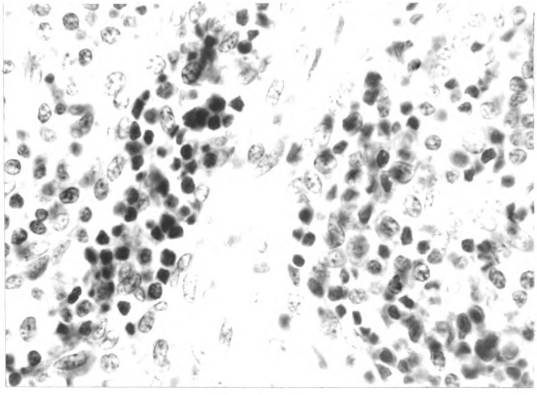


Figure 30

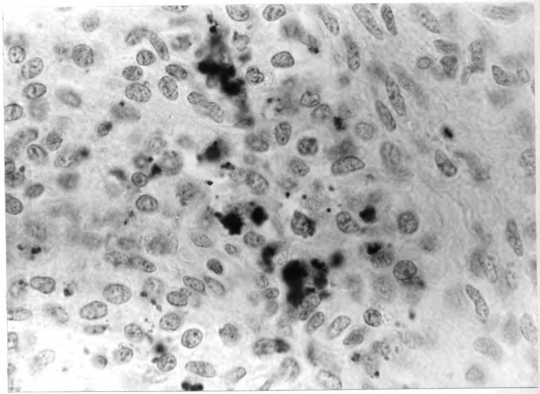


Figure 31. Iron-containing pigment in medulla of lymph node of farm-raised pig G-11

Gomori's iron stain. x 960

The Heidenhain aniline blue stain and the Foot's modification of Bielschowsky's stain failed to reveal any differences in the connective tissue framework of the lymph nodes, but the latter stain emphasized the reticular framework of the different parts of the node. As Figure 32 shows, there were very few fibers in the center of the lymphatic nodules, but these fibers formed a slight capsule around the exterior of the nodule. The fibers in the remainder of the cortex were relatively coarse and widely spaced, while those in the medulla were fine and more closely spaced.

The histological picture of the spleen was much more uniform than that found in the lymph nodes. As can be seen from Figure 33, the red pulp occupied a larger part of the volume of the spleen than did the white pulp. Mitotic activity was noted in the lymphocytes of the splenic corpuscles. Sometimes the mitotic figures were scattered in the relatively mature lymphocytes. At other times light-centered areas were found in one portion of the splenic corpuscle. These light centers were usually smaller than those seen in the lymph nodes, but their cellular make-up was quite similar.

No definite differences in the structure of the white pulp were noted between the various groups of animals.

The ellipsoids were well-marked in the red pulp of the spleen. Some eosinophils and neutrophils were noted in the red pulp, but their number was quite small in comparison to that found in the lymph nodes. A few small collections of cells similar to cells of the hematopoietic centers of the liver were seen in the red pulp along with an occasional megakaryocyte.

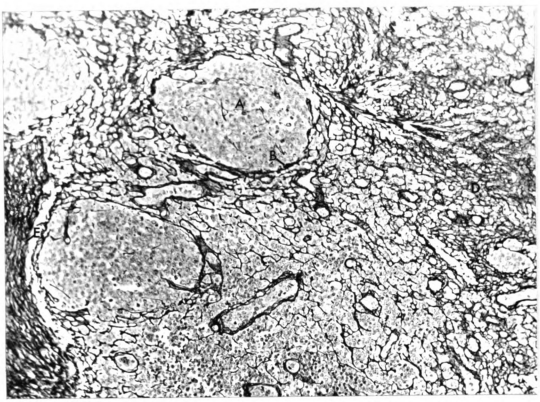


Figure 32. Reticular framework of prefemoral lymph node of farm-raised pig G-4

Foot's modification of Bielschowsky's stain for reticulum. x 215

A. Germinal center

B. Reticular fibers surrounding A

C. Cortex

D. Medulla

E. Trabecula

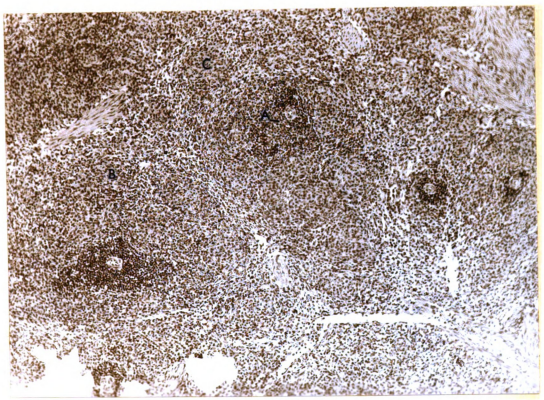


Figure 33. Spleen of germfree animal E-1

Hematoxylin and eosin. x 105

A. Splenic corpuscle with light-centered area

B. Red pulp

C. Ellipsoid

The amount of iron-containing pigment, presumably hemosiderin, found in the spleens was quite variable. Pigs of group A, group C, and group D, all raised artificially, exhibited quite significant amounts of this pigment in the cytoplasm of cells of the reticulo-endothelial system. The pigment granules were rather small when compared to those seen in the lymph nodes of pigs G-10 and G-11. Pigs of groups A and E, also raised artificially, had very little of this pigment in the spleen, and the same was true for the farm-raised pigs of group G. Figure 34 demonstrates this material.

The aniline blue and reticular stains revealed no significant differences between the various groups. The white pulp was sometimes surrounded by an accumulation of reticular fibers, but more often these fibers were arranged concentrically within the splenic corpuscle itself. The red pulp contained fibers similar to those seen in the cortex of the lymph nodes, but they were finer. The ellipsoids contained reticular fibers so small as to not be noticeable except under very high magnification.

4. Histology of the Liver

The outstanding histological difference between the livers of the various groups of pigs involved the connective tissue septa between the lobules. In the farm-raised pigs these septa were clear and distinct as in Figure 35 and were composed of from one or 2 to several rows of fibroblasts. There often appeared to be slight separations between the rows of fibroblasts and also between the fibroblasts and the hepatic cells themselves. This tended to emphasize the septa.

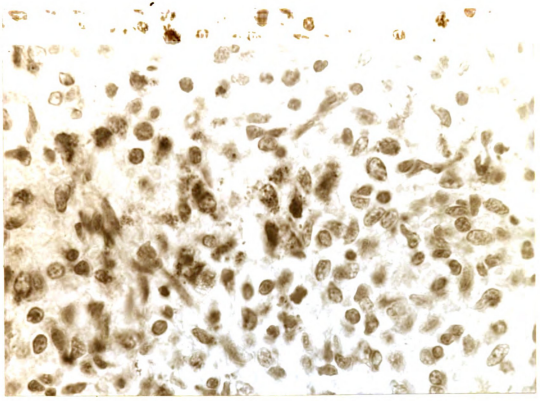


Figure 34. Iron-containing pigment in spleen of gnotobiotic pig A-1

Gomori's iron stain. x 960

The interlobular septa were less distinct in the artificially-raised pigs, and in many cases the only indications of the borders of the lobules were a somewhat more foamy appearance of the cytoplasm of the peripherolobular hepatic cells and a tendency for the cells to line up in rows in these areas. Figure 36 illustrates this arrangement.

The connective tissue stains emphasized this difference in the degree of development of the septa. Figures 37 and 38, both stained for reticulum, demonstrate the band of connective tissue in the farm-raised animal and the lining up of the reticular fibers between the hepatic cells at the edge of the lobule in the germfree animal.

The underdevelopment of the septa was less distinct in the group F animals (those raised in the presence of Achromobacteriaceae and an unidentified anaerobe). Although there was some development of the septa in these pigs, it was not as distinct as in the farm-raised animals.

Best's carmine stain revealed the presence of glycogen in all the sections stained. The glycogen appeared to be in heavier concentrations in the cells of the peripherolobular areas. The fat stains failed to demonstrate lipids in any of the sections stained. However, the hematoxylin and eosin stain of the liver of pig B-3 revealed numerous vacuoles diffusely spread throughout the lobules. These were quite suggestive of fatty changes, but formalin-fixed tissue was not available for lipid staining.

Most of the liver sections examined contained several collections of small, intensely-staining cells (Figure 39). These were found in the sinusoids and were presumed to be remaining evidence of the hematopoietic function of the fetal liver.

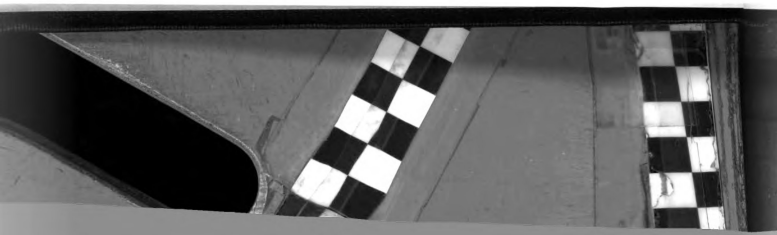


Figure 35. Liver of farm-raised pig G-9

Hematoxylin and eosin. x 105

Note clearly-defined interlobular septa.

Figure 36. Liver of germfree pig E-2

Hematoxylin and eosin. x 105

A. Lining up of hepatic cells in peripherolobular area

B. Hematopoietic centers

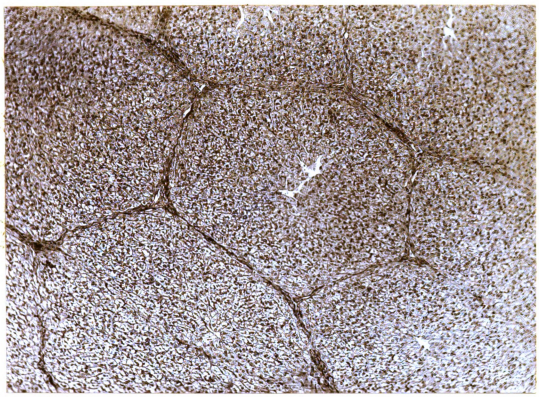


Figure 35

Figure 36

Figure 37. Liver of farm-raised pig G-8

Foot's modification of Bielschowsky's stain for reticulum. x 105

Note well-developed interlobular septa.

Figure 38. Liver of germfree pig E-2

Foot's modification of Bielschowsky's stain for reticulum. x 105

Note lining up of reticular fibers in peripherolobular areas.

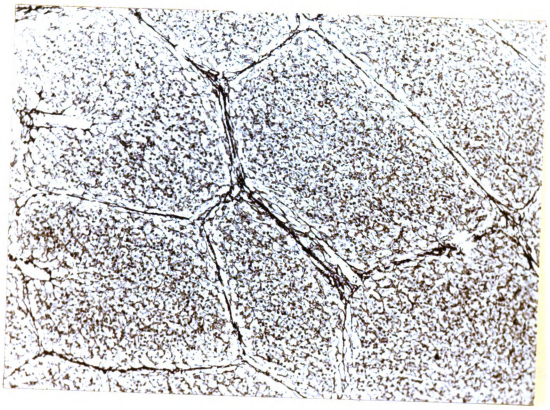


Figure 37

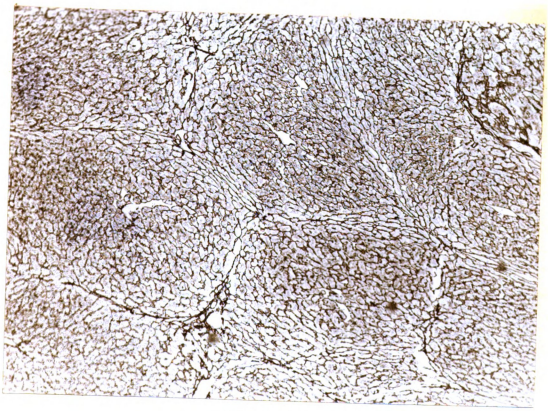


Figure 38

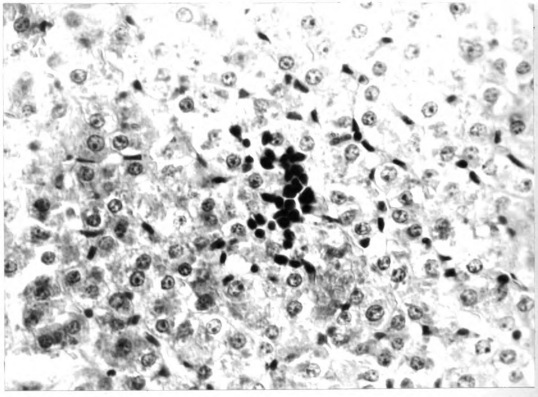


Figure 39. Hematopoietic cells in hepatic sinusoid of germfree pig E-1

Hematoxylin and eosin. x 700

5. Microscopic Evidence of Post-Mortem Degeneration in Germfree Pigs

Microscopic examination of the tissues of the 2 germfree animals left in the isolator after death, one for 3 days and one for 5 days, revealed varying degrees of degeneration. In the lymph nodes and spleens of both animals, the capsules and trabeculae were clearly visible (Figure 40), but no structural detail of the remaining tissue was evident except for pyknotic nuclei.

The adrenals appeared quite degenerate, with the cortex containing only debris, while the medulla contained a few pyknotic nuclei. The glandular arrangement of the thyroid could still be distinguished, but again the nuclei were quite pyknotic.

The glomeruli were still visible in the kidneys of both animals although they were more numerous in the animal dead 5 days. The convoluted tubules were difficult to see, but rows of pyknotic nuclei marked the straight tubules.

The hepatic cells of the pig dead 3 days showed degeneration as evidenced by pyknosis, karyorrhexis, and karyolysis (Figure 41). The outlines of the hepatic cells of the other animal were visible, but nuclei were not.

The myocardium of the animal dead 3 days showed little degeneration. The cross striations appeared to be absent, but in a few areas they were faintly visible. In the animal dead for 5 days the nuclei of the myocardium were showing evidence of pyknosis.

The erythrocytes within the lumen of the atria and in some other organs, were still quite distinct. In the lymph nodes, spleen, and adrenals this was not the case. Figure 42 shows the atrium from the animal dead 5 days.

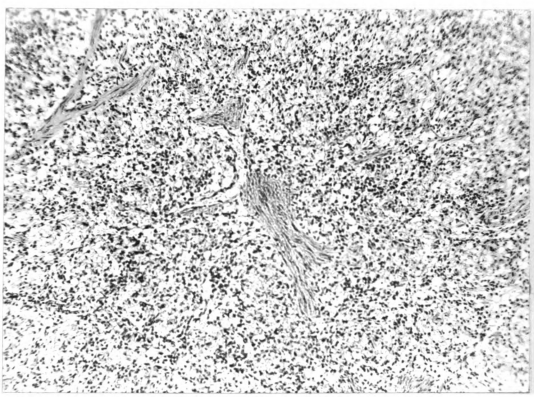


Figure 40. Spleen of germfree pig 3 days after death

Hematoxylin and eosin. x 175

Note pyknosis and lack of cellular detail in parenchyma, along with relatively good preservation of trabeculae.

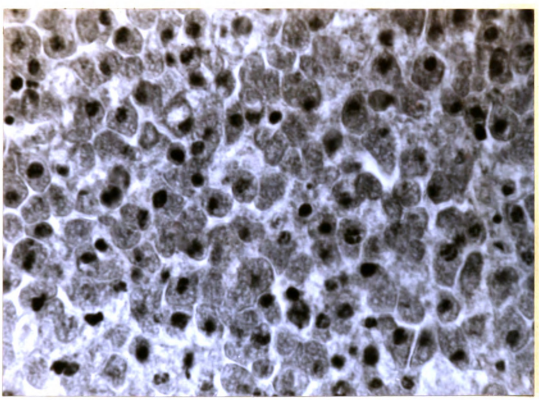


Figure 41. Liver of germfree pig 3 days after death

Hematoxylin and eosin. x 615

Note pyknosis, karyolysis, and karyorrhexis of nuclei.

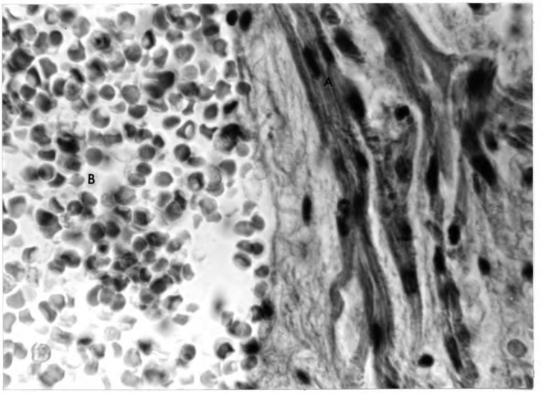


Figure 42. Atrium of germfree pig 5 days after death

Hematoxylin and eosin. x 615

A. Myocardial fibers with some pyknosis of nuclei.

B. Erythrocytes showing little evidence of degeneration.

IV. DISCUSSION

A. Body and Organ Weights

As was expected, the farm-raised pigs were significantly heavier than those raised artificially. The fact that the gnotobiotic pigs of the last group (group F) were heavier than those of previous groups is encouraging although the difference was not significant except when compared to group B at the 5 per cent level.

If one follows the suggestion of Gustafsson (1946-1947) that little value can be drawn from the comparison of germfree animals to control animals raised on the mother, it logically follows from these results that the absence of bacteria, in itself, did not significantly affect the body weights at 3 weeks of age.

The organ weights, as expressed in grams, correlated quite well with the body weights, and the farm-raised pigs of group G had heavier weights than the other groups in every organ weighed. In most cases this difference was significant at the 5 per cent level and often at the 1 per cent level. In the majority of organs the next heavier group of animals, group F, had the greatest organ weights of the artificially-raised animals.

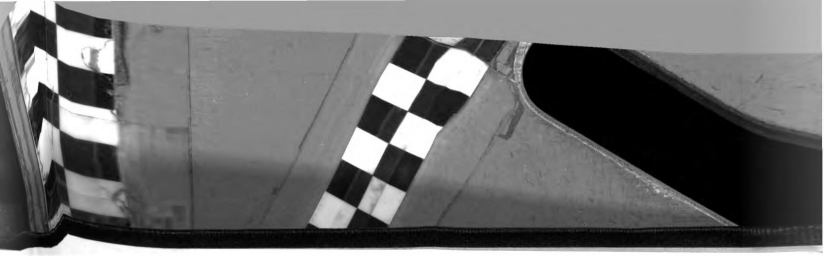
With regard to the relative organ weights, it was difficult to find any definite pattern. The mandibular nodes of the farm-raised pigs were significantly heavier (at the 5 per cent level) than were those of the germfree animals or the group C gnotobiotes. However, in the external inguinal nodes the germfree animals displayed the greatest weight. No differences existed in the prefemoral nodes. This does not follow the pattern found by Gordon (1959) and Glimstedt (1936) in which

there was an inhibition of development of the lymphatic organs. It may be of significance that the germfree conditions appear to have influenced the development of the mandibular lymph nodes (as measured by relative weights) to a greater degree than the nodes of the other groups studied. Since the mandibular nodes receive lymph from the tissues surrounding the oral cavity, they may be considered as having closer contact with the external environment than do the external inguinal or prefemoral nodes. It might therefore be expected that any influence of the germfree rearing would be reflected in the mandibular lymph nodes or the mesenteric lymph nodes rather than the other 2 groups studied. The feasibility of dissecting out and weighing the mesenteric lymph nodes should be investigated.

It was interesting to note that the kidneys of all artificially-raised animals were relatively heavier than those of the farm-raised group. This would suggest the desirability of a histological study to attempt to determine a reason for this increased weight. The possibility exists that the sterilized diet may contain some factor, perhaps toxic in nature, which causes renal hypertrophy or hyperplasia.

Another finding was that the relative adrenal weights of the germfree animals and the gnotobiotes of group A were significantly greater than those of the farm-raised animals. Gordon (1959) found the same with rats. If adrenal enlargement is associated with stress factors, it may be logically assumed that young pigs kept in a germfree environment are subjected to stress, even though this stress is of a different nature than that encountered by the farm-raised animal.

The lack of any clear-cut trend in the comparison of the relative organ weights of gnotobiotic animals and those raised on the



sterilized diet in open cages does not bear out the results reported by Gordon (1959), Glimstedt (1936), and Thorbecke and Benacerraff (1959). Before one could safely conclude, however, that the presence of bacteria, in itself, had no influence on relative organ weights, it would be necessary to examine considerably more animals.

B. Histology of the Lymph Nodes and Spleen

The histology of the lymph nodes and spleens of the pigs studied followed closely the descriptions found in the literature for this species. However, if one took the statement by Calhoun and Smith (1958) that the spleen of swine contains considerable white pulp and relatively little red pulp to mean that the former predominates over the latter in amount, this was not found to be true in the spleens studied. It is possible that their statement refers to the relative amounts as compared to other species. Another possibility is that age may influence the relative amounts of red pulp and white pulp, and that the latter may increase in amount as the animals grow older.

The finding of germinal centers or reaction centers in the lymph nodes and spleens of the germfree animals examined in this investigation is in contrast to the reports of Glimstedt (1936), Miyakawa (1959b), and Miyakawa et al. (1957) for the guinea pig. Instead, the results seem to be more comparable to the findings of Thorbecke (1959) on the germfree rat. The failure to demonstrate definite differences in the histological picture of these organs in gnotobiotic animals and those raised on sterilized diets in open cages suggested that any differences in the lymphatic system were probably not due to the presence or absence of bacteria. These differences seemed to depend more upon the diet and the method of rearing.

The large numbers of granulocytic cells, especially eosinophils, found in the lymph nodes may have been a carry-over from the hematopoietic function of these organs in the fetus. It is also possible that the eosinophils are related to antibody production as suggested by Speirs (1958). In contrast to the findings by Bouwman (1959), there was no correlation between the numbers of eosinophils and the rearing of the animals under conditions where they may have been more likely to harbor intestinal parasites. The finding of neutrophils in the germfree animals makes it seem unlikely that these cells were the result of bacterial infections.

Some of the small, spherical, intensely-staining cells found in the lymph nodes and spleens could have resulted from degeneration of lymphocytes as described by Maximow (1927). However, the appearance of a definite rim of eosinophilic cytoplasm in many cells, along with the locations of these cells, makes it improbable that very many of them came from lymphocytes. Tompkins (1958, 1959, and 1960) has described atypical small lymphocytes whose nuclei had an undue affinity for nuclear stains. From the study of these cells, she concluded that they were stages in the transformation of small lymphocytes to plasma cells. The virtual absence of mature plasma cells in the lymph nodes studied in this investigation would indicate that few if any of these darkly-staining cells corresponded to the cells described by Tompkins. The most logical explanation may be that these cells were remnants of the hematopoietic function of the lymph nodes and spleen in the embryo and that these remaining cells were no longer active but were undergoing degeneration.

The absence of plasma cells in the lymph nodes and spleens of the pig is difficult to explain in view of the emphasis placed on the presence of these cells in lymph nodes and spleens of other species by various authors. It is possible that the use of special staining techniques would have demonstrated plasma cells, or at least immature forms. The present findings are in agreement with those of Bouwman (1959) in this respect.

The iron-containing pigment seen in the lymph nodes of pigs G-10 and G-11 was probably related to the administration of the injectable iron compound. No logical explanation can be offered for the variability in the amount of iron-containing pigment found in the spleens.

C. Histology of the Liver

The stage of development of the interlobular septa in the artificially-raised pigs seems to fit into the pattern of the formation of these structures as described by Johnson (1916-1917, 1919) and White (1938-1939). It appeared that the septa were at an earlier stage of development than in the livers of the farm-raised animals. This, again, seemed to be more related to the diet and rearing techniques than to the presence or absence of bacteria. No evidence was found to support or deny the contention of Elias et al. (1954) that these septa represent hepatic cirrhosis.

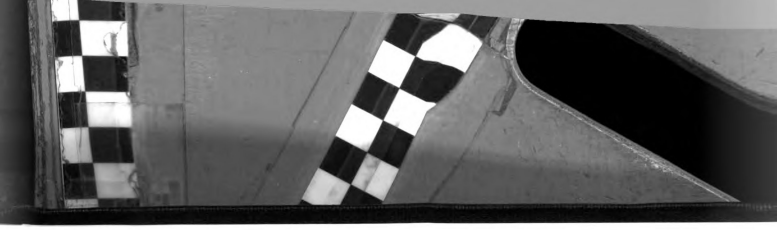
The presence of hematopoietic cells in the sinusoids of the liver was not unexpected, as such have been previously described. Maximow and Bloom (1957) stated that the hematopoietic activity of the liver gradually subsides so that only small foci of erythroblasts can be found in the liver of the newborn. These, too, soon disappear.

The vacuoles found in the liver of pig.B-3 may have been the result of fatty degeneration due to anemia, since the hemoglobin of this animal was rather low. The diarrhea present in this animal may also have been a factor.

D. Microscopic Evidence of Post-Mortem Degeneration in Germfree Pigs

The degree of degeneration found in these 2 pigs, kept at 90° F. for 3 and 5 days, respectively, after death, was much less than would ordinarily be expected in animals raised in contact with bacteria. The variation in the rate of degeneration between different organs may have been related to the enzyme content of these organs. This may also account for the fact that the erythrocytes remained intact in some organs longer than in others.

Although the evidence is limited, the results of the present work indicate that there are no marked differences between gnotobiotic and farm-raised pigs. The pigs kept under these conditions of artificial rearing did not show as great a growth rate as did the pigs raised on the sow. In addition, there was some evidence that the lymph nodes were not as highly developed and the interlobular septa of the liver were at an earlier stage of development in the artificially-reared animals as compared to the farm-raised pigs.

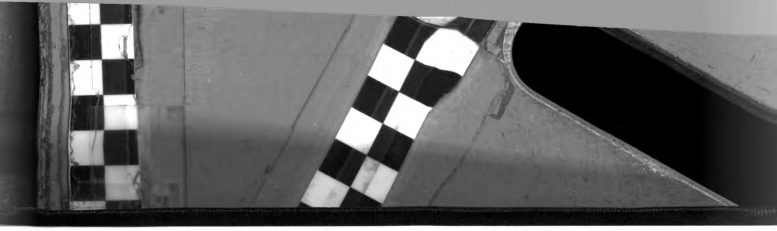


V. SUMMARY

A comparison of the body weights of the 18 pigs discussed in Part One and 11 animals raised by the sow to an age of 3 weeks under ordinary farm conditions revealed a significantly higher value for the latter animals.

The weights of various body organs, including 3 groups of lymph nodes, heart, lungs, liver, spleen, kidneys, pancreas, thyroid, and adrenals, in general correlated with the body weights. When these organ weights were expressed as relative weights (milligrams of tissue per 100 gm. body weight), no definite pattern was found in comparing the various groups of animals. The mandibular lymph nodes of the farm-raised pigs were significantly heavier than those of the germfree animals, but this relationship did not hold true for the relative weights of the other lymph nodes weighed. The kidneys of all artificially-reared animals were relatively heavier than those of the farm-raised pigs, and the relative adrenal weights of the germfree pigs and the gnotobiotes reared in the presence of Staphylococcus aureus were greater than those of the farm-raised animals.

Histological examination of lymph nodes from the various groups of animals revealed extreme variability in the degree of fusion of the smaller nodes and the relative amounts and location of cortex and medulla. In general the cortex was centrally located, while the medulla was found at the periphery of the node. Light-centered nodules, corresponding to the usual germinal or reaction centers were found in the lymph nodes of the germfree animals, but these did not appear as large or numerous as in the farm-raised pigs. The germfree pigs usually



displayed more solid-centered primary nodules than did those animals raised under farm conditions. The histological picture of the lymph nodes seemed to be influenced more by the diet and rearing conditions under which the animals were kept than by the presence or absence of bacteria in the environment.

Numerous immature eosinophils, neutrophils, and small, darkly-staining cells resembling hematopoietic cells were found in the lymph nodes. Iron-containing pigment was found in the lymph nodes of 2 farm-raised animals given parenteral iron injections at 2 or 3 days of age. Typical mature plasma cells were not noted in the lymph nodes of any of these animals.

No significant differences were found in the histology of the spleens of the various groups of animals, except for the fact that the artificially-reared animals appeared to have somewhat more iron-containing pigment in this organ. Light-centered areas were found in the splenic corpuscles of the various groups.

The hepatic interlobular septa of the artificially-reared animals were less well-developed than in the farm-raised pigs, and in many cases the only indication of the borders of the lobules was a lining up of the hepatic cells and reticular fibers in this area. Hematopoietic centers were found in the livers of all groups of animals.

Examination of 2 germfree pigs left in the germfree environment 3 and 5 days, respectively, after death revealed less degenerative changes than would be expected under conditions in which the animals were in contact with bacteria. Of the tissues examined, the myocardium and erythrocytes showed the least degeneration, the lymphatic tissue and the adrenals the most.

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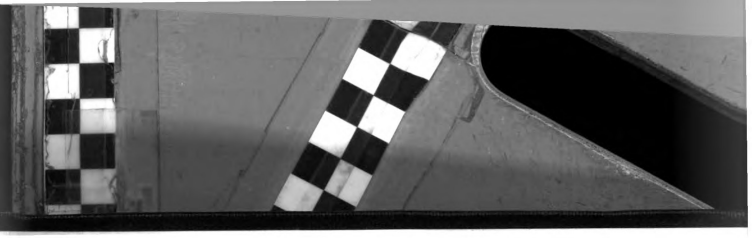

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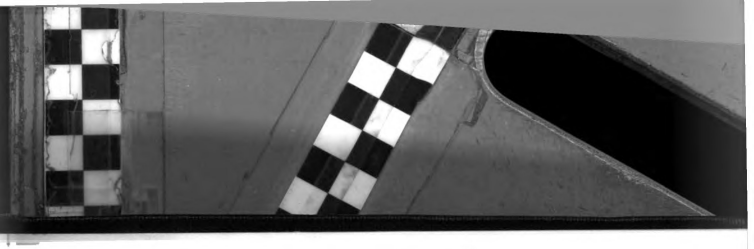

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APPENDIX

Necropsy Numbers of Animals

Group	Pig No.	Necropsy No.
A	1	D6261
A	2	D6262
A	3	D6263
A	4	D6264
B	1	D6249
B	2	D6250
B	3	D6251
C	1	E647
C	2	E648
C	3	E649
D	1	E650
D	2	E651
E	1	E1704
E	2	E1705
F	1	E3739
F	2	E3740
F	3	E3741
F	4	E3742
G	1	E723
G	2	E724
G	3	E1331
G	4	E1332
G	5	E1812
G	6	E1985
G	7	E1986
G	8	E1987
G	9	E1988
G	10	E3770
G	11	E3771