SOME MICROMORPHOLOGIC FEATURES OF THE INTESTINAL TRACT OF THE YOUNG PIG

I. MITOTIC ACTIVITY OF THE EPITHELIUM

II. A MICROMORPHOLOGIC COMPARISON OF GNOTOBIOTIC AND FARM-RAISED PIGS

Thesis for the Degree of Ph. D.

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Donald A. Schmidt

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This is to certify that the

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Some Micromorphologic Features of the Intestinal Tract of the Young Pig.

I. Mitotic Activity of the Epithelium.
II. A Micromorphologic Comparison of Gnotobiotic and Farm-Raised Pigs.

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ABSTRACT

SOME MICROMORPHOLOGIC FEATURES OF THE INTESTINAL TRACT

OF THE YOUNG PIG

I. MITOTIC ACTIVITY OF THE EPITHELIUM

II. A MICROMORPHOLOGIC COMPARISON OF GNOTOBIOTIC

AND FARM-RAISED PIGS

by Donald A. Schmidt

I. Mitotic Activity of the Epithelium

Utilizing colchicine, which arrests mitosis in metaphase, the mitotic activity in 9 regions of the digestive tract was studied. Thirty-six 5-week-old Yorkshire pigs of both sexes were utilized in this study. The colchicine was injected intraperitoneally into the pigs at the rate of 0.25 mg./kg. body weight after a 6-hour fast. Four hours later the pigs were killed and samples of tissues fixed in Heidenhain's SUSA fixative, after which paraffin sections were made. Stain was Harris' hematoxylin and eosin. Fifteen hundred resting epithelial cells plus the cells in mitosis were counted for each level of the intestine studied.

The study showed that there was no diurnal variation in mitotic activity for the levels of the intestine studied. The percentages of epithelial cells in mitosis 4 hours following colchicine injection were: gastric cardia, 2.50; gastric fundus, 2.22; gastric pylorus, 2.88; duodenum, 3.60; jejunum, 4.45; ileum, 3.68; cecum, 5.01; spiral colon, 5.21; and terminal colon, 4.79.

The numbers of days required for complete replacement of the lining epithelium of various levels of the digestive tract are as follows: gastric cardia, 6.66; gastric fundus, 7.50; gastric pylorus, 5.78; duodenum, 4.63; jejunum, 3.74; ileum, 4.52; cecum, 3.27; spiral colon, 3.20; and terminal colon, 3.48.

The length of the mitotic cycle varied from 38 to 52 minutes for different levels of the intestinal tract.

II. A Micromorphologic Comparison of Gnotobiotic and Farm-raised Pigs

Baby pigs were obtained from pregnant sows at term by hysterectomy and the uterus transferred through an antiseptic lock into a sterile plastic surgical isolator where the pigs were removed. They were then transferred to cages in a sterile plastic rearing isolator. The pigs were fed sterile cows' milk with added vitamins and minerals. In 2 experiments monocontaminated pigs were obtained and in 1, the pigs were germfree. The contaminants in the first 2 experiments were Staphylococcus aureus and a Bacillus sp. respectively. Growth responses were poor, probably due to unsatisfactory nutrition.

Following a period of 17-21 days, the pigs were euthanized and tissues saved for study. Little morphological difference was noted between the monocontaminated pigs and the germfree pigs.

The gnotobiotic (monocontaminated and germfree) pigs had less connective tissue in the submucosa of the intestines and it was more loosely arranged than that in the farm-raised pigs. The amount of lymphatic tissue in the small intestines of the gnotobiotic pigs was much less than that seen in the farm-raised pigs.

Marked vacuolization of the epithelial cells of the small intestine and a golden-brown pigment in the epithelial cells of the cecum were present in the gnotobiotic pigs and their littermate controls. These were not present in the farm-raised pigs.

Post-mortem decomposition is greatly retarded in germfree animals. Intact erythrocytes were present in blood vessels of the cecum from a pig which was allowed to remain in a sterile environment at room temperature for 3 days following death.

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PART I. MITOTIC ACTIVITY OF THE EPITHELIUM

PART I. MITOTIC ACTIVITY OF THE EPITHELIUM

INTRODUCTION

Cells which constitute the tissues of the intestinal tract do not grow to maturity and then become static. Ramond, as early as 1904, observed the desquamation of the epithelium which occurs in the small intestine incident to the digestion of food. There is constant activity of the epithelial cells with new cells being formed in some parts of the intestinal glands and the old or worn-out cells being lost in areas designated by Leblond and Stevens (1948) as "cell extrusion zones". Fig. 1-1 is a photograph of the ileum of a normal 5-week-old pig from the research to be reported in this thesis showing this "cell extrusion zone". The epithelial cell at the very tip of the villus is about to be extruded and lost into the lumen of the intestine.

Our basic knowledge of enteric disturbances is far from complete, yet these disturbances are frequently encountered by the practicing veterinarian. More research is needed to supply the necessary information for proper diagnosis, treatment, and control.

One might question whether there is any difference between the cellular activity of the intestinal epithelium in the "normal" pig and in the pig with enteritis. Does the presence of the increased peristalsis often present in enteritis have any effect on the rate of replacement of this epithelium? Do the different areas of the intestinal tract of the baby pig have the same or similar cellular activity, or do they vary greatly? These are interesting questions. Their answers are

Fig. 1-1.--- Tip of villus of ileum of a normal baby pig showing the cell extrusion zone as described by Leblond and Stevens (1948). H & E X 640.

either unknown or incompletely known. Their elucidation depends upon knowing the conditions which exist in the normal pig concerning the cellular activity of this epithelium.

Similar questions might be asked about the effect of the bacterial flora of the intestine on the integrity and health of the epithelial lining.

This thesis reports research on a limited segment of the basic studies necessary to a better understanding of the digestive tract. It deals primarily with the intestinal epithelium of the apparently normal pig. Part I is concerned with the mitotic activity of the epithelium of the digestive tract of the pig raised under typical farm conditions, and part II deals with the micromorphology of the digestive tract of pigs raised under gnotobiotic and germfree conditions and a comparison is made to the digestive tract of pigs raised under farm conditions.

REVIEW OF LITERATURE

The study of mitotic activity of the intestinal tract is facilitated by employing a drug which will stop or inhibit mitosis at some specific phase of karyokinesis. Such drugs are colchicine and trypaflavin (Bucher, 1939) and aminopterin, fluoride, cyanide, chloroacetophenone, and urethane (Hughes, 1950).

Since the work of Dustin (1934) and Lits (1934) on the use of colchicine for the purpose of producing metaphasic arrest in animals, the number of papers dealing with the use of this drug in the study of mitotic activity has increased enormously. The bibliography on colchicine compiled by Eigsti and Dustin (1947, 1949) contains about 2,000 references. Actually, as pointed out by Eigsti et al. (1949), the credit for first observing the action of colchicine on mitosis should go to Pernice (1889) who described in considerable detail the mitotic picture seen in the stomach of a dog to which tincture of colchicum bulbs had been administered. Why the delay occurred in applying this knowledge concerning the action of colchicine to cell study is not known, but a period of 45 years elapsed before Dustin and Lits were to again use the drug for this purpose. The book by Eigsti and Dustin (1955) covers most of the important work regarding the use of colchicine as a mitotic inhibitor.

Eigsti and Dustin (1955) state that colchicine acts on the cell in the mitotic cycle by inhibiting the formation of a spindle at prophase, precludes a nuclear mitosis, delays chromosome separation, inhibits daughter nuclei and effectively blocks cleavage processes. This causes an accumulation of cells which are in metaphase. Tennant and Liebow (1940) have shown with tissue cultures of mammary carcinoma of mice that this accumulation of metaphasic mitotic figures was apparent within 30 minutes after colchicine was introduced into the culture. Törö and Vadasz (1935), in their study of cardiac muscle in tissue culture, reported that the effect of colchicine on mitosis was apparent within one hour after adding it to the culture. These authors suggest that the reason for metaphasic arrest is that at this stage the cell is the most sensitive to external influences probably due to the lack of a nuclear membrane at this phase of karyokinesis. Increased metabolic activity at this stage of the process might also be a factor in the increased sensitivity.

Sentein (1943), Hughes (1950), Tennant and Liebow (1940), Bucher (1939), Ludford (1936), Verne and Vilter (1940), Brues (1936), and Buschke <u>et al</u>. (1943) conclude that the effect of colchicine is purely inhibitory, acting at metaphase, and that it does not influence the rate at which cells enter mitosis.

Since colchicine disrupts normal mitosis by interfering with spindle formation it might be suspected that there would be some morphological alterations in the mitotic figures produced at metaphase. This is indeed true. Eigsti and Dustin (1955) call attention to the star type mitotic figures, the distorted star types, the exploded type, and the ball type. Hughes (1950) mentions that following metaphasic arrest there is "clumping" of the chromosomes which then cease to be separately distinguishable. Miszurski and Doljanski (1949) call attention to pathological mitotic figures following the use of colchicine in their studies of mouse sarcoma #180, and in rat liver following subtotal hepatectomy.

The exact nature, however, of the mechanism of colchicine in inhibiting mitosis is not known. Chargaff et al. (1948) have shown that the inhibitory effect of colchicine at metaphase in Alium cepa was prevented by meso-inositol. The mechanism of this inhibitory action of inositol was not explained.

While colchicine is useful in stopping mitosis at a definite and easily ascertainable stage of the mitotic cycle thus enabling the determination of the percentage of resting and dividing nuclei, other important factors must be considered to make this enumeration valid. Some cells show periodicity in their mitotic activity depending upon the time of day. In other words, there is a diurnal variation in cellular activity. Thus, Blumenfeld (1938a, 1938b) shows that there is a significant diminution in the rate of mitosis in the submaxillary salivary gland of the rat between 2:00 A. M. and 4:00 A. M. with a fairly constant level of mitotic activity during the rest of the 24-hour period. The maximum activity of the epidermis occurred at 8:00 A. M. to 10:00 A. M. and that of the renal cortex at 2:00 P. M. to 4:00 P. M. The minimal activity occurred at 6:00 P.M. to 12:00 midnight and from 10:00 P.M. to 12:00 midnight respectively. Mühlemann et al. (1955) found a high point of mitotic activity of the thyroid and oral epithelium of the rat during the day and a low point of activity at night. The adrenal cortex, on the other hand, had a high mitotic activity during the night and a low activity during the day. Klein and Geisel (1947), in studies of the intestinal gland cells of the rat and mouse, found two periods of high activity, at 12:00 noon and at

4:00 A. M., and two periods of low activity, at 8:00 A. M. and at 4:00 P. M. Klein (1951) also mentions the presence of two periods of high activity and two periods of low activity of the epithelial cells of the duodenal mucosa of the rat and mouse. Mühlemann et al. (1956), on the other hand, were unable to demonstrate any significant difference between the rate of mitoses in the duodenum of the rat at different times of the day.

Another factor that may cause variation in the mitotic activity is the nutritional state of the animal and the activity of the intestines incident to the digestive processes. Although Klein and Geisel (1947) found no relationship between the taking of food and its digestion and the mitotic activity of the digestive tract of the rat and mouse, other investigators reported such a relationship in their studies of tissues other than from the digestive tract. Leduc (1949), studying the mitotic activity of the liver of weanling mice, noted that this activity disappeared on prolonged fasting and reappeared on refeeding. The amount of protein in the diet was a factor which determined the time of appearance of the peak mitotic activity. Blumenthal (1950), studying the cyclical variation in mitotic activity in guinea pigs fasted for 24 hours and sacrificed at intervals during the next 24 hours, showed that there was a progressive decline in the number of mitotic figures in the thyroid and parathyroid, while in the adrenal cortex there was a progressive rise in the number of cells undergoing mitosis. Bullough and Eisa (1950) concluded that nutrition must be considered when studying mitotic activity of cells. In their work with mitosis in the epidermis of mice, they found a marked depression of activity when the feed intake of the animal was reduced from 80% to 70% of that normally eaten.

Mitotic activity has been studied in many different tissues of the body by using colchicine. Some recent studies have been concerned with the lung (Bertalanffy and Leblond, 1953), epidermis (Bullough, 1949), sebaceous glands (Bertalanffy, 1957), corneal epithelium (Buschke _et al_., 1943), and urinary bladder (Leblond, 1955). Most of the studies of the intestinal tract have been with the rat and mouse, some with the guinea pig, and a few with dogs and cats. Literature concerning mitotic activity in the pig was not found. Two reports concerning the use of colchicine to alter the chromosome number in the pig are available (Haggqvist, 1951, and Melander, 1951).

Hunt (1952), in studying the activity of the gastric glands in mature rats, concluded that there was no diurnal rhythm in the cellular activity. The average number of mitoses was 15 per lineal millimeter of section cut at 6 microns with a variation of from 16 to 82 mitoses per millimeter in different parts of the stomach. Stevens (1952) and Stevens and Leblond (1953) determined the renewal time of the surface epithelial cells of the stomach of the rat to be 2.84 days and that of the mucous neck cells to be 6.52 days. Bertalanffy (1960) found that the renewal time for the epithelium of the cardiac region of the stomach of the rat was 9.1 days. In the cat, Grant (1945) studied the replacement of the stomach epithelium that had been practically denuded of cells by treatment with ethyl alcohol. As long as the underlying gland cells were not damaged, the denuded surface epithelium was replaced within a few hours. He concluded that this ability to replace itself was a fundamental property of the gastric mucosa.

The mucosal epithelium of the small intestine has also been studied in detail in the smaller animals. Stevens and Leblond (1947) determined the half life of the epithelium of the ileum of the rat to be between 1 and 2 days. The half life of the epithelium of the duodenum was somewhat longer. The cells of the intestinal epithelium originate from mitoses that take place throughout the length of the Lieberkühn glands. The cells are gradually pushed along the sides of the villi and appear to be eliminated at the tips of the villi. Leblond and Stevens (1948) determined the renewal time for the epithelial cells of the rat duodenum to be about 1.6 days and that of the epithelial cells of the ileum to be about 1.4 days. Bertalanffy (1960) found the renewal time of the jejunal epithelium of the rat to be about 1.3 days. McMinn (1954), in studying the rate of renewal of the duodenal and ileal epithelium of the cat, showed that in the cat not treated with colchicine, 0.95% of the epithelial cells were in mitosis. In cats to which colchicine had been administered 5 hours before, 3.77% of the epithelial cells were in mitosis. This means that, in the 5-hour period, 3.97 mitotic cycles had taken place and that the time for one mitotic cycle was, therefore, 1.26 hours or 75 minutes. The renewal time calculated from these figures is 5.53 days. McMinn and Mitchell (1954), in their studies of healing of artificially produced lesions of the small intestine of the cat, also determined that 3.77% of the epithelial cells of the small intestine were in mitosis 5 hours after the administration of colchicine. McMinn (1958) found that, in the dog, 5 hours after the administration of colchicine, 17.12% of the epithelial cells of the small intestine were in mitosis. Comparing this to the 3.77% of the cells in mitosis in the cat and assuming that the duration of the mitotic cycle was the same in both the dog and the cat, he concluded that the colchicine appears to stimulate the epithelial cells to enter mitosis. He noted increasing numbers of mitoses with increasing dosage of colchicine in the dog and at the dose of 0.25 milligrams of colchicine per kilogram of body weight which was the amount administered, toxic manifestations were observed.

Bertalanffy (1960) found that the renewal time of the epithelium of the colon of the rat was about 10 days and the renewal time for the rectal epithelium in the same animal was about 6.2 days.

It can be seen that this important aspect of intestinal dynamics has been studied primarily in the laboratory animal. Studies in the pig are conspicuous by their absence. The complete elucidation of enteric diseases depends upon the better understanding of the basic changes taking place in the cells making up the intestinal tract.

MATERIALS AND METHODS

A total of 36, five-week-old Yorkshire pigs from 6 litters were used in this study. They were of both sexes. The animals were allowed to nurse the dam and were provided with supplementary feed via creep-feeding facilities as soon as they showed a desire for it. Supplementary iron was provided by the oral administration of a commercial iron, copper, and trace mineral preparation.

The experimental animals were observed frequently and always appeared to be in good health. Blood samples taken at or near the termination of the experiment were normal in the level of hemoglobin and in the number and kinds of leukocytes present.

Since the degree of activity of the digestive epithelium might be dependent upon the stage of the digestive process, a procedure was established to standardize this aspect. All animals were removed from the environs of the dam exactly 6 hours preceding the administration of the colchicine. They were then placed into an empty stall which was devoid of all bedding and were provided with neither feed nor water for the duration of the experiment. At the termination of the 6-hour fast period they were weighed and the calculated dose of colchicine was injected intraperitoneally. The colchicine employed was USP colchicine obtained from Nutritional Biochemicals of Cleveland, Ohio. The colchicine was used without purification although it had been shown by Horowitz and Ullyot (1952) that the USP grade might not be entirely pure. It was prepared for injection by making a solution in distilled water in the concentration of 1 milligram of USP colchicine per milliliter of solution. Aseptic technique was employed in preparing the solution and sterile bottles were used for mixing the colchicine with the freshly distilled, though nonsterile, water.

Five animals were used in a preliminary study to determine the amount of colchicine to use in arresting mitosis. Since all cell division would not be arrested if an insufficient amount of colchicine were used and since too large a dose might cause too much damage to the mitotic figures or prove toxic to the animal, it was decided that a gradation of doses be injected into several animals and the dose which gave the highest number of distinguishable mitotic figures in a stated period of time without significant evidence of toxicity would be the amount used in the study. The dose for the rat and mouse was 1 milligram per kilogram of body weight in investigations by Tier et al. (1952) and Bertalanffy and Leblond (1953). One milligram per kilogram of body weight was used for the pigeon by Leblond and Allen (1937). For the cat the dosage was 0.25 milligrams per kilogram of body weight in work by McMinn (1954,1958) and McMinn and Mitchell (1954). A dose of 0.25 milligrams per kilogram of body weight gave some toxic manifestations in the dog although still inhibiting mitosis as reported by McMinn (1958). Thus, it appeared that the amount of colchicine needed per kilogram of body weight varied inversely with the size of the experimental animal.

It was decided that the dosage in the preliminary trial should start at 0.25 milligrams per kilogram of body weight. The 5 pigs were accordingly injected intraperitoneally with the drug: 2 at the rate of 0.25 milligrams, 1 at the rate of 0.5 milligrams; and 2 at the rate

TABLE 1-1.---Determination of dosage of colchicine. Colchicine injected 4 hours before. Dosage is that giving the greatest number of mitotic figures in the stated period.

pig number	sex	colchicine dosage mg/kg body weight	percent epithelial cells in mitosis in duodenum
23	F	0.25	5.23
27	M	0.25	4.28
26	M	0.50	1.81
19	M	1.00	1.06
22	M	1.00	2.21

of 1.0 milligram per kilogram of body weight respectively. This is shown in Table 1-1. The animals were killed 4 hours following injection with colchicine. From the results of this preliminary work a dosage of 0.25 milligrams of colchicine per kilogram of body weight was decided upon as the dosage to be employed in this study.

Another factor of importance in this work is the length of time to wait following the injection of the colchicine before selecting tissues for examination. This is of importance because if the interval between injection of colchicine and the selection of tissue is too long, there will be disintegration of the mitotic figures followed by their complete disappearance. This was reported by Bertalanffy and Leblond (1953) and was apparent in the work of Dustin (1934). Seven pigs were accordingly injected intraperitoneally with colchicine at the rate of 0.25 mg./kg. of body weight and killed at intervals varying from 1 to 8 hours following the injection. Examination of the tissues from the duodenum showed an increase in mitotic figures up to 4 hours with variability at 5, 6, and 8 hours. In the cecum, an increase in mitosis was noted up to 6 hours with a decrease at 8 hours. (See Table 1-2).

TABLE 1-2.---Determination of time required for colchicine effect using dosage of 0.25 mg./kg. of body weight.

pig number	sex	hours post-injection	percent cells in mitoses duodenum	cecum
30	M	1	0.36	0.96
36	M	2	1.14	4.64
32	M	3	1.21	16.23
33	M	4	5.62	10.93
34	M	5.3	1.04	1.19
35	M	6	1.40	18.94
31	F	8	5.08	11.79

Bertalanffy and Leblond (1953), in their study of the alveolar cells in the lung of the rat stated that a time for collection of tissues should be selected at which the number of mitoses arrested by colchicine is still increasing. Four hours following the injection of colchicine was accordingly selected as the time that the tissues would be collected.

It had been demonstrated by various authors (Blumenfeld, 1938a, 1938b; Hunt, 1952; Klein and Geisel, 1947; Klein, 1951; Leblond and Stevens, 1948; Mühlemann et al., 1955) that a diurnal variation existed in the mitotic activity of some cells. It was not known whether a diurnal variation existed in the mitotic activity of the epithelium of the digestive tract of the pig. To determine this it was necessary to study pigs at different periods of the day. Twenty-four pigs were divided into 4 groups. Each group contained 6 pigs of which 4 were injected intraperitoneally with colchicine at the rate of 0.25 mg./kg. body weight after a 6-hour fast. Two pigs were not injected with colchicine and served as controls. Four hours following the injection of the colchicine, the pigs were anesthetized by intravenous injection of sodium pentobarbital and exsanguinated. The protocol of this part of the experiment is given in Table 1-3.

TABLE 1-3.---Protocol for determination of the mitotic activity of the epithelial cells of the intestinal tract.

pig number	sex	body wt. (kg.)	time off feed	mg. colchicine I-P*	time of injection	time of necropsy
46	F	11.36	Noon	2.85	6:00 P.M.	10:00 P.M.
52	F	7.05	"	1.77	"	"
5	M	6.81	"	1.71	"	"
11	F	9.09	"	2.28	"	"
56	M	7.73	"	--	---	"
42	F	9.32	"	--	---	"
43	M	10.45	6:00 P.M.	2.62	Midnight	4:00 A.M.
51	F	6.81	"	1.70	"	"
6	M	9.09	"	2.28	"	"
7	F	9.09	"	2.28	"	"
3	M	9.09	"	--	---	"
41	F	10.45	"	--	---	"
44	F	9.09	Midnight	2.28	6:00 A.M.	10:00 A.M.
54	F	7.50	"	1.88	"	"
2	M	7.95	"	2.00	"	"
8	M	7.27	"	1.82	"	"
50	F	7.95	"	--	---	"
4	M	10.00	"	--	---	"
40	M	10.00	6:00 A M.	2.50	Noon	4:00 P.M.
53	F	7.27	"	1.82	"	"
1	M	7.73	"	1.94	"	"
9	F	5.90	"	1.48	"	"
45	F	12.05	"	--	---	"
55	M	7.27	"	--	---	"

* intraperitoneally

After exsanguination the intestinal tract was removed and the intestine separated from its mesenteric attachment and measured. Tissues were saved from the stomach, including the cardiac, fundic, and pyloric portions, from 10 equidistant parts of the small intestine, from 5 equidistant parts of the spiral colon and from 3 levels of the terminal colon. All tissues were removed shortly after the death of the animal and fixed in Heidenhain's SUSA fixative (Davenport, 1960).

Figure 1-2 shows a diagrammatic representation of the digestive tract of the pig. The numbered designations are the approximate areas from which the tissue samples were taken. Tissues used in this study are the cardiac (5), fundic (6), and pyloric (7) areas of the stomach, the duodenum (8), the jejunum (13), the ileum (17), the cecum (20), the spiral colon (23) and the terminal colon (27). The tissues were embedded in paraffin according to the usual histopathological practices, cut at 6 microns, and stained with Harris' hematoxylin and eosin (Davenport, 1960).

A minimum of 1,500 resting nuclei was counted from each tissue using the oil immersion objective. A Howard mold-counting disc was placed in the eyepiece of the binocular microscope to facilitate counting. The number of mitotic figures seen while counting the 1,500 resting nuclei was added to the resting nuclei and the percentage of cells in mitosis calculated.

Nuclei were counted in glands only if the gland were cut longitudinally throughout its entire length. The reasons for this are two-fold. First, it was observed that the major number of mitotic figures were in the lower portion of the gland and second, there was a tendency for the mitotic figures to be found closer to the lumen of the gland than were the resting nuclei of the same gland. (See Figures 1-3 and 1-4). If glands had been included that were not cut longitudinally throughout their entire length, there would have been a greater likelihood of an inaccurate representation of the distribution of the mitotic figures.

Once a count was begun in a specific gland, the epithelial cell nuclei in the entire gland were counted even though a total of 1,500 resting nuclei was reached soon after beginning the count in the gland.

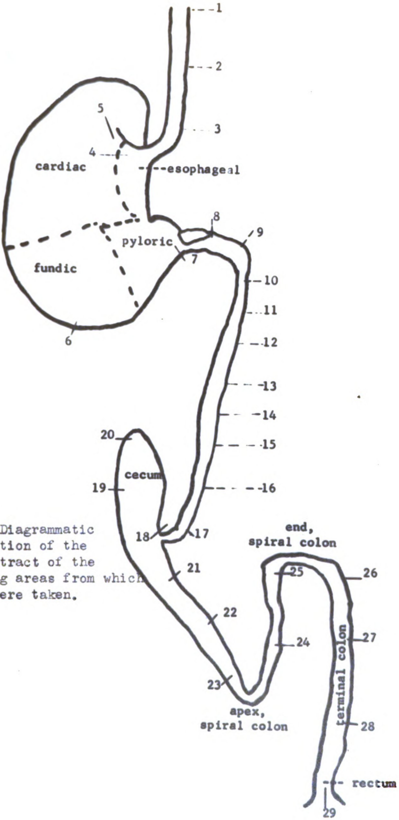

Fig. 1-2 Diagrammatic representation of the digestive tract of the pig showing areas from which sections were taken.

Prophase nuclei were included with the resting nuclei and no anaphase or telophase nuclei were seen in tissues from animals injected with colchicine. In the control pigs, the anaphase and telophase nuclei were included with the metaphasic nuclei in determining the number of cells in mitosis.

Analysis of variance using the F test as described by Batson (1956) was applied to the results of this study.

Fig. 1-3---Mitotic figures in glands in cecum of pig injected with colchicine four hours before. Note that the mitotic figures are located in the cytoplasm of the cell nearer the lumen of the gland than are the resting nuclei. Note also the round shape of the nuclei, the so-called ball nuclei, which is evidence, according to some authors, for the toxic action of colchicine. H & E X 640.

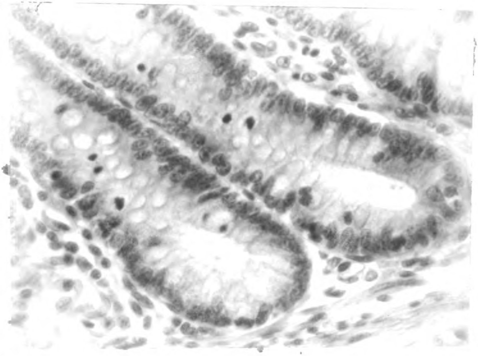

Fig. 1-4---Mitotic figures from cecum of pig which had not been injected with colchicine. Compare with figure 1-2 and note the smaller number of cells in mitosis. Note also that mitosis is taking place near the lumen. H & E X 640.

RESULTS AND DISCUSSION

The amount of colchicine to be given to the pig was determined in a preliminary experiment the results of which are given in Table 1-1. The greatest numbers of mitotic figures were seen in the animals injected with colchicine in the amount of 0.25 mg./kg. of body weight. Since the two values obtained with this amount of colchicine agreed within 1%, and since these values were twice as great as the next highest value, it was decided that 0.25 mg./kg. of body weight be used as the dosage for further work. This value agrees with the amount administered to the dog and cat in other investigations. Toxic manifestations were not observed clinically in the animals, but 4 hours may be too short a period for toxic manifestations to become apparent. Most of the metaphasic arrests noted in examining the tissue sections were of the ball type, Figures 1-3 and 1-5, which, according to Eigsti and Dustin (1955), are indicative of toxic action of the colchicine. Note also in Figure 1-6, in an animal which had not been given colchicine, that the mitotic figures are not ball shaped but assume a more angular configuration. Whether the decreased numbers of mitoses noted in the pigs given the higher doses of colchicine were due to damage or destruction of the mitotic figures by the increased amount of colchicine was not determined. This finding does contradict the suggestion of McMinn (1958) that colchicine in increasing doses causes an increase of mitoses in the duodenal epithelium of the dog. This apparently is not true for the duodenal epithelium of the baby pig.

Fig. 1-5---Jejunum of pig which had received colchicine. Note the ball mitoses in the glands of Lieberkühn. Compare with Fig. 1-6 for the number of mitotic figures in a comparable area in a pig which had not received colchicine. H & E X 640.

Fig. 1-6.---Glands of Lieberkühn from the jejunum of a pig which had not received colchicine. Note the small number of mitotic figures. The gland at the left has one metaphasic mitotic figure and one anaphasic figure. The gland at the right has one mitotic figure in metaphase. H & E X 640.

With the dosage of colchicine determined, it now became necessary to ascertain the optimal time for allowing the colchicine to act. Table 1-2 gives the results of a study to determine this factor. A time of 4 hours was selected for further studies with colchicine because there was a constant increase in the number of mitoses up to this time in the epithelial cells of the duodenum as shown in the table. The peak mitotic activity for the duodenal epithelium and the peak activity for the cecal epithelium do not fall on the same hour following the injection of colchicine. In the case of the duodenum, the peak mitotic activity occurred at 4 hours and, in the case of the cecum, the peak activity occurred at 5 hours. Since it was not possible to select two time periods for the same experiment and since it had been stated by Bertalanffy and Leblond (1953) that a time should be selected in which the numbers of mitotic figures are still increasing, it was decided to use 4 hours as the time period. This time is at or near the peak activity for the duodenal epithelium and is in the period in which the mitoses in the cecum are still increasing. It would probably not be possible to select a time which would be ideal for all tissues. If work were to be done involving a specific tissue of the body, a preliminary study should be done to determine the ideal time for the tissue in question.

Examination of Table 1-2 suggests another interesting possibility. There appears to be a cyclical pattern in the number of mitoses in the duodenum with a high point of mitotic activity at 4 hours and another high point at 8 hours. In the cecum the same picture obtains but with the high points of mitotic activity at 3 and 6 hours. While the number of observations is too small to be of significance, the possibility exists that the mitotic activity of the epithelial cells is cyclic. Chodkowski (1937) describes phases of karyokinesis following administration of mitotic poisons in which one wave of mitotic activity is followed by a second. However, the intervals between the waves as described by him are measured in days rather than hours.

The reasons for the decreased numbers of mitotic figures in the period between the two apparent highs is not explained. It would seem improbable that the dosage of colchicine was so high that the damaging effect of excessive amounts had destroyed the mitotic figures and that the increase seen at the next peak is due to previously resting cells now undergoing cell division.

Table 1-4 gives the mitotic activity of 9 areas of the digestive tract of the baby pig during 4 periods of the day. Four pigs were studied for each of the 4 time periods. Analysis of variance using the F test was applied to the data for each area of the intestinal tract to determine if there was significant variation in the activity at any of the 4 periods of the day. Results of this analysis showed no significant variation between the mitotic activity during the 4 periods of the day for any of the 9 areas of the digestive tract studied. Accordingly, the percentages for the 4 periods were averaged giving the percentage of cells undergoing mitosis during any four-hour period of the day.

Examination of this table indicates that the 9 areas of the intestinal tract studied can be placed into 3 groups. The cellular activity of the 3 areas of the stomach is somewhat similar. Mitoses varied from 2.22% to 2.88%. A somewhat intermediate group includes the 3 areas of the small intestine which have from 3.60% to 4.45% of the epithelial cells in mitosis. The third group is composed of the cecum, spiral

TABLE 1-4.---Percentage of epithelial cells in mitosis at various levels of the intestinal tract of the baby pig during four periods in a day. Colchicine injected 4 hours before.

Interval	stomach cardiac	stomach fundic	stomach pyloric	duo-denum	jeju-num	ileum	cecum	spiral colon	terminal colon
6-10 P.M.	2.38	2.69	3.24	4.53	4.88	3.11	5.27	5.25	4.80
12-4 A.M.	2.47	1.85	2.95	2.80	4.74	3.61	5.10	5.40	4.25
6-10 A.M.	2.74	1.77	2.64	3.85	4.00	3.79	4.59	4.92	4.72
12-4 P.M.	2.42	2.45	2.70	3.22	4.18	4.23	5.08	5.25	5.38
Average	2.50	2.22	2.88	3.60	4.45	3.68	5.01	5.21	4.79
S.E.*	0.11	0.17	0.12	0.28	0.16	0.17	0.17	0.18	0.20

* Standard error

colon and terminal colon with a range in the percentage of epithelial cells in mitosis of 4.79% to 5.21%.

If one now calculates the time required for a complete replacement of epithelial cells in these areas of the intestinal tract, it can be seen (Table 1-5) that it takes from 3 to 7 days to accomplish this. These results do not compare too closely with those obtained by other authors in work done with other species of animals. Stevens (1952) and Stevens and Leblond (1953) in studies on the stomach of the rat determined the renewal time for the surface epithelial cells to be 2.84 days and for the mucous neck cells to be 6.52 days. Bertalanffy (1960) determined the renewal time for the epithelium of the cardiac region of the stomach of the rat to be 9.1 days as contrasted to the 6.66 days determined in this study of the baby pig.

In the case of the small intestine, Leblond and Stevens (1948) determined the renewal time of the epithelium of the duodenum and ileum of the rat to be 1.6 and 1.4 days respectively. The comparable renewal times for these areas in the baby pig as determined in this study are 4.63 and 3.74 days respectively.

The renewal times for the epithelium of the spiral colon and the terminal colon of the baby pig as determined in the present study are 3.20 and 3.48 days respectively. A comparable value for the epithelium of the colon of the rat as reported by Bertalanffy (1960) is 10 days.

The percentage of cells in mitosis at different levels of the digestive tract from 8 pigs which had not been given colchicine is depicted by Table 1-6. These pigs were of the same age and treated in a similar manner as were those animals which had been given colchicine. Two pigs were used for each interval of the day shown in the table.

TABLE 1-5.---The renewal time in days for the epithelium of various levels of the digestive tract of the baby pig

	stomach			duo-	jeju-			spiral	terminal
	cardiac	fundic	pyloric	denum	num	ileum	cecum	colon	colon
Percentage of cells in mitosis in 4 hrs.	2.50	2.22	2.88	3.60	4.45	3.68	5.01	5.21	4.79
Percentage of cells in mitosis in 24 hrs.	15.00	13.32	17.28	21.60	26.70	22.08	30.06	31.26	28.74
Days required for 100% renewal of cells	6.66	7.50	5.78	4.63	3.74	4.52	3.27	3.20	3.48

Analysis of variance showed a significant difference between the numbers of mitoses at the different time intervals in the instance of both the cardiac region of the stomach and the cecum. This difference was significant at the 5% level in both instances. In all other areas studied, there were no significant differences between the numbers of mitoses at the 4 time intervals. The difference in the case of the cardiac region of the stomach and the cecum, although showing a low level of significance, does not necessarily mean that there are diurnal differences in the mitotic activity of these two organs. The number of animals studied is small, being composed of 4 groups of 2 animals each. If a larger number had been used, these differences might not have existed. In support of this is the fact that the groups of animals that had received colchicine (see Table 1-4) showed no significant variation between groups.

TABLE 1-6. --- Percentage of epithelial cells in mitosis at various levels of the intestinal tract of the baby pig during 4 periods in a day. These pigs had received no colchicine.

Interval	stomach cardiac	stomach fundic	stomach pyloric	duodenum	jejunum	ileum	cecum	spiral colon	terminal colon
6-10 P.M.	0.26	0.46	0.53	1.31	0.84	0.77	0.89	0.96	0.93
12-4 A.M.	0.57	0.46	0.57	0.54	0.95	0.82	0.89	0.82	0.86
6-10 A.M.	0.63	0.44	0.69	1.00	1.01	1.11	1.23	0.99	0.85
12-4 P.M.	0.58	0.44	0.66	0.72	0.74	0.90	0.89	1.03	0.85
Average	0.51	0.45	0.61	0.89	0.88	0.90	0.97	0.95	0.87
S.E.*	0.06	0.03	0.05	0.15	0.07	0.06	0.06	0.04	0.03

* Standard error

The average number of epithelial cells in mitosis was determined for each area of the intestinal tract studied in the animals that were not given colchicine. If the percentage of cells in mitosis after colchicine administration is divided by the percentage of cells in mitosis from an animal which did not receive colchicine, the number of mitotic cycles taking place during the interval in which colchicine is acting will be determined. If one conservatively uses a time interval of 30 minutes for the colchicine to be absorbed from the peritoneal cavity and to be distributed by the blood to all cells of the body, then the time that colchicine was acting in the animals in this experiment was $3\frac{1}{2}$ hours or 210 minutes. If one now divides the time that the colchicine was acting by the number of mitotic cycles taking place in that time interval, the length of time for one mitotic cycle will be determined. This is shown in Table 1-7. It can be seen that the duration of the mitotic cycle varies from 38 to 52 minutes. This is somewhat longer than the 27.5 minutes determined for the rat jejunum by Widner et al. (1951) or the 23.9 minutes reported for the rat jejunum by Knowlton and Widner (1950).

It is interesting to consider this great activity of the cell. The cells lining the intestines complete the mitotic cycle in less than one hour. In one week, the lining epithelium of some areas of the intestinal tract is completely replaced, not once, but twice. Thus, the intestine is far from being a simple tube, playing a passive role in the nutrition of the animal but rather a dynamic organ, constantly undergoing change.

TABLE 1-7.---Number of mitotic cycles in a four-hour interval and the length of the mitotic cycle for the epithelial cells of various levels of the digestive tract of the baby pig.

	Percentage of cells in mitosis								
	stomach			duo-	je ju-			spiral	terminal
	cardiac	fundic	pyloric	denum	num	ileum	cecum	colon	colon
No colchicine	0.51	0.45	0.61	0.89	0.88	0.90	0.97	0.95	0.87
Colchicine	2.50	2.22	2.88	3.60	4.45	3.68	5.01	5.21	4.79
Number of mitotic cycles	5.02	4.94	4.73	4.05	5.06	4.09	5.17	5.48	5.51
Length of mitotic cycle in minutes	41.8	42.6	44.4	52.0	41.6	51.4	40.6	38.4	38.1

SUMMARY

Using the colchicine technique, the mitotic rates and the renewal times for the epithelium of nine areas of the digestive tract of the 5-week-old baby pig were determined. The time required for the complete renewal of the epithelium of the stomach varied from 5.78 to 7.50 days, depending on the area; for the small intestine, the time varied from 3.74 to 4.63 days; for the cecum, 3.27 days; and for the spiral colon and the terminal colon, the times were 3.20 and 3.48 days respectively.

An estimation of the length of the mitotic cycle was made for the nine areas studied. This varied from 38 to 52 minutes for different levels of the intestinal tract.

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PART II. A MICROMORPHOLOGIC COMPARISON OF GNOTOBIOTIC AND FARM-RAISED PIGS

INTRODUCTION

It is of fundamental interest to know what effect, if any, microbial life has on the intestinal tract. The intestine of the normal animal has been extensively studied; that of the germfree animal is largely unknown.

Much work has been done with the propagation and study of germfree laboratory animals. Morphological studies on the intestinal tract of the germfree pig have not been reported.

It is the purpose of this study to compare the micromorphologic features of the intestine of the germfree or gnotobiotic pig to that of the pig raised under farm conditions. Certain other aspects of the gnotobiotic pig will also be considered.

At this point, a few words on terminology are in order. The term gnotobiote is from the Greek language and means "known life". The term was first suggested by Reyniers et al. (1949). By the expression gnotobiotic pig, therefore, is meant a pig, the microbial flora of which is known. The term can be used to designate pigs which are completely free from microbial life. It can also be used for pigs which have one or more contaminating organisms as long as the identity of these organisms is known. For example, in experiments 1 and 2 to be reported in this thesis, the contaminating organisms of the gnotobiotic pigs are Staphylococcus aureus and a member of the Bacillus genus, respectively.

The expression "gnotobiotic control" will be used to designate those pigs, usually littermates of the gnotobiotic pigs, but which are not kept under gnotobiotic conditions. They are animals that are raised outside the isolators in open cages but within the same room and fed the same diet as the gnotobiotic pigs.

By farm-raised pigs or farm-raised control pigs is meant those pigs born by natural means and raised with the sow under normal farm conditions.

By disease-free pigs is meant those pigs which are obtained by hysterectomy and raised in a previously cleaned and disinfected environment away from contact with other swine. The original purpose of disease-free pigs was to break the infection cycle of certain common swine diseases such as viral pneumonia of pigs (VPP), atrophic rhinitis, and transmissible gastro-enteritis (TGE) of pigs. They are therefore "free" of certain specific diseases. The term specific-pathogen-free (SPF) is often applied to these animals.

REVIEW OF LITERATURE

Historical Aspect

Historically the idea of growing plants and animals free from living microorganisms received its initial impetus from the work of Duclaux (1885), who attempted to grow beans in sterile soil. In the same journal, Louis Pasteur (1885), in commenting on Duclaux's paper, expressed the opinion that higher life without bacteria would be impossible. He suggested that the chicken be used to prove his point since the egg would lend itself nicely to such an experiment.

Nencki (1886) disagreed with Pasteur and predicted that when the chicken was raised free of microbial life, as Pasteur had proposed, it would be more active than the one with bacteria in its digestive canal. His opinion was that bacteria produced toxic materials which were harmful to the growing animal.

The transition from the theoretical to the practical in this controversy was made by Nuttal and Thierfelder (1895-96, 1896-97), who succeeded in raising guinea pigs obtained by Caesarean section for a period of 8 days in a modified bell jar apparatus. These guinea pigs were determined to be free of microbial contamination by direct examination of excreta and intestinal contents and by the culture techniques then available. The same authors (1897) failed in attempts to raise chickens free from bacteria. They were the first to express the possibility that this technique might be used to study the effect of pure cultures of pathogenic and non-pathogenic bacteria on the otherwise germfree animal.

Schottelius (1899, 1902, 1908, 1913), following experiments in which he attempted to raise chickens without bacteria, took issue with Nuttal and Thierfelder and again championed the idea that life without bacteria was impossible.

Cohendy (1912), working in the laboratory of Metchnikoff, succeeded in raising chickens free of bacteria for periods up to 45 days and Cohendy and Wollman (1914) raised germfree guinea pigs for 29 days.

Küster (1913,1915) who was a student and assistant of Schottelius at the University of Freiburg, succeeded in raising a goat obtained by Caesarean section for 35 days in the absence of bacteria. Attempts were being made at this time to raise other forms of life free from microbial contamination. Metchnikoff (1901) and Wollman (1913) experimented with germfree tadpoles.

In 1922, Cohendy and Wollman used the germfree guinea pig to study a nutritional entity, scurvy, and an infectious entity, cholera.

In 1928, workers at the University of Notre Dame began experimenting with germfree life and their interest and work in this field has continued unabated since that time. Reyniers (1943) described this early work in a symposium held at the University of Notre Dame in 1939.

Numerous species of animals have now been raised free of bacterial contamination, ranging from the platyfish propagated by Baker and Ferguson (1942) to the monkey which was reared under germfree conditions by Reyniers (1942).

The Microflora of the Intestine

The main habitat for bacteria in an animal is the intestinal tract. Thus it is this tract and its contents which serve as a source of inoculum for the determination of the presence or absence of microbial life.

Several papers have appeared giving the normal microflora of the intestinal tract of the pig. Table 2-1 gives the bacterial counts of swine feces as compiled from the recent literature. It can be seen that the numbers of aerobic and anaerobic forms each vary from 10^8 to 10^9 organisms per gram of feces. The other groups vary from 10^4 to 10^9 organisms per gram of feces.

Other workers have made studies of specific groups of organisms of the intestinal tract and have determined the most prominent species in the particular group of bacteria that they were studying. Raibaud and Caulet (1957a, 1957b) characterized the lactobacilli and streptococci which they found in swine feces. Raibaud, Caulet, and Mocquot (1957) studied the lactobacilli and the coliforms and Fewkins _et al_. (1957) investigated the lactobacilli and the streptococci found in swine feces.

The flora of the intestinal tract is not constant but is subject to variations. The diet may affect the kinds and numbers of bacteria found as shown by Gall _et al_. (1948a) in mice and even the genetic make-up of the animal may have an effect on this bacterial population as shown by the same authors in 1948b. Wilbur and associates (1960) have also demonstrated that the diet fed pigs may affect the bacterial population of the feces.

Whether the flora of the intestinal tract is harmful or advantageous to the animal has been and is still controversial. This problem has more than one facet and cannot be answered simply.

TABLE 2-1. --- Microbial flora of swine feces as compiled from the literature.

Investigator	Age of pigs*	Aerobes	Anaerobes	Coliforms	Enterococci	Shigella	Proteus	Lactobacilli	Clostridium
		Counts expressed as logarithm of number of bacteria per gram of feces							
Briggs *et al.* (1954)	var.	8-9	8-9						
Bridges *et al.* (1953)	1-9			6		7.6	7.5		
Bridges *et al.* (1952)	8	7.7							
Larson *et al.* (1955)	1-8	9.2	9.4	9.1	8.4			4.6	6.7
Quinn *et al.* (1953a)	4	9.0							
Quinn *et al.* (1953b)	8	9.1		6.3					
Sieburth *et al.* (1951)	8								7.5
Wilbur *et al.* (1960)	2-6	8.6	9.3	7.3	7.5			8.9	
Willingale and Briggs (1955)	var.	8-9	8-9	6-8					

* Age of pigs in weeks

Johansson and Sarles (1949), in a review paper, discuss the biological importance of the intestinal micro-organisms. Suffice it to say that, as early as 1914, Cooper observed that alcoholic extracts of feces from chickens and rabbits would cure the polyneuritis of pigeons fed polished rice. This was confirmed by Portier and Randoin in 1922. This demonstrated that the fecal microflora produces something of nutritional value for the animal. On the other hand, it was shown by Coates et al. (1951) that antibiotics fed to chickens in a "contaminated" environment resulted in an increased rate of growth. When antibiotics were fed to chickens raised in a "clean" environment, there was not an increased rate of growth. This implied that the antibiotics modified the intestinal flora in a manner which prevented the growth depression due to the "contaminated" environment. The growth rate of the chickens raised in the "clean" environment was superior to that of the chickens raised in the "contaminated" environment and was not affected by antibiotics.

Some Nutritional Aspects

The feeding of the germfree animal is not a simple undertaking. Although much is known regarding the nutrition of the usual animal-microflora complex, very little is known about feeding the animal without its usual complement of intestinal microbes. It is probable that the poor growth responses obtained by the germfree animals of Schottelius, Nuttal and Thierfelder, and Cohendy already cited previously, were due to poor nutrition.

One of the problems encountered is the destruction of vitamins by the sterilization process. Luckey et al. (1955a) reported an 80% destruction of thiamine by steam sterilization and a 70% destruction of it when sterilized by cathode rays. Losses of riboflavin, niacin, pantothenic acid and folic acid were 8, 3, 31, and 9.5%, respectively, when these vitamins were subjected to steam sterilization.

Reyniers and associates (1950) stated that there is considerable destruction of the vitamins, particularly thiamine, by steam sterilization. They also demonstrated a 7.5% loss of tryptophane following steam sterilization.

Wostmann (1959) noted a loss of 80% to 90% of the thiamine by steam sterilization and probably not more than 40% to 60% loss of the other water soluble vitamins. He stresses the dangers of over-fortification with vitamins, however, since some of the degradation products of sterilization may be potent antivitamins. This is stated to be particularly true in the case of thiamine since oxythiamine may be one of the products formed. Sterilization by gamma radiation results in the loss of ammonia as reported by Groninger and Tappel (1957). This loss is probably brought about by the destruction of the 6-amino group of the pyrimidine portion of the thiamine molecule. This may or may not be the mechanism of breakdown when thiamine is subjected to steam sterilization.

There is evidence that at least some of the B-vitamins are synthesized and excreted into the intestine in the absence of bacteria. Luckey _et al_. (1955b) observed that germfree chickens on a thiamine-deficient diet were dying from the deficiency while excreting into the lower intestine enough thiamine to save their lives. Luckey _et al_. (1955c) also found that the germfree rat, while receiving only 0.03 micrograms of folic acid per gram of feed eaten and having in its entire body less than one microgram of folic acid per gram of body tissue, was excreting 100 to 500 micrograms of folic acid per day. This could only have come from tissue synthesis of folic acid.

Morphologic and Other Characteristics of Germfree Life

Since the germfree animal is different from the conventional or non-germfree animal by virtue of the absence and presence, respectively, of microbial life in the digestive tract and elsewhere in the body, one wonders whether other changes in the physiological and morphological make-up might also be apparent. Externally the two animals look alike and the growth rates and blood values are normal as shown by Gordon (1955) in working with the germfree chick. Wostman (1959) reports, on the other hand, that the growth rate in the rat and mouse is retarded in the germfree state, particularly in the male animal. He attributes this effect on growth to improper nutrition.

Some internal changes have been observed both macroscopically and microscopically. Rodents have been observed to develop an enlarged cecum in the germfree state. This characteristic change which is the bane of germfree research with the rodent was first observed by Nuttal and Thierfelder (1896-97, 1897-98), who described the inflated or puffed up cecum in their guinea pigs. Other changes that have been observed are also associated with the digestive tract or with organs not far removed from the vicinity of the digestive tract. Gordon (1959) reports that the intestinal tract of the germfree animal weighs significantly less than the digestive tract of the conventional animal. He states that preliminary evidence indicates that the amount of connective tissue in the intestine is partially responsible for this difference. He also states that some of this difference in weight might be caused by a slightly lower water content of the tissues of the intestinal tract of the germfree animal. A slight difference in water content was demonstrated in both the chicken and the rat.

Another observation which is important and may be a contributing factor in causing lesser weight of the intestines of the germfree animal is the smaller amount of lymphatic tissue in the germfree animal. This hypoplasia is reported by Gustaffson (1948) and Miyakawa (1959). Gordon (1959) describes both a lesser weight of the lymphatic tissues and a smaller concentration of lymphocytes in the lymphatic organs closely associated with the digestive tract.

The change in weight of the intestinal tract, if due to the absence of bacteria, might also be expected in those animals in which the bacterial population is reduced or altered by other means such as by the administration of antibiotics. This has indeed been found to be true by Coates _et al_. (1955), Hill _et al_. (1957), Jukes _et al_. (1956), and Pepper _et al_. (1953) in the chicken, and by Taylor and Harrington (1955) in the pig. That this change is not caused by the antibiotics themselves is demonstrated by Gordon and coworkers (1957), who administered antibiotics to germfree chickens. They found that there was no alteration in the morphologic picture or weights when the intestine of a germfree bird fed antibiotics was compared to the germfree bird that had not received them.

MATERIALS AND METHODS

In rearing the germfree animal it is necessary to remove the fully developed fetus from the dam and transfer it to a sterile environment using aseptic technique. Provisions must be made for supplying sterile food and for repeatedly determining the bacterial status of the equipment and animal. A sterile surgical isolator and rearing unit were used to maintain sterility. An antiseptic lock provided an entrance for the baby pig to the surgical isolator at the time of the hysterectomy.

The Rearing Unit

The rearing unit (Figures 2-1 and 2-2) was approximately 6 feet long, 2 feet high and 2 feet deep. The plastic was vinyl film 8 mils (0.008 inch) in thickness. A plywood base was used to give the unit greater strength and stability. A large plastic ring placed in the plastic wall on one side of the isolator provided an access door to the interior of the unit. On the inside of the plastic ring, a plastic cover, held on the flange of the ring with a heavy rubber band, served as a door to seal the isolator from outside contamination. On the outside of the plastic ring a vinyl sleeve was attached; to this was also attached a plastic cover. The sleeve provided an antechamber which could be easily sterilized with peracetic acid vapor. It was used to transfer sterile material into and remove waste materials from the isolator. When used for this purpose the item to be admitted was

Fig. 2-1. --- The rearing unit showing the air filter at A, the air supply tube at B, and the vinyl sleeve at C.

Fig. 2-2. --- The rearing unit showing the reverse side of that in Fig. 2-1. The two arm-length rubber gloves can be seen in the side. On the floor in the background can be seen a Spencer Turbo Compressor for supplying air to the unit.

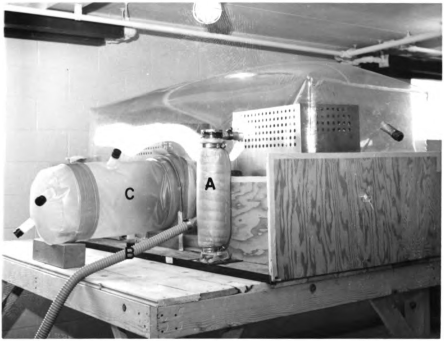

FIG. 2-1.

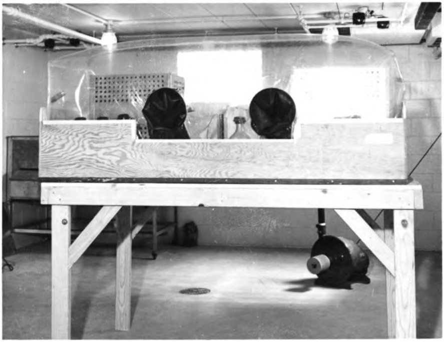

FIG. 2-2.

sprayed with peracetic acid solution and placed within the sleeve. The cover was placed on the sleeve and the interior of the sleeve filled with peracetic acid vapor. After 30 minutes, the inside door was removed and the item transferred into the isolator. The peracetic acid was used as a 2% solution made up in distilled water.

On the opposite side of the isolator (Figure 2-2) a pair of long rubber gloves was provided which were used by the operator for necessary manipulations within the isolator. Methods for attaching the gloves to the isolator are given by Trexler and Reynolds (1957).

Air enters the isolator through a glass-wool filter as described by Trexler and Reynolds (1957) and leaves through an air outlet trap similar to that described by Trexler (1959).

Four stainless steel cages were wrapped in paper and sterilized in an autoclave. They were then placed within the isolator, the paper removed and the interior of the isolator and the cages sprayed with the peracetic acid solution. Other materials were placed within the isolator at the time of the initial sterilizing of the unit. This added material was sterilized by steam and transferred directly from the autoclave to the isolator through a connecting lock. The material added at this time included towels, cotton swabs for sterility testing, a supply of milk, vitamins and minerals, a pair of cotton gloves to be placed over the rubber gloves when handling animals or any material within the isolator, paper sacks for storing empty vitamin and mineral vials, a file for nicking the glass vials prior to opening, and hemostats, scissors, string, and clamps used in the surgical isolator at the time of the hysterectomy.

All sterilizing of the isolators, the surgical isolators, and the materials that were placed within them as described in the previous paragraph, was done by the staff of the Lobund Institute, University of Notre Dame for the first 3 experiments described herein.

The surgical isolator and the rearing unit isolator were constructed by Professor P. Trexler and Mr. Robert Hakes of the University of Notre Dame.

The Surgical Isolator

The surgical isolator (Figures 2-3 and 2-4), also made of vinyl plastic, was somewhat larger than the rearing unit. It was mounted on a plywood base and equipped with an air filter and an air outlet. It was provided with three pairs of shoulder-length rubber gloves for use at the time of hysterectomy. A door was provided at one side for attaching a sleeve through which the pigs, after separation from the fetal mebranes, were passed into the rearing unit.

On the bottom and at one end was attached a rather rigid plastic tube which extended into a tank of germicidal solution. This tank of germicide provided an antiseptic lock for the transfer of the uterus of the sow to the interior of the surgical isolator.

The unit was prepared for use by covering the lower opening of the ventral plastic tube with a sheet of plastic film. The door at the side was provided with a plastic cover which was attached to the flange of the door with a heavy rubber band as described for the rearing unit. The interior of the unit was sterilized with a solution of peracetic acid. The spraying unit for applying the solution was brought into the isolator through the side door of the unit. After thoroughly spraying all internal surfaces the plastic cover was placed over the opening

Fig. 2-3. --- The surgical isolator showing the door at A to which is attached the rearing unit for the purpose of transferring the baby pigs at the time of the hysterectomy. At B is seen the rigid plastic tube which dips down into the antiseptic lock.

Fig. 2-4. --- The surgical isolator showing the antiseptic lock in place. At A can be seen the air filter and, at B, the air outlet trap.

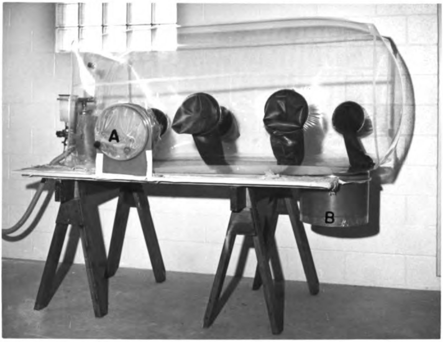

FIG. 2-3.

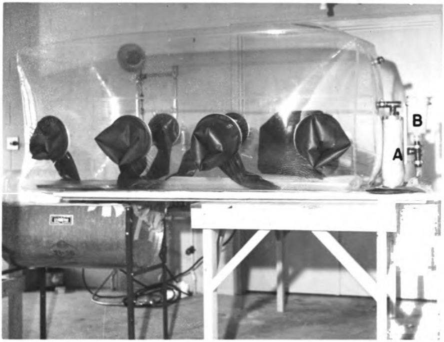

FIG. 2-4.

and the rubber band placed over it and around the inside flange of the door, thus securely holding the cover in place.

The isolator was then filled with peracetic acid vapor through a small, rigid tube set in the end of the plastic isolator. Following inflation of the isolator, the opening in the tube was closed with a rubber stopper. Prior to sterilizing the unit, the air inlet had been covered with a plastic film after thoroughly spraying it with the peracetic acid solution. Before using the isolator, the cover to the air inlet tube is punctured with a sterile instrument from inside the isolator.

The Antiseptic Lock

The antiseptic lock (Figure 2-5) was prepared by lining a steel Jamesway milk-can-washing tank with padding material and polyethylene plastic film. The polyethylene film was attached to the rigid plastic sleeve which extends from the bottom of the surgical isolator.

Twenty gallons of water, 1 gallon of Clorox bleach and 12.5 ml. of 40% peracetic acid were placed within the plastic-lined tank.

When all preparations for the hysterectomy were completed, the plastic film which previously had been placed over the bottom of the ventral sleeve of the isolator was punctured,making the passageway from the disinfecting solution in the antiseptic lock to the interior of the surgical isolator complete.

Germfree Technique

Baby pigs were obtained from healthy Yorkshire sows by hysterectomy using aseptic technique and transferring the pigs so obtained to a sterile plastic isolator. (See Figure 2-6)

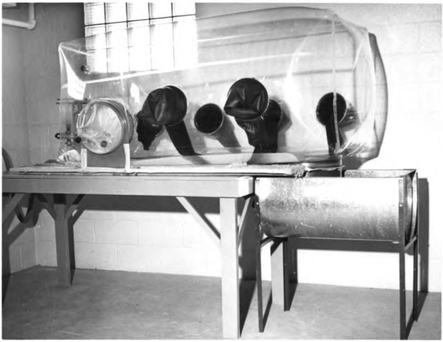

Fig. 2-5. --- The antiseptic lock is a Jamesway milk-can-washing tank. In use, this tank is lined with plastic and filled with germicidal solution.

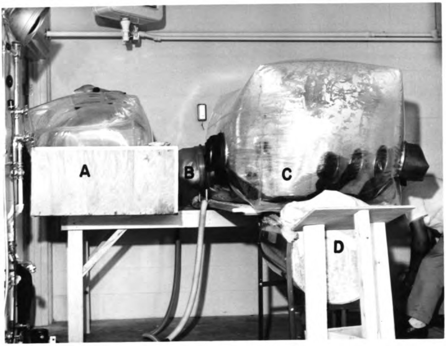

Fig. 2-6. --- The isolator equipment connected together preparatory to the hysterectomy. At A is the rearing unit, at B the connecting sleeve, at C the surgical isolator and at D, the antiseptic lock.

The breeding date of the sow was usually known and near the termination of the gestation period she was carefully watched for signs of parturition. When milk could be obtained from the mammary gland or at the end of 112 days of gestation, the hysterectomy was performed.

The sow was brought into the laboratory a day or two before the surgery was to be performed. On the day of surgery she was scrubbed with soap and warm water using a brush for the purpose of removing the gross filth. When preparations for the hysterectomy were completed, the animal was hoisted by the rear legs till her head just cleared the floor. The abdomen was scrubbed with soap and water using first a brush and later sterile gauze squares. The skin was dried with pieces of gauze and a solution of Weladol (Pitmann Moore Company) applied to disinfect the skin.

Directly after the application of the disinfectant to the abdomen, the head and anterior part of the body of the animal were lowered into a large tile sunken into the concrete floor. To the tile had previously been added several pounds of crushed dry ice to supply CO_2 for general anesthesia. The sow was kept suspended with her head in the tile for one minute. Some struggling was noted at first but already at one-half minute the anesthesia seemed quite complete. After one minute she was raised to the previous position.

During the one-minute period of anesthesia induction, the disinfected abdominal area was sprayed with a surgical adhesive preparation, ViHesive (Aeroplast Corporation, Dayton, Ohio), and a large piece of sterile Mylar film was applied. The length of the Mylar film was such as to allow the end to extend from the abdomen of the sow into the tank of germicidal solution. This allowed the uterus, when removed from the sow, to slide easily from the abdominal incision into the tank of germicide without touching the skin of the sow or any other non-sterile surface.

Employing aseptic technique, an incision was made through the Mylar film, the skin, fascia, muscles of the abdominal wall, and the peritoneum. The horns of the uterus were brought out through the incision and a pair of Carmalt-type forceps was clamped across the neck of the uterus just anterior to the cervix. The uterus was then removed by incising just posterior to the attached forceps. The uterus, including the attached forceps, was then gently guided down the Mylar film into the solution of the antiseptic lock. In the first attempt to obtain germfree pigs the uterus was tied with sterile cord at the cervix instead of using the abdominal forceps before severing it from the sow.

The uterus was submerged in the antiseptic lock for 30 seconds. It was then raised through the inlet tube of the surgical isolator with the combined assistance of the gloved arms of the operator working in the isolator and the gloved arms of the worker pushing up on the uterus from within the tank of disinfectant.

In the isolator, the uterine wall was opened by tearing the muscles with the gloved fingers and with the aid of scissors or preferably the not-as-sharp tips of a pair of hemostats. All the pigs were rapidly taken from the uterus and the placental membranes removed. The mouths were opened and the throats cleared of the viscous amniotic fluid with the fingers. Artificial respiration was applied to each pig by alternately pressing and releasing the rib-cage.

Clamps were applied to the umbilical cords several inches from the body shortly after the placental membranes were removed from the pigs. After it was ascertained that the pigs were alive and breathing, the umbilical cord of each pig was tied an inch from the body with sterile string and the cord severed.

When the umbilical cords had been ligated and severed, the uterus, placental membranes, and dead pigs were dropped through the rigid tube in the floor of the isolator and a cover placed over the opening.

The live pigs were wiped with dry towels and particles of adhering membranes were removed from the skin. Just before passing the animals through the connecting sleeve into the rearing unit, they were wiped with a towel soaked in Hyamine (Rohm and Haas, Philadelphia, Pennsylvania). They were then passed to the rearing unit where they were individually confined in the stainless steel cages.

After placing the cover over the inner opening of the connecting sleeve and securing it with a heavy rubber band, the sleeve was removed from the surgical unit and a plastic cover also placed over the end of the sleeve. The interior of the sleeve was then sterilized by spraying peracetic acid vapors into it through small tubes set into the side of the sleeve; the tubes were subsequently closed with rubber stoppers.

Feeding the Germfree Pig

The pigs were fed within several hours after placing them in the cages. They were fed 4 times a day: at 8:00 A.M., 12:00 noon, 5:00 P.M. and 10:00 P.M. The amount of milk fed depended on how much they were consuming. The initial feeding was usually about 100 ml. If milk was left in the pan,at the next feeding, less milk was offered.

When the amount given was consumed, it was gradually increased. At about 1 week to 10 days of age, feedings were reduced to three by discontinuing the one at 10:00 P.M. At the termination of the experiment at 21 days, the pigs were eating about 700 ml. or more of milk a day.

The diet fed consisted of commercial, vitamin-D fortified, pasteurized cows' milk obtained from the university dairy. This milk was sterilized in two-quart quantities in two-liter Square-Pak bottles obtained from the American Sterilizer Company. The caps on these bottles allowed the escape of the expanding gases during the steam sterilization but prevented the influx of air after the steam pressure was reduced. The milk was sterilized for 28 minutes at 17 psi (pounds per square inch) steam pressure. Some boiling over of milk was encountered which necessitated washing the outside of the bottles to remove the dried milk solids prior to spraying them with peracetic acid at the time of introduction into the isolators. It would have been better, perhaps, to use less milk in each Square-Pak when sterilizing to decrease the problem of boiling over.

Fortification of the diet with supplementary vitamins was necessary since it had been shown by Luckey (1959), Luckey et al. (1955) and Reyniers et al. (1950) that up to 90% of the thiamine and considerable proportions of other vitamins were destroyed during the sterilization process. The vitamin mixture used in this study (Table 2-2) is a modification of that used by Reber et al. (1953). It was modified to assure that enough vitamins would be present after allowing for 90% destruction of the thiamine and 50% destruction of all other vitamins. The solution of vitamins was placed into glass ampules in 10 ml. quantities, sealed with a burner and sterilized with steam for 15 minutes at a pressure of 15 psi.

TABLE 2-2. --- Vitamin Mixture used to supplement milk diet

Vitamin[1.]	amount/5 ml.
Vitamin A[2.]	1600 I.U.
Vitamin D[3.]	240 I.U.
Vitamin E[4.]	2.0 mg.
Vitamin K	0.5 mg.
Thiamine Hydrochloride	3.0 mg.
Riboflavin	1.2 mg.
Pyridoxine	4.0 mg.
Nicotinic Acid	5.2 mg.
Calcium Pantothenate	4.0 mg.
Inositol	20.0 mg.
Para-aminobenzoic Acid	5.2 mg.
Choline	260.0 mg.
Folic Acid	0.1 mcg.
Vitamin B_{12}	5.2 mcg.
Biotin[5.]	47.2 mcg.

1. All vitamins from Merck and Company, Rahway, N. J. unless otherwise indicated.
2. PGB-250 Dry Vitamin A. Distillation Products Industries.
3. Viosterol, Nutritional Biochemicals.
4. Alpha Tocopherol Acetate.
5. Hoffmann-La Roche Inc. New York.

TABLE 2-3. --- Salts solution 15[1]

Component	mg./ml.
Solution A	
KH_2PO_4	176.0
Na_2HPO_4	180.0
KI	0.4
Solution B	
$MgSO_4$	14.0
$MnCl_2 . 4H_2O$	4.0
Ferric Ammonium Citrate	24.0
$CuCl_2$	2.4
$ZnSO_4 . H_2O$	5.2
$CoCl_2 . 6 H_2O$	0.8

1. From Pleasants, J.R., Annals N. Y. Acad. Sci. 78: 116, 1959.

TABLE 2-4. --- Vitamin B mixture 103[1]

Component	mg./3 ml.
Thiamine Hydrochloride	3.0
Riboflavin	1.0
Pyridoxine Hydrochloride	1.0
Niacinamide	2.5
Calcium Pantothenate	12.5
Choline Dihydrogen Citrate	100.0
Biotin	0.025
Folic Acid	0.25
Inositol	100.0
Vitamin B_{12}	0.025

1. From Pleasants, 1959.

A mineral supplement was also added to the milk. In the first experiment in this study, both the vitamins and minerals were supplied by Professor Trexler of the Lobund Institute, University of Notre Dame. These are described by Pleasants (1959) and their compositions are shown in Tables 2-3 and 2-4. The supplements for Experiment 1 were supplied in sterile ampules as solution A and solution B for the mineral supplement and in single sterile ampules in the case of the B-vitamin mixture. These were added to the milk in amounts of 7.5 ml. of solution A and 15 ml. of solution B and 15 ml. of the vitamin B mixture to each quart of milk.

In Experiments 2 and 3 the vitamin mixture given in Table 2-2 was used. In these experiments only solution B of the mineral mixture, to which was added the potassium iodide, was used. Solution A was discontinued since it was felt that the large quantities of phosphorous salts present might be at least partially responsible for the semi-fluid consistency of the feces observed in the pigs in Experiment 1. In view of the fact that the pigs in these studies were to be sacrificed at the end of 3 weeks, it was felt that no serious phosphorous deficiency would result. The mineral mixture was placed into ampules in 15 ml. quantities, sealed with a burner and sterilized with steam for 15 minutes at a pressure of 15 psi. The supplements were added to the milk within the isolators at the rate of 5 ml. of the vitamin mixture and 7.5 ml. of the mineral mixture to each quart of sterilized milk.

Bacteriological Methods

Examination of animals raised under farm conditions

Methods for obtaining and culturing the feces of farm-raised pigs were essentially those of Larson and Hill (1955). Fecal samples were collected in the morning from 5 pigs chosen at random from each litter examined. Aliquots from each sample were mixed and from this composite a 1-gram sample was taken and used for preparing appropriate dilutions in sterile water blanks.

All plates were prepared in triplicate and tubes for the estimation of most probable numbers were inoculated in a single series of 5 tubes per dilution. The following differential media were used: for coliforms, violet red bile agar (Difco); for total anaerobic and total aerobic counts, Eugon agar (BBL) to which was added cows' blood in proportion of 10 ml. of blood to 100 ml. agar; for lactobacilli, LBS medium (BBL) of Rogosa et al. (1951); for clostridia, a modified Wilson-Blair medium (Thompson, 1939); for fecal streptococci, SF medium (Difco); and for yeasts, potato dextrose agar (Difco).

Cultures for total anaerobes were incubated in a Brewer anaerobic jar. Following the insertion of the cultured petri plates into the jar, it was exhausted of air and the air replaced with nitrogen.

Examination of germfree animals

Except for the first attempt to obtain germfree pigs, the media and techniques used for bacteriological examination of germfree animals were the same as those described for the pigs raised under farm conditions. A difference was the source of material used for the inoculation of the culture media. During the course of the 21-day experiments a source of inoculum was the mixture of feces, urine, and spilled milk in the tray beneath each cage. This was collected in sterile screw-capped vials of about 10 ml. capacity and passed out of the isolator at the time it was entered for the purpose of introducing milk. In addition to the sample from the tray, a sterile swab was inserted into the rectum of each pig and then placed along with the adhering feces into another screw-capped vial. All tubes containing material for culture were placed within the empty Square-Paks on which the cover was then secured before the Square-Pak was placed into the connecting sleeve of the isolator. The purpose was to protect the material to be cultured from the germicidal effect of the peracetic acid fumes with which the sleeve was filled before opening it from the outside.

The inocula were taken to the laboratory where the fluid materials from the trays were inoculated without dilution into the various media described for the examination of animals raised under farm conditions. The swabs were streaked on Eugon agar blood plates which were incubated at 37° C. under both aerobic and anaerobic conditions for at least 2 weeks before discarding them as negative.

In addition to bacteriological cultures, smears and wet mounts were prepared from all material taken from the isolator including the rectal swabs. The smears were stained with Gram's stain and the wet mounts were examined unstained for the presence of motile organisms.

At the end of the 21-day experimental period, at the time of necropsy, fecal material was obtained from all pigs and examined by the procedures described.

The cultural procedures used in Experiment 1 were not as elaborate as those used in Experiments 2 and 3 or for the farm-raised pigs. Cultures were made on Eugon agar blood plates which were incubated both aerobically and anaerobically at 37°C. In addition, tubes of thioglycollate medium without indicator (Difco) were inoculated with both feces and materials from the trays. These tubes were incubated at 37°C. for a period of 2 weeks during which interval, several Gram-stained preparations from each tube were examined for the presence of bacteria. At the end of the 2-week period, subcultures were made on Eugon blood agar and incubation carried out under both aerobic and anaerobic conditions.

Pathological Methods

Blood samples were taken from the pigs prior to necropsy. An ammonium-potassium oxalate mixture was used as the anticoagulant. Hemoglobin values were determined using the cyanmethemoglobin method and hematocrits were determined using the micro-hematocrit method. Total leukocyte and erythrocyte counts and differential leukocyte counts were made.

Pigs were anesthetized by the intravenous injection of pentobarbital and exsanguinated. Following death, the intestinal tract was removed and the intestine freed from its mesenteric attachment by cutting the latter close to the intestine. Pieces of tissue were saved from the same areas as in Part I of this thesis (see Figure 1-2). Specimens were fixed in both Zenker's fluid and 10% formalin. Bouin's fluid was also used in a few instances.

The tissues were prepared for sectioning by dehydrating with alcohol, clearing with xylene, and infiltrating with and embedding in paraffin. Sections were cut at 6 microns and 4 to 5 serial sections were affixed to each slide. Hematoxylin and eosin were used as the routine stains. Special stains were used on selected tissues. All histopathological procedures are described in the Manual of Histologic and Special Staining Technics of the Armed Forces Institute of Pathology, Washington, D. C. 1957.

RESULTS

Fecal Flora of Young Pigs

The microbial flora present in the feces of 12 litters of farm-raised pigs is shown in Table 2-5. The total numbers of aerobic and anaerobic forms present were in each instance in excess of one billion organisms per gram of fresh, moist feces. The numbers of enterococci and clostridia present per gram of feces were each in excess of one million organisms. Coliforms numbered about one hundred million organisms per gram and lactobacilli about one billion per gram. Yeasts were present in only small numbers but in many instances the culture plates were impossible to read because of overgrowth with molds. It is interesting to note that the average count on the twenty-first day was not too different from that obtained on the tenth day.

One litter of disease-free pigs (5 animals) was examined and the number of organisms present in the feces at 21 days determined. The number of organisms present is quite similar to that found in the pigs raised under farm conditions: the number of lactobacilli was less by a factor of 100 and the number of clostridia was less by a factor of more than 1000. It is not possible, however, to place much significance on these values since these results are from a single litter and might have been quite different if more litters had been examined.

Table 2-5 also shows the results of the examination of gnotobiotic control animals in the germfree experiments. Fecal cultures were made of 2 animals derived by the technique used for obtaining germfree

TABLE 2-5. --- Fecal microflora of young pigs[1]

Litter	Age[2]	Aer-obes	Anaer-obes	Coli-forms	Entero-cocci	Lacto-bacilli	Clos-tridia	Yeasts
		Log of number of organisms/gram of fresh sample						
H-13	10	10.272	10.677	8.876	---	10.019	5.130	3.522
	21	9.199	9.568	8.167	5.278	8.540	6.693	---
H-14	10	8.962	9.000	8.431	6.708	8.248	6.041	3.113
	21	9.279	9.389	8.620	6.347	9.086	6.164	3.367
H-15	10	9.529	9.448	9.009	7.206	9.312	5.101	---
	21	9.272	9.204	8.591	7.708	8.481	5.653	---
H-16	10	9.765	9.625	9.349	6.347	9.250	6.374	---
	21	8.634	9.053	8.305	3.301	8.529	6.672	---
H-18	10	9.426	9.176	8.804	6.347	9.619	5.568	---
	21	9.420	9.349	8.935	4.708	---	5.653	---
H-19	10	9.404	9.447	8.839	6.347	8.037	5.817	2.518
	21	10.027	10.024	8.776	6.347	9.626	6.865	---
BH-90	10	9.467	9.447	9.113	6.347	9.271	5.552	---
	21	9.598	9.482	7.509	5.708	9.333	6.841	---
BH-91	10	9.271	9.732	8.787	5.347	8.678	5.271	---
	21	9.613	9.505	7.881	6.708	9.235	6.755	---
CDY-104	10	9.465	9.225	8.707	6.206	9.184	8.488	2.875
	21	8.028	8.585	7.504	5.962	7.696	6.832	1.220
CDY-106	10	9.888	9.444	8.713	---	9.913	5.579	1.301
	21	8.591	8.243	6.859	---	7.656	5.486	2.000
DHY-107	10	9.312	9.008	7.675	6.206	9.035	7.753	2.114
	21	8.535	8.243	8.514	---	8.355	6.702	1.000
CHX-117	10	9.915	9.980	8.824	---	9.637	7.207	---
	21	10.263	8.591	8.135	---	9.583	6.539	---
Average	10	9.698	9.882	8.896	6.571	9.484	6.814	---
	21	9.586	9.385	8.372	6.884	9.119	6.598	---
Disease-free	21	9.083	9.267	8.961	---	7.785	3.000	---
Gnotobiotic controls	21	9.039	9.022	8.684	6.347	5.800	6.247	---

[1]All litters are farm-raised with the exception of the disease-free and the gnotobiotic controls. All litters represent 5 pigs with the exception of gnotobiotic control group which represents 2 pigs.

[2]Age in days.

animals but raised in open pens. These animals were fed the same feed as were the gnotobiotic animals. The microflora present in these animals was essentially the same as that present in the animals reared under farm conditions. An exception to this is noted in the case of the lactobacilli, which are less by a factor of 10,000 when compared to the pigs raised under farm conditions and by a factor of 100 when compared to the disease-free pigs.

Experiment 1. Gnotobiotic Pigs, *Staphylococcus aureus* Contaminated. Macroscopic and Hematological Observations

The first attempt to obtain germfree pigs was made on May 17, 1960. With the aid of personnel from the Lobund Institute, University of Notre Dame, and employing the equipment and techniques described above, 7 baby pigs were obtained and placed into the previously sterilized plastic isolator. On May 19, fecal smears from the pigs showed the presence of Gram-negative rods. Three of the pigs were taken from the isolator and placed into open cages where they were fed the same diet that was being fed to gnotobiotic litter mates and served as controls for the gnotobiotic pigs. All pigs were fed 3 times a day. They showed variable interest in the milk, some days eating well and on others eating only a portion of that fed. The amount fed varied from 100 to 150 ml. per feeding depending upon whether the portion fed at the previous feeding had been completely consumed or not.

On the fourth day, a somewhat sour odor was detected at the air outlet of the isolator. This sourness disappeared after several days and the spilled milk which was found in the pans below the animals and the milk which remained in the feed pans from time to time was never coagulated.

The posterior parts of the legs and thighs of the gnotobiotic pigs were covered with fluid feces which had a dark reddish-brown color. The feces were never formed but throughout the period of the experiment were consistently fluid in nature.

One of the gnotobiotic control pigs had an intermittent diarrhea throughout the experiment. Its feces had a yellowish color and contained yellowish semi-solid material somewhat suggestive of coagulated milk. The other 2 gnotobiotic control pigs had well-formed feces of a normal brown color.

At 17 days of age the pigs were removed from the isolator, blood samples were taken for morphologic studies, and the pigs were anesthetized with intravenous sodium pentobarbital and exsanguinated.

Fecal samples were taken from each pig by means of a sterile swab inserted into the rectum and samples of the contents of each tray of excreta were taken prior to the removal of the pigs from the isolator. Cultures made from the feces and from the contents of each pan of excreta on blood agar and in thioglycollate broth and incubated for 2 weeks at 20, 37, and 59°C. showed the presence of an organism which was classified as Staphylococcus aureus.

Re-examination of the slides made on May 19 failed to reveal the presence of Gram-negative rods as originally reported. It is conjectured that needles of crystallized safranin which were present might have been mistaken for the Gram-negative rods which were reported at that time.

Results of the blood studies on the gnotobiotic pigs, the gnotobiotic controls, and the farm-raised pigs are given in Table 2-6. Two samples of blood from the gnotobiotic pigs clotted due to insufficient

TABLE 2-6. --- Hematological values on gnotobiotic pigs (S. aureus contaminated) and their controls at 17 days of age.

Pig. no.	Wt.[1]	Sex	Hb[2]	Hct[3]	RBC[4]	WBC[5]	N[6]	L	M	B	E
				Gnotobiotic pigs, S. aureus infected							
4-D6261	3.7	F		Sample clotted							
5-D6262	4.5	M	10.9	34	6.05	7.60	39	60	0	1	0
6-D6263	4.3	F	11.3	38	5.72	4.80	27	72	1	0	0
7-D6264	3.8	M	Sample clotted				56	43	1	0	0
				Gnotobiotic control pigs							
1-D6249	2.8	M	10.3	27	3.55	9.70	50	49	1	0	0
2-D6250	4.2	M	12.5	37	5.08	10.05	51	49	0	0	0
3-D6251	3.5	M	7.2	24	2.84	29.6	67	32	1	0	0
				Farm-raised control pigs							
13-D1471	12.3	M	9.0	32	4.62	6.9	17	83	0	0	0
11-D1477	13.3	F	10.9	37	4.74	6.6	24	74	0	0	2
15-D1489	12.0	M	11.2	38	5.16	6.0	18	80	0	0	2

[1]Weight in pounds.

[2]Hemoglobin in grams per 100 ml. of blood.

[3]Hematocrit in cell volumes per 100 volumes of blood.

[4]Erythrocytes in millions per cubic millimeter.

[5]Leukocytes in thousands per cubic millimeter.

[6]Percentages of neutrophils (N), lymphocytes (L), monocytes (M), basophils (B), and eosinophils (E).

agitation following the placing of the blood into the vials, making it impossible to get blood counts on these animals.

Post-mortem examination of the gnotobiotic pigs revealed what appeared to be a hemorrhagic exudate in the posterior third of the small intestine. The affected area of the intestine was covered with this reddish-brown exudate which could not be readily washed off with a gentle stream of water, but could be scraped off quite easily, revealing normal-appearing intestinal mucosa beneath.

The wall of the spiral colon appeared to be quite thin. When cutting the connective tissue between the coils of the colon with scissors for the purpose of extending it for measurement, the wall of the intestine was penetrated in numerous places. This did not occur when cutting the connective tissue between the coils of the colons of the gnotobiotic control pigs or of the farm-raised pigs.

None of the pigs raised under farm conditions showed any macroscopic abnormalities at the time of necropsy.

<u>Observations at the Microscopic Level</u>

a. <u>The esophagus</u>

The esophageal lumens of the gnotobiotic pigs were devoid of bacteria whereas in both the littermate controls and the farm-raised pigs, bacteria were present. A colony of staphylococcus-like organisms was noted in the keratinized layer of the esophagus of one of the gnotobiotic pigs (Figure 2-7). No inflammatory reaction was noted around this bacterial focus.

b. <u>The stomach</u>

No differences were noted among the stomachs of the gnotobiotic pigs, the gnotobiotic controls, and the farm-raised control pigs.

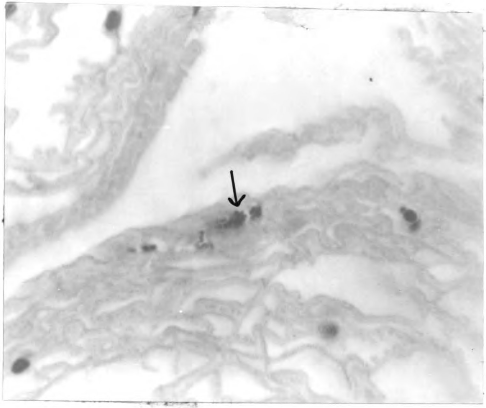

Fig. 2-7. --- Colony of staphylococci (arrow) in the superficial layer of keratin near the lumen of the esophagus in a gnotobiotic pig (S. aureus contaminated). H & E X 1600.

c. The duodenum

Fewer goblet cells were present in the duodenal glands of Lieberkühn of the gnotobiotic pigs and the gnotobiotic control pigs than in the farm-raised pigs.

d. The jejunum

No differences were noted in the jejunums of any of the pigs.

e. The ileum

In the ilea the major difference was in the lymphatic tissue. The gnotobiotic pigs (Figure 2-8) and their littermate controls had relatively smaller amounts of lymphatic tissue in the Peyer's patches than did the farm-raised pigs (Figure 2-9). The lymphatic tissue was arranged in somewhat discrete nodules in the gnotobiotic pigs and the gnotobiotic controls. In the farm-raised pigs it was present in much greater quantities and was more confluent.

Another difference noted was extensive vacuolization in the epithelium of the villi of the gnotobiotic pigs (Figure 2-10) and their littermate controls (Figure 2-11). The vacuoles had not been filled with mucin because neither the PAS reaction nor Heidenhain's mucicarmine stain indicated the presence of mucin here although the goblet cells present reacted well to these stains. The ilea of the pigs raised under farm conditions did not show such vacuolization (Figure 2-12).

f. The cecum

A golden-brown pigment was noted in the cytoplasm of the epithelial cells which lined the lumens of the ceca of the gnotobiotic pigs (Figure 2-13) and to a lesser extent in the cecal epithelium in the gnotobiotic control pigs. The pigment was noted only in those

Fig. 2-8. --- Peyer's patch in ileum of a gnotobiotic pig (S. aureus contaminated). Note the somewhat nodular arrangement of the lymphatic tissue. H & E. X 30.

Fig. 2-9. --- Ileum of farm-raised control pig showing similar area to that in Fig. 2-8. Note the large amount of lymphatic tissue and its tendency to be confluent rather than nodular in arrangement. H & E X 30.

Fig. 2-8

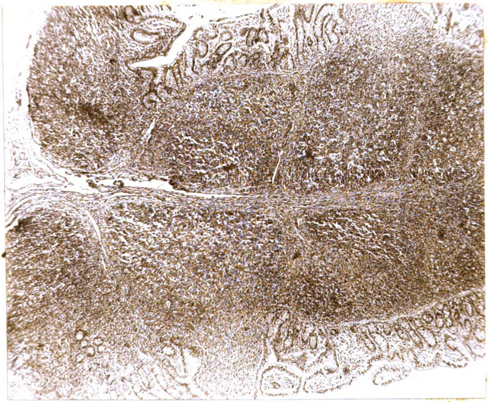

Fig. 2-9

Fig. 2-10. --- Ileum from gnotobiotic pig (S. aureus contaminated) showing the extensive vacuolization involving the epithelial cells of the villi. H & E X 80.

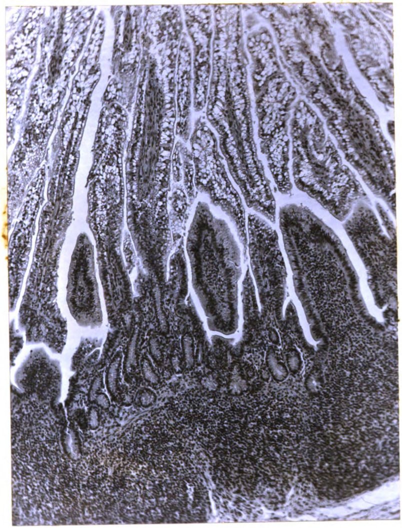

Fig. 2-11. --- Ileum from gnotobiotic control pig showing vacuolization of epithelial cells of villi. H & E X 80.

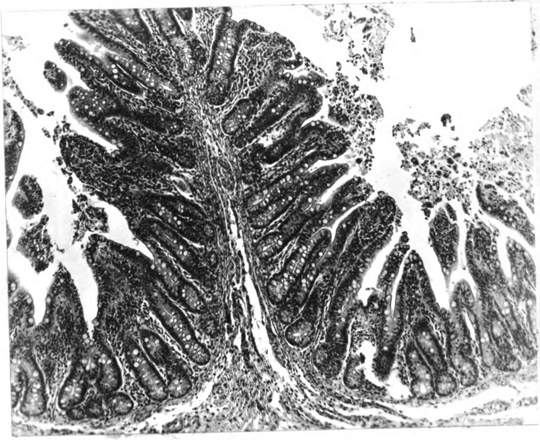

Fig. 2-12. --- Ileum from farm-raised pig showing absence of vacuolization of epithelium. H & E X 80.

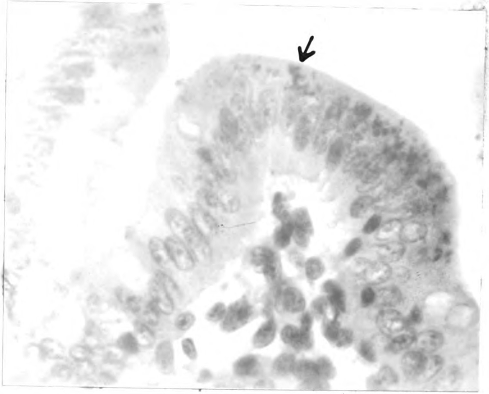

Fig. 2-13. --- Pigment in the epithelium of the cecum. Note (arrow) the pigment in the cytoplasm of the epithelium nearest the lumen of the intestine. Prussian blue stain followed by H & E X 1100.

epithelial cells which had intimate contact with the contents of the cecum. Those cells which lined the middle or fundus of the intestinal gland did not contain it. The pigment was not noted in the farm-raised control pigs. It gave a positive test for iron by the modified Mallory's stain for iron and a negative stain for bile when stained by the method of Stein.

More deeply situated in the mucous membrane of the cecum in the connective tissue stroma were noted a few macrophages which contained a similar brownish pigment. This pigment also gave a positive reaction for iron and a negative reaction for bile. The pigment-containing macrophages were seen only in the gnotobiotic pigs and in the gnotobiotic control pigs, but never in the farm-raised pigs.

g. The spiral colon

No differences were noted in the spiral colons of any of the 3 groups of pigs.

h. Terminal colon

A brownish pigment similar to that seen in the cecum was present in the epithelial cells lining the lumen of the terminal colon in the gnotobiotic pigs but was not present in the gnotobiotic controls nor in the farm-raised pigs. In all instances when the pigment was noted in the epithelial cells, it was also present in the more deeply situated macrophages.

Experiment 2. Gnotobiotic Pigs, *Bacillus* sp. Contaminated

Macroscopic and hematological observations

The second attempt to obtain germfree pigs was made on July 25, 1960. Using the technique previously described, 6 baby pigs were obtained from a Yorkshire sow. Four of them were placed in cages in a sterile rearing isolator and 2 were placed in Horsfall-Bauer units. One of the pigs in the rearing isolator accidentally bled to death within the first 2 hours from a tear in the umbilical cord.

For the first 10 days the animals were fed 4 times a day in amounts varying from 120 to 150 ml. per feeding. At 6 days of age the 2 control pigs were removed from the Horsfall-Bauer units and placed in open cages in the same room as the pigs in the plastic isolators. Beginning at 10 days of age, the pigs were fed 3 times a day and the amount of milk fed was increased until each was receiving 200 ml. per feeding.

These pigs appeared well at all times with the exception of one control pig which ate poorly for the first 7 or 8 days. At about 1 week of age, this animal started to eat and, to all outward appearances, was similar to the other control pig.

The feces of the pigs within the isolator were fluid to semi-fluid at all times and were somewhat reddish-brown in color. The feces of the 2 control pigs were usually formed and quite dry in appearance. The color was usually dark brown and never the reddish-brown seen in the gnotobiotic pigs.

At 17 days of age the pigs were removed from the isolator and following collection of blood samples were anesthetized with sodium pentobarbital and exsanguinated. Fecal samples were taken from the pigs and from the trays beneath the cages for bacteriological examination. Fecal samples were also taken from the gnotobiotic control pigs.

Cultures were made on Rogosa's agar, potato dextrose agar, SF media, violet red bile agar, and on modified Wilson-Blair medium with negative results. Cultures were also made on blood agar and incubation carried out at 37°C. The blood plates were incubated under both aerobic and anaerobic conditions.

Bacterial growth occurred on the blood agar under aerobic conditions and to a slight extent under anaerobic conditions. The organism growing under anaerobic conditions grew better when placed under aerobic conditions. The organism was isolated from all the trays and from all of these gnotobiotic pigs. It was considered to be one organism because the different organisms isolated were the same morphologically and gave identical biochemical reactions. This organism fermented glucose, mannitol, and sucrose but not lactose, glycerol, or xylose. It caused liquefaction of gelatin, produced acetyl-methyl-carbinol and catalase. It did not grow in litmus milk nor did it use citrate as the sole source of carbon. It was nonmotile, Gram-positive, and produced cylindrical, centrally-located spores. It was classified as belonging to the genus Bacillus.

Table 2-7 gives the results of the hematological examination of the gnotobiotic pigs, the gnotobiotic controls, and the farm-raised control pigs.

Postmortem examination of the gnotobiotic pigs revealed no differences from the farm-raised pigs with the exception that the distal one-third of the small intestine of the gnotobiotic pigs contained reddish-brown material which appeared to contain some blood. This reddish brown material could be removed by gentle scraping, leaving fairly normal appearing mucosa beneath. The contents of this portion of the small intestine in the farm-raised pigs were light brown in color. The contents of the colon and rectum were semi-liquid in consistency and reddish-brown to brown in color in the gnotobiotic pigs and the posterior portion of the pig including the hind legs and thighs were covered with this brownish, fluid, fecal material.

TABLE 2-7. --- Hematological values on gnotobiotic pigs (Bacillus sp. contaminated) and their controls at 17 days of age.

Pig no.	Wt.[1]	Sex	Hb[2]	Hct.[3]	RBC[4]	WBC[5]	N[6]	L	M	B	E
			Gnotobiotic pigs, Bacillus sp. contaminated								
647	5.2	F	8.4	29	5.83	6.05	33	64	2	1	0
648	4.6	M	8.8	32	5.85	3.80	30	70	0	0	0
649	4.9	M	8.8	30	5.71	4.75	33	66	1	0	0
			Gnotobiotic control pigs								
650	5.0	M	7.5	30	5.98	18.65	78	19	3	0	0
651	5.0	F	8.1	29	6.16	4.80	31	67	1	0	1
			Farm-raised control pigs								
D1471	12.3	M	9.0	32	4.62	6.9	17	83	0	0	0
D1477	13.3	F	10.9	37	4.74	6.6	24	74	0	0	2
D1489	12.0	M	11.2	38	5.16	6.0	18	80	0	0	2

[1] Weight in pounds.

[2] Hemoglobin in grams per 100 ml. of blood.

[3] Hematocrit in cell volumes per 100 volumes of blood.

[4] Erythrocytes in millions per cubic millimeter.

[5] Leukocytes in thousands per cubic millimeter.

[6] Percentages of neutrophils (N), lymphocytes (L), monocytes (M), basophils (B), and eosinophils (E).

The gnotobiotic controls showed the presence of darkened intestinal contents but it was not reddish but rather brown in color. The contents of the colon and particularly the rectum were in a semi-formed to formed state and fluid or semi-fluid feces were not noted.

The connective tissue between the coils of the spiral colon was noted to be quite edematous in one of the gnotobiotic control pigs. It was this animal which was noted to be doing poorly during the first week of life.

None of the pigs raised under farm conditions showed any abnormalities at the time of necropsy.

Observations at the Microscopic Level

a. The esophagus

The esophageal lumens of the gnotobiotic pigs were free from bacteria or any debris whereas those of the gnotobiotic control pigs and the farm-raised pigs contained much debris and many bacteria. Between the layers of desquamating keratin in the esophagus of one of the gnotobiotic pigs was noted a colony of bacillus-like organisms.

b. The stomach

No differences were noted in the esophageal portion of the stomachs among the 3 groups of pigs. The cardiac and fundic portions of the stomach of the gnotobiotic control pigs and the farm-raised control pigs appeared to have more connective tissue in the submucosa than did the stomachs of the gnotobiotic pigs. It is possible that this difference was due to differences in the state of distention of the stomach although no marked difference in this respect had been noted at the time of necropsy.

In the pyloric portion of the stomach, blood vessels in the muscle layers showed the presence of cuffs of lymphocytes. These perivascular cuffs were present in the muscle layers of the gnotobiotic pigs (Figure 2-14) and also in the gnotobiotic control pigs (Figure 2-15). In one of the farm-raised pigs, in an animal that was in no way related to or in contact with the gnotobiotic pigs or their gnotobiotic controls, a few of the blood vessels in the muscle layers also showed the presence of small cuffs of lymphocytes (Figure 2-16). Differences in the amount of connective tissue present in the submucosa of the pyloric portions of the stomachs of the 3 groups of pigs were not noted.

c. The duodenum

The duodenum showed no clearly significant differences between the gnotobiotic pigs or their controls. There was a suggestion of an increased amount of connective tissue in the submucosa of the gnotobiotic controls and to a greater extent in the farm-raised controls when these were compared to the gnotobiotic pigs.

d. The jejunum

Vacuoles were present in most of the epithelial cells of the villi of 2 of the gnotobiotic pigs and in the gnotobiotic control pigs. The epithelium of the villi of the farm-raised pigs and of 1 of the gnotobiotic pigs did not show this vacuolization. The stroma of the villi of the farm-raised pigs was more cellular than that of the gnotobiotic pigs or the gnotobiotic controls. This increased cellularity was due to the presence of greater numbers of fibroblasts, eosinophils, and large mononuclear cells.

e. The ileum

In the ileum, the epithelial cells of the villi were extensively

Fig. 2-14. --- Gnotobiotic pig (Bacillus sp. contaminated) showing the presence of a perivascular cuff of lymphocytes involving a vessel in the muscle layer of the pyloric region of the stomach. H & E X 450.

Fig. 2-15. --- Gnotobiotic control pig, a littermate to the one represented in Figure 2-14, showing presence of perivascular cuffs involving blood vessels in muscle layer of the pyloric region of the stomach. H & E X 450.

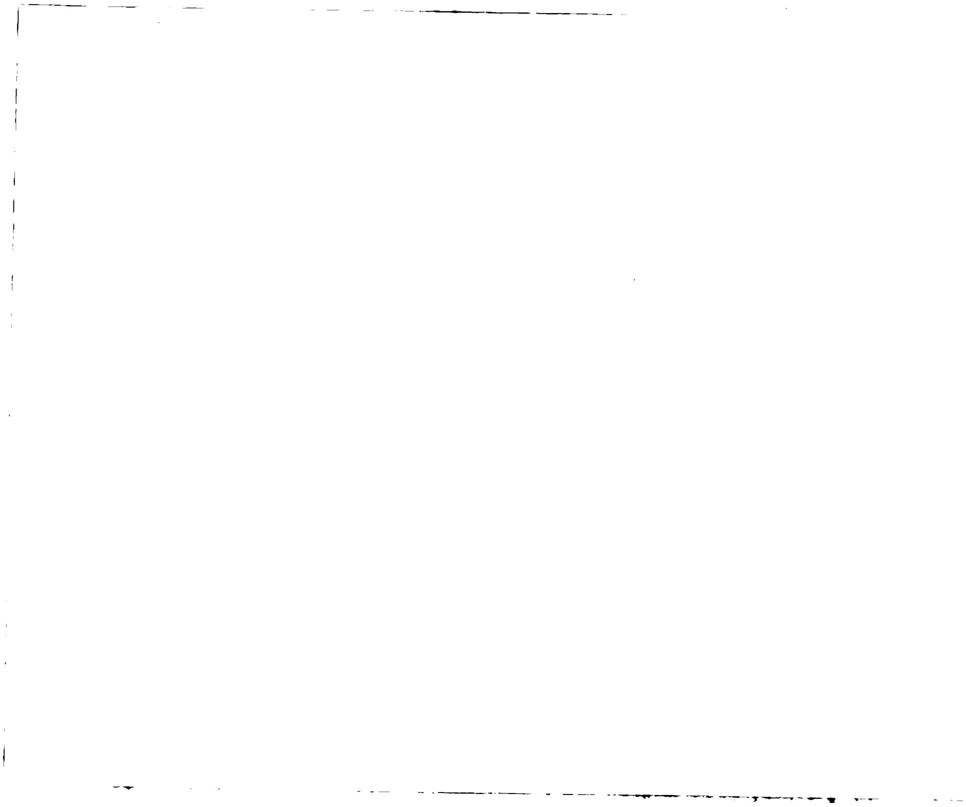

Fig. 2-14

Fig. 2-15

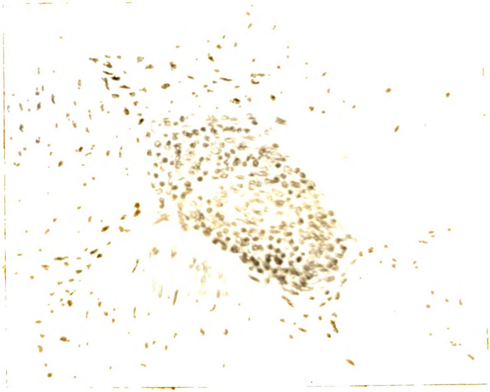

Fig. 2-16. --- Farm-raised pig showing presence of perivascular cuff of lymphocytes in the muscle layers of the pyloric region of the stomach. This pig was in no way related to those represented in the previous 2 photographs. H & E X 450.

vacuolated in both the gnotobiotic pigs and the gnotobiotic control pigs. No vacuolization was noted in the epithelial cells of the villi in the farm-raised pigs.

The most notable finding in the ileum was the variation in the amount of lymphatic tissue present. The smallest amount was present in the gnotobiotic pigs and the greatest amount in the farm-raised pigs. An intermediate amount was present in the gnotobiotic control pigs (See Figure 2-17). The lymphatic tissue present in the gnotobiotic pigs was arranged in relatively discrete nodules. Some of these nodules contained foci of lightly stained cells which were reminiscent of germinal centers. Mitotic figures, however, which are characteristically found in germinal centers, were absent. The lymphatic tissue present in the farm-raised pigs was less clearly nodular in arrangement and was present in much greater amounts than in the gnotobiotic pigs.

The epithelial cells of the villi in both the gnotobiotic pigs and the gnotobiotic control pigs showed the presence of extensive vacuolization. Vacuoles were not present in the epithelium of the farm-raised pigs.

f. The cecum

The cecum showed the presence of a brown pigment in the epithelial cells and in the macrophages present in the stroma of the mucous membrane. This pigment seemed to be the same as that already described for the animals in Experiment 1. No pigment was observed in the epithelial cells of the cecum in the gnotobiotic control pigs but it was present in macrophages within the stroma of the mucous membrane. No pigment was observed in the cecal tissues of the farm-raised pigs.

Fig. 2-17. --- Comparative amounts of lymphatic tissue in the ilea of gnotobiotic pigs and their controls. At the left is shown the ileum of a germfree pig; next is the ileum of a monocontaminated pig (Bacillus sp.); next, the ileum from a gnotobiotic control pig; and at the right, the ileum from a farm-raised pig. H & E X 1.4.

The amount of connective tissue present in the cecum of the gnotobiotic pigs appeared to be somewhat less than in the farm-raised pigs. It appeared to be more loosely arranged whereas that in the farm-raised pigs appeared to be more compact. The situation was somewhat intermediate in the gnotobiotic control pigs.

g.,h. The spiral and terminal colons

The spiral colon and the terminal colon were quite similar in microscopic morphology in all groups of animals.

Experiment 3. Germfree Pigs

Macroscopic and Hematological Observations

The third attempt to obtain germfree pigs was made on September 19, 1960. Using the techniques previously described, 13 pigs were taken from a Yorkshire sow. For reasons not exactly known, only 4 living pigs were obtained in this attempt. Three of the dead pigs were mummified and the other 6 were alive when removed from the uterus but could not be induced to breathe.

The 4 pigs obtained were small and weak. They weighed from 1.4 to 2.0 pounds at birth. When handled they squealed excessively. They ate so poorly that for the first 2 days the amount of milk fed was only 50 to 60 ml. per feeding. Two of the pigs, in particular, ate very poorly and became progressively weaker and died on the third and fifth days of life, respectively.

The 2 remaining pigs slowly improved in appearance and in vitality although they never ate as well as pigs in the previous experiments. At 21 days of age the pigs were removed from the isolator, blood samples were taken and, following anesthesia with sodium pentobarbital, they were exsanguinated. Samples of feces and other excreta from the trays were aseptically collected for bacteriological examination. Examinations of wet mounts and Gram-stained preparations of the feces and tray contents were negative for the presence of bacteria. Gross contamination is readily detected by the examination of Gram-stained smears of the feces. Figures 2-18 and 2-19 show comparable pictures of fecal smears from a germfree pig and from a farm-raised control pig.

Bacteriological cultures, made on blood agar, Eugon agar, SF media, violet red bile agar, modified Wilson-Blair medium, and Rogosa's medium were negative for bacterial growth.

Table 2-8 shows the results of the blood examinations of these animals and the gnotobiotic control pigs from Experiment 1. These animals are compared to the gnotobiotic control pigs from Experiment 1 because there were no pigs from the same litter which could be used for control purposes.

Postmortem examination showed no gross abnormalities except in the small intestine. The distal one-third of the small intestine contained a reddish-brown exudate which appeared hemorrhagic. The exudate could be scraped off leaving a fairly normal appearing mucous membrane beneath. No other gross abnormalities were noted.

Observations at the Microscopic Level

Since there were no pigs from this litter that could be used as gnotobiotic controls, those from Experiment 1 are used for comparison.

a. The esophagus

The esophageal lumens of the germfree pigs were free from debris whereas those of the gnotobiotic control pigs and the farm-raised pigs contained a considerable amount of debris and bacteria. No other

Fig. 2-18. --- Photograph of a fecal smear from a germfree pig at 21 days of age. No bacteria are seen. Gram stain X 1600.

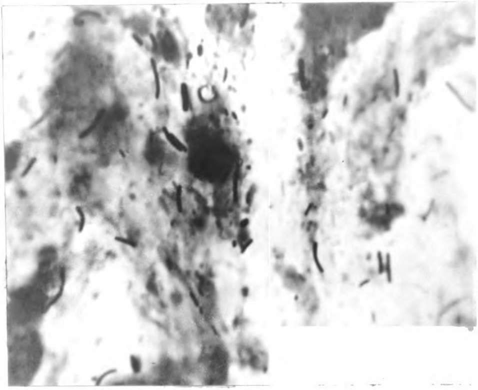

Fig. 2-19. --- Photograph of a smear made from the feces of a farm-raised pig at 21 days of age. The dark organisms are Gram-positive and the lighter ones are Gram-negative. Gram stain X 1600.

TABLE 2-8. --- Hematological values on gnotobiotic pigs (germfree) and their controls at 3 weeks of age.

Pig no.	Wt.[1]	Sex	Hb.[2]	Hct.[3]	RBC[4]	WBC[5]	N[6]	L	M	B	E
Germfree pigs											
1704	4.4	M	10.6	38	4.05	9.35	16	81	3	0	0
1705	4.1	M	12.8	37	5.61	6.00	47	50	3	0	0
Gnotobiotic control pigs											
6249	2.8	M	10.3	27	3.55	9.70	50	49	1	0	0
6250	4.2	M	12.5	37	5.08	10.05	51	49	0	0	0
Farm-raised control pigs											
1471	12.3	M	9.0	32	4.62	6.9	17	83	0	0	0
1477	13.3	F	10.9	37	4.74	6.6	24	74	0	0	2
1489	12.0	M	11.2	38	5.16	6.0	18	80	0	0	2

[1] Weight in pounds.

[2] Hemoglobin in grams per 100 ml. of blood.

[3] Hematocrit in cell volumes per 100 volumes of blood.

[4] Erythrocytes in millions per cubic millimeter.

[5] Leukocytes in thousands per cubic millimeter.

[6] Percentages of neutrophils (N), lymphocytes (L), monocytes (M), basophils (B), and eosinophils (E).

differences were noted among the germfree pigs and their gnotobiotic and farm-raised controls.

b. The stomach

No marked differences were noted in the different areas of the stomach. In the cardiac level of one of the germfree pigs, the connective tissue in the submucosa appeared to be somewhat more loosely arranged than that in the gnotobiotic control or farm-raised pigs. The other germfree pig did not show such loosely arranged connective tissue.

c. The duodenum

No differences were noted in the duodenums from any of the pigs.

d. The jejunum

In the jejunum, more goblet cells were present in the epithelium of the villi in the germfree pigs than in the gnotobiotic control or the farm-raised pigs. There appeared to be less connective tissue and that which was present appeared to be more loosely arranged in the germfree pig when compared to the gnotobiotic control pigs or the farm-raised pigs.

e. The ileum

In the ileum of the germfree pig, there were no foci of lymphocytes as in the gnotobiotic control pigs or in the farm-raised pigs. Typical Peyer's patches were present in the gnotobiotic control pigs and the farm-raised pigs but the germfree pigs showed only scattered lymphocytes at this level of the intestine. The epithelium of the intestinal mucosa at this level was greatly vacuolated as was that in the gnotobiotic controls. The epithelium of the villi in the ileum of the farm-raised pig was not vacuolated.

f. The cecum

The germfree pig showed the presence of more goblet cells (Figure 2-20)

Fig. 2-20. --- Cecum of germfree pig showing numerous goblet cells in the epithelium and loose connective tissue in the submucosa. H & E X 80.

Fig. 2-21. --- Cecum of gnotobiotic control pig showing fewer goblet cells in the epithelium and denser connective tissue in the submucosa. H & E X 80.

Fig. 2-20

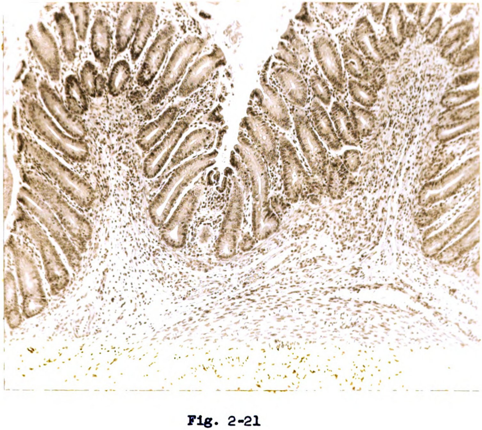

Fig. 2-21

at the cecal level than did the gnotobiotic controls (Figure 2-21) or the farm-raised pigs (Figure 2-22). The connective tissue in the submucosa was less dense and more loosely arranged than it was in the gnotobiotic controls or the farm-raised pigs. The epithelial cells in the germfree pigs and in the gnotobiotic controls contained a yellowish-brown pigment. This pigment was also seen in macrophages in the stroma of the mucous membrane. It was not seen in the farm-raised pigs.

g. The spiral colon

In the spiral colon of the germfree pig the epithelium contained many vacuolated cells; these were fewer in number in the gnotobiotic control pigs and absent in the farm-raised pigs. The connective tissue in the submucosa appeared to be more loosely arranged and less dense than in the gnotobiotic control pigs and the farm-raised pigs.

h. The terminal colon

No marked differences between the terminal colons of the germfree pigs and their controls were noted. The brownish pigment noted in the cecum was present in the epithelium of the terminal colon of the germfree pig but it was not noted in the gnotobiotic control pigs or in the farm-raised pigs.

Autolysis in the Dead Germfree Pig

The 2 germfree pigs which died in Experiment 3 were allowed to remain in their cages for 3 and 5 days, respectively, following death. The purpose was to determine changes which take place in the digestive tract following death of the animal in the absence of bacteria. Since digestive enzymes are present in the tract, digestion of the tissues of the intestine should take place in the absence of the protective

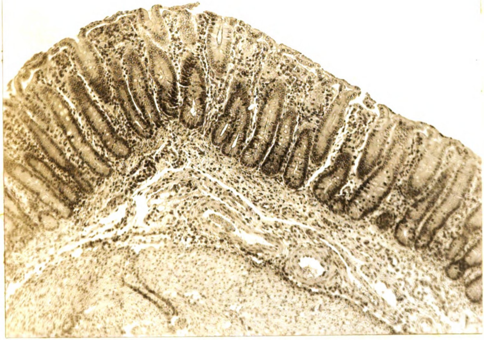

Fig. 2-22. --- Cecum of farm-raised control pig showing fewer goblet cells in epithelium and dense connective tissue in the submucosa. H & E X 80.

substances which must be produced during life to prevent auto-digestion of the digestive organs.

Gross examination of these pigs indicated dehydration as evidenced by the presence of sunken eyes and dry, tight, closely adherent skin. Offensive odors were absent. The digestive organs were quite soft and slimy to the touch.

a. The esophagus

In the animal which had been dead for 3 days, the epithelium of the esophagus was in a fairly good state of preservation. The nuclei did not show evidence of pyknosis. The mucous glands in the submucosa were quite badly degenerated. Most of the nuclei of these glands were pyknotic and some had undergone complete lysis. The muscles were in a fairly good state of preservation. Cross striations were still visible and some of the nuclei were intact, although most had undergone some degree of pyknosis. (See Figure 2-23). The erythrocytes within blood vessels were still intact.

In the animal which had been dead for 5 days, the epithelium was markedly degenerated although some distinguishing characteristics were still apparent. Cross striations were still visible in the muscles (Figure 2-24) but most of the nuclei were markedly pyknotic. Intact erythrocytes were still visible within some blood vessels.

b. The stomach

In the pyloric region of the stomach of the germfree pig which had been dead for 3 days, the mucosa had undergone some degree of disruption but most of the gland cells still were in their normal relationships. Nuclei in the glandular epithelium had undergone some degree of karyorrhexis. Most of the connective tissue elements in the stroma of the

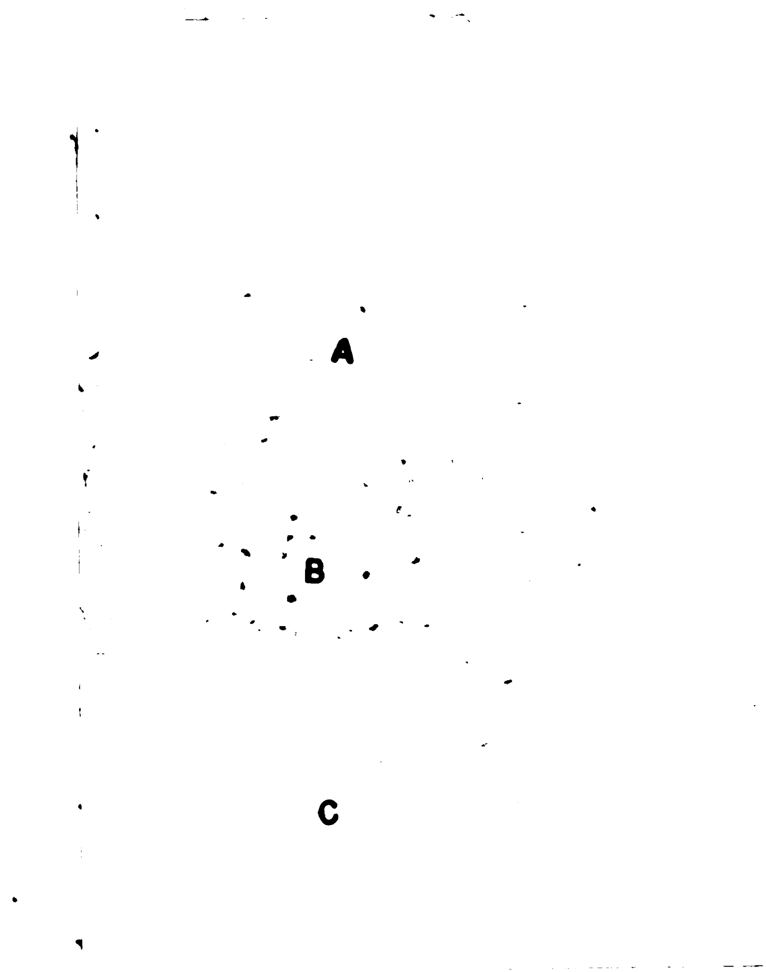

Fig. 2-23. --- Esophagus of germfree pig dead for 3 days. Note the relatively intact epithelium at A, the degenerating mucous gland at B and the intact arrangement of the muscles at C. H & E X 450.

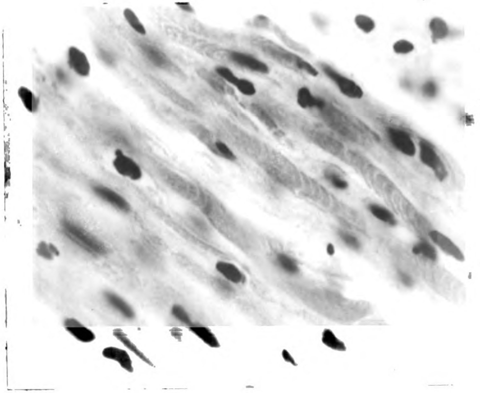

Fig. 2-24. --- Esophagus of germfree pig dead for 5 days. In spite of some post-mortem change, there are still demonstrable cross striations in the muscles.
H & E X 1600.

mucosa exhibited pyknotic changes in their nuclei. Muscle layers were fairly well preserved but here, too, the nuclei were pyknotic. Erythrocytes in some of the blood vessels were still intact.

At 5 days following death, degenerative changes in the stomach had made quite extensive progress. The mucous membrane was badly disrupted and most of the nuclei had undergone varying degrees of pyknosis and/or karyorrhexis. Submucosal connective tissue showed marked autolysis. The cells making up the blood vessels appeared to be the best preserved tissue in the entire stomach. The muscle layers of the stomach were fairly well preserved but the nuclei were quite pyknotic.

c. The small intestine

The small intestine of the germfree pig dead for 3 days showed marked disruption and autolysis of the mucous membrane. Despite the marked degeneration, some intact epithelial cells with well preserved nuclei were still found in the debris within the lumen of the intestine. It appeared as if some glue-like substance which in life had held the epithelial cells in place had dissolved away, setting free the intact epithelial cell which now floated with other epithelial cells in the lumen of the intestine.

Only remnants remained of the glands of Bowman in the duodenum and the few nuclei which remained were markedly pyknotic. The muscle layers were intact but most of the nuclei were pyknotic.

At 5 days following death, the autolytic changes seen at 3 days were more advanced. Surprisingly enough, although the disruption of the mucous membrane was marked, some few well-preserved epithelial cells with quite well preserved nuclei were still visible. The muscle layers were rather well preserved although all nuclei were pyknotic.

d. The spiral colon

In the spiral colon of the pig dead for 3 days, the autolytic changes in the mucous membrane were extensive. Some of the epithelial cells were still intact and in some places, there was a suggestion of glandular arrangement remaining. The muscle layers were quite intact and most of the nuclei were pyknotic but there was a discernible gradation in the severity of the pyknosis. Some of the larger blood vessels contained intact erythrocytes (Figure 2-25).

In the animal dead for 5 days, the spiral colon showed a greater degree of autolysis than at 3 days. Most of the nuclei had disappeared and most of those remaining were pyknotic. A few epithelial cells were surprisingly well preserved (Figure 2-26). The muscle arrangement was rather well preserved but most of the nuclei were pyknotic.

e. The terminal colon

The stratified squamous epithelium at the recto-anal junction was very well preserved in the pig which had been dead for 3 days. The cytoplasm of the epithelium stained somewhat more red than is normal for the hematoxylin and eosin stain on this tissue but the nuclear staining was very good, indicating very good preservation of this tissue.

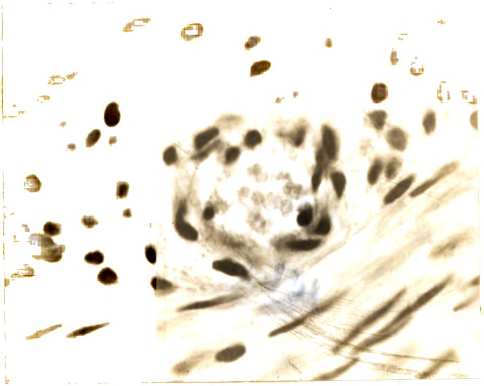

Fig. 2-25. --- Blood vessel containing intact erythrocytes from the spiral colon of a germfree pig dead for 3 days. H & E X 1600.

Fig. 2-26. --- Degenerating epithelium in spiral colon of a germfree pig dead for 5 days. Note the still discernible gland-like arrangement of some of the epithelium.
H & E X 450.

DISCUSSION

General Procedure

One of the difficulties in germfree work is to be able to collect the young as close to the end of the gestation period as possible. Gustaffson (1946-1947, 1948) discussed in detail the determination of the time of parturition in the rat and Glimstedt (1936) devoted a considerable amount of discussion to the problem in the guinea pig.

A probable explanation for the weak, small and squealing pigs obtained in Experiment 3 is that they were taken too early in the gestation period. It is best if the investigator has control of the swine-herd from which he is to derive the germfree animals. He can then know the exact breeding date and the time of expected parturition can be accurately determined. If one depends on the swine raiser in the community to supply the pregnant animals, disappointment is often the result because the swine raiser either does not knowthe exact breeding date or, for the sake of a sale, gives an erroneous date. This results in frustration for the investigator. One is greatly chagrined to prepare for the hysterectomy and find that during the preparation the sow delivers a litter of pigs. On the other hand, it is just as frustrating to perform a hysterectomy on the calculated date and find that the pigs were taken too early as evidenced by small, weak and squealing pigs.

Determination of the presence of milk in the udder was used as an indicator of imminent parturition, but even here, despite the frequent observations carried out, one was often greeted by the sound of squealing pigs before the hysterectomy could be done.

Nutrition

Another difficulty in the raising of germfree pigs is the problem of nutrition. It can be seen from the examination of Tables 2-6, 2-7, and 2-8 that the growth responses of the gnotobiotic pigs and the gnotobiotic control pigs were not good. These pigs, fed cows' milk which had been fortified with vitamins and minerals, did not grow as well as pigs raised under farm-conditions.

The frequency of feeding may be a factor in the poor growth rate. The pigs in these experiments were fed 4 times a day in amounts which they would consume in a reasonable period of time. The amount of milk consumed in a day was small. More frequent feeding would undoubtedly have increased the amount consumed and probably would have given a better growth response.

A big inhibitor to a proper start is the fact that these pigs did not receive colostrum. Sows' colostrum, according to Braude _et al_. (1947), contains more than 3 times as much protein as does sows' milk. In addition, the colostrum contains larger quantities of the B vitamins than are present in whole milk. Heidebrecht _et al_. (1951) have shown that the amount of vitamin A in colostrum is much higher than in whole sows' milk.

In addition, not only were the pigs deprived of colostrum but the injury was compounded by feeding cows' milk, which does not approach sows' milk in nutritional value for the baby pig. According to Eckles and associates (1951), cows' milk is deficient in fat, protein, and minerals when compared to the milk of the sow. For proper growth response, the diet of the baby pig fed cows' milk should be fortified with protein, in particular, and probably also with fat. These matters of milk differences may be primarily a difference in energy supply. Sows' colostrum and milk supply more energy than that from the cow.

Another factor that may have affected the growth response is the possibility of vitamin deficiencies. Even though the diet was fortified with vitamins in amounts so as to provide the requirement despite a possible 50% or more destruction during sterilization, the possibility of antivitamin formation during the sterilization process is present. Figures 2-20, 2-21, and 2-22 show the cecum from a germfree pig, a gnotobiotic control pig, and a farm-raised pig, respectively. There are more goblet cells present in the germfree pig than there are in the farm-raised pig. The gnotobiotic control pig occupies a somewhat intermediate position. It is possible that the increased numbers of goblet cells indicate a mild niacin deficiency. If this is true, it is hard to explain why the gnotobiotic pig does not show a similar picture since it was fed the same sterilized milk diet.

Bacteriology

The determination of the germfree state was by cultural methods and by examination of both Gram-stained fecal smears and the observation of wet mounts of feces. The incubation of cultures, with the exception of those in Experiment 1, was done at 37°C. The special media were used to facilitate growth of the more common organisms expected from the digestive tract of the pig. The animals from Experiment 3 were considered to be free of microbial contamination under the conditions used in this study to determine sterility. Tissue cultures and egg inoculation to determine the presence of viruses as suggested by Wagner (1955) were not done.

The examination of Gram-stained fecal smears readily shows the presence of gross contamination. In the germfree animal, the fecal smear rarely showed the presence of an occasional organism following the Gram stain. These organisms were thought to be those killed during the sterilization of the milk but passing through the digestive tract of the pig in an unchanged form.

In Experiment 1, a sour odor was noted at the air outlet of the isolator early in the experiment. This sour odor disappeared after a few days. The cause of the acid fermentation was not determined. Although Staphylococcus aureus, the only organism isolated from this group of pigs, ferments lactose, one would expect that if the acid production was of an amount sufficient to generate an odor, it should have been present in sufficient quantities to cause coagulation of the milk. Coagulation of the milk was never observed. It is also hard to explain the reason for the disappearance of the sour odor after a few days. Was there a species of bacteria present early in the experiment which subsequently died out? This seems unlikely. At the end of the experiment, only Staphylococcus aureus was demonstrated to be present. It is possible that the media used were not able to support the growth of this postulated organism. This also seems unlikely since Eugon agar contains dextrose which should serve as a source of energy for any organism which can ferment lactose. Furthermore, only Gram-positive cocci were ever observed in fecal smears, with the exception of the possible Gram-negative rods observed on the third day. These rods were never again observed and re-examination of the original preparation disclosed only the presence of needle-like crystals of the counterstain safranin which may have been mistaken for Gram-negative rods in that first examination.

Another possibility, however remote it may be, is that there was another organism present which could ferment only glucose. This organism could have multiplied for a time in the milk deriving its energy from the fermentation of the small amount of glucose which must have been present in the milk as a result of the slight hydrolysis of lactose during the steam sterilization of the milk. When this glucose was consumed by the organism, the organism died out. This might mean that only a very small amount of acid was produced, not enough to coagulate the casein in the milk but enough to give a slight acid odor at the air outlet of the isolator.

With reference to odor it might be mentioned that at the termination of each of the 3 experiments, one was surprised at the pleasant odor of the gnotobiotic pigs. They never had an unpleasant odor nor did they remind one of farm-raised pigs which exhibit a typical "piggy" odor. The odor noted in all three groups of gnotobiotic pigs was somewhat reminiscent of that noted in a candy shop and made one think of sweet chocolates. Gustafsson (1948) notes that the cecal contents of the germfree rat exhibited a peculiar sweet smell very different from the penetrating stench noted in cecal contents of normal control rats.

<u>Fecal color and epithelial pigmentation</u>

The feces of the gnotobiotic pigs were consistently quite soft and were never formed. Glimstedt (1936) noted that a soft condition of the feces appeared to be a peculiarity of germfree animals. He gave no reason for this.

The color of the feces in these animals was somewhat reddish-brown. In the gnotobiotic control pigs the feces were dark brown in color.

It is possible that at least one of the factors in this difference in color was the presence of hydrogen sulfide producing bacteria in the gnotobiotic control pigs. Black iron sulfide could very readily cause a darkening of the feces.

Another factor which may be involved in causing the reddish color is the absence of bacteria in the intestinal tract which are necessary for the breakdown of bilirubin. Hoffman (1954) states that as bilirubin passes down the intestine to the colon it is reduced by anaerobic bacteria to two colorless compounds of similar composition, mesobilirubinogen and stercobilinogen. Since anaerobic bacteria were absent in these pigs, the bilirubin passed on through the intestinal tract unchanged, maintaining its reddish color.

In the cecum and colon, a golden-brown pigment was noted in the cytoplasm of the epithelial cells which lined the lumen and in macrophages located more deeply in the stromal tissue of the intestine. This pigment was not noted in the farm-raised pigs but was present in the gnotobiotic pigs and the gnotobiotic controls. It was present in larger amounts in the gnotobiotic pigs than in the gnotobiotic controls. Its source is unknown. It was thought that it might be related to the reddish-brown intestinal contents since it was found only in those epithelial cells in intimate contact with the intestinal lumen. It was first thought that this pigment might be bilirubin, but the Stein stain for bile pigments was negative. Since it was also present in the gnotobiotic control pigs, the possibility of bilirubin should be discarded because these pigs had an almost normal complement of intestinal bacteria including anaerobes as shown in Table 2-5, and bilirubin would no longer have been present in appreciable amounts in the cecum and colon.

Application of Mallory's Prussian-blue stain indicated the presence of ferric iron in this pigment, which more or less rules out the possibility that this pigment is a bilirubin-related substance.

Another possibility is that this pigment is the iron component of the mineral supplement, ferric ammonium citrate. In the gnotobiotic pig this iron probably would not be converted to the black, insoluble iron sulfide because of the absence of hydrogen sulfide-producing bacteria. The absence of these bacteria would allow the iron-containing compound to reach the lower intestine in a soluble form, where it would be available for absorption by the epithelium of the intestine.

The mechanism of the absorption of the iron-containing compound is not explained, nor is it explained why the iron is in the ferric form. Ferric iron is reduced to the ferrous form in the digestive tract in a reaction associated with the hydrochloric acid of the stomach. It is the ferrous form which is absorbed in the small intestine for incorporation into ferritin. The iron of ferritin is ferric. The oxidation of the absorbed ferrous iron to the ferric form presumably takes place within the absorbing epithelial cell of the intestine.

It is not known whether the ferric ammonium citrate reaches the cecal cells in an unchanged form or whether the form of iron in the cecal lumen is ferrous which is changed to the ferric form after absorption into the epithelial cell of the cecum. The nature of the compound which makes up the iron-containing pigment is also unknown.

The fact that a small amount of this pigment was also present in the epithelial cells of the cecum and colon in the gnotobiotic control pig is difficult to explain since sulfide-producing bacteria were present here. It is possible that some of the iron in the lower intestinal tract escaped the action of the hydrogen sulfide.

Whether these explanations adequately tell the story of the pigment or not, the fact remains that only the gnotobiotic pigs and the gnotobiotic control pigs received ferric ammonium citrate in their feed and the farm-raised pig did not. The pigment was never noted in the epithelial cells of the intestine of the farm-raised pig.

The presence of edema in the intestinal wall of one of the gnotobiotic control pigs of Experiment 2 can possibly be explained by the lack of gamma globulin in the serum caused by the fact that these pigs had been deprived of colostrum. This possibility was reported by Beran et al. (1960) who observed this in twelve colostrum-deprived pigs. The fact that this was observed in only one colostrum-deprived pig in the present study may be due to an individual idiosyncrasy or it may be associated with the fact that this pig had not been eating well during the first week of life, thereby aggravating a deficiency so far as serum proteins are concerned.

The significance of the presence of the perivascular lymphocytic cuffs in the muscle layers of the pyloric region of the stomach is not known. The fact that they were found, not only in the gnotobiotic pigs and the gnotobiotic controls which were littermates, but also in a completely unrelated farm-raised pig, decreases the possibility that it was due to a viral infection although this cannot be ruled out.

Morphology

The intestinal walls of the gnotobiotic pigs appeared to be thinner than those of the gnotobiotic controls and the farm-raised pigs. This lesser thickness was probably due to a lesser amount of connective tissue in the submucosa. A lesser amount of connective tissue in the germfree chicken and rat was reported by Gordon (1955 and 1959). The thinness may also be due in part to the lesser amount of lymphatic tissue present, demonstrated in Figures 2-8, 2-9, and 2-17.

The large terminal Peyerian gland that was reported by Chauveau (1873), but which was not observed in the pigs studied by Titkemeyer and Calhoun (1955), was present in all the farm-raised pigs examined in this study. It was not easily detected by gross examination in the gnotobiotic pigs or the gnotobiotic control pigs, but in none of these did random sectioning of the terminal ileum fail to disclose the presence of at least some lymphocytes, even though these were not in nodular arrangement.

The significance of the vacuoles seen in the epithelial cells of the small intestines of the gnotobiotic pigs and their gnotobiotic controls as shown in Figures 2-10 and 2-11 is unknown. The change was apparently a hydropic degeneration. Mucin was not demonstrable here with special stains.

It is interesting to note the effect that the presence of bacteria must exert on the post-mortem decomposition of the digestive tract. The fact that intact erythrocytes were still present within blood vessels of a germfree pig which had been allowed to remain at room temperature for 3 days following death indicates that bacteria play an important role in post-mortem decomposition. The epithelium of the intestine, although no longer attached to its normal position on the basement membrane, was fairly well preserved. The nuclei were not even pyknotic in some instances 5 days after death. Thus it appears that, although autolytic enzymes are active in decomposition after the death of an animal, the role of bacteria in these processes probably is much more important.

SUMMARY

Three experiments were conducted in attempts to rear gnotobiotic pigs for the purpose of studying the digestive tract and making a morphological comparison of it to that in the farm-raised pig. The pigs were obtained by hysterectomy and housed in sterile plastic isolators. The farm-raised pigs were obtained by natural birth in typical farm surroundings. The first two attempts were unsuccessful and the animals obtained were contaminated. One of the experimental groups was contaminated with Staphylococcus aureus and the other with a member of the genus Bacillus. In the third experiment, germfree animals were obtained. All pigs were raised for 17-21 days.

Growth responses of the pigs in the three experiments were poor. This was probably due primarily to qualitative and/or quantitative nutritional deficiencies.

The intestinal tracts of the gnotobiotic pigs were compared to those of littermate controls and to farm-raised pigs of the same age. On necropsy, the only macroscopic difference which was noted between the gnotobiotic pigs and their controls (besides differences in condition and weight) was the presence of brownish-red fecal material in the intestine of the gnotobiotic pigs which was not present in the control pigs. This material was ascribed to the presence of unaltered bilirubin in the intestines.

Microscopic comparison showed that the gnotobiotic pigs had less connective tissue in the submucosa of the intestines. This connective tissue was also more loosely arranged in the gnotobiotic pigs than in the littermate control pigs or the farm-raised pigs. The amount of lymphatic tissue present in the small intestine of the gnotobiotic pig was less than that present in the littermate controls or in the farm-raised pigs. The epithelium of the ileum was greatly vacuolated in the gnotobiotic pigs and in the gnotobiotic control pigs. The ileum of the farm-raised pigs showed no such vacuolization. In the cecum, more goblet cells were present in the mucosa in the gnotobiotic pigs than in their controls. In one experiment (Bacillus sp.) the arterioles in the pyloric region of the stomach showed the presence of cuffs of lymphocytes. This was seen in the gnotobiotic pigs as well as in their controls, including the farm-raised pigs.

The epithelial cells of the cecum and colon of the gnotobiotic pigs and the gnotobiotic control pigs contained a brown, iron-containing pigment which had as its possible source the ferric ammonium citrate present in the mineral supplement fed the pigs. This pigment was also present in the macrophages situated deeply in the intestinal wall.

The monocontaminated pigs were similar in most respects to the germfree pigs except for the presence of bacteria.

Tissues from the intestines of germfree pigs dead for 3 to 5 days showed much less decomposition than one might expect in dead animals which have a normal complement of bacteria in the digestive tract.

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