EXPERIMENTAL COLIBACILLOSIS IN GNOTOBIOTIC PIGS

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ABSTRACT

EXPERIMENTAL COLIBACILLOSIS IN
GNOTOBIOTIC PIGS

by Bruce Robert Christie

Research was conducted using a total of 62 gnotobiotic pigs in 3 experiments to determine the clinical effects, pathogenesis and lesions produced by 2 closely related serotypes of Escherichia coli--O138:K81:NM (Michigan origin) and O138:K81 (Minnesota origin).

It was determined that both serotypes readily colonized in the intestinal tract from experimental infection simulating neonatal contamination of the umbilical stump. Oral exposure was precluded.

A bacteremia resulted within 1 1/2 hours after injection, and by 24 hours the infection was generally established in the mucosa of the gastrointestinal tract. By 48 hours after injection the bacteremia had subsided so that only an occasional isolation from organs other than the gastrointestinal tract was made.

The clinical signs of profuse watery diarrhea, dehydration and elevated haircoat characteristic of colibacillosis in the neonatal pig were not apparent until the organism became well established in the gastrointestinal tract. The

clinical signs of infection with E. coli O138:K81 (Minn.) were significantly milder than those resulting from infection with E. coli O138:K81:NM (Mich.), but edema of the subcutis was a constant feature of infection with the former.

Oral exposure of 1 germfree litter to E. coli O138: K81:NM (Mich.) produced both heavy colonization of the entire intestinal tract and diarrhea within 4 hours. Intermittent bacteremia was evident in this litter.

No definite trends in body temperature or hematologic findings were observed, although in some animals there was a marked reduction in leukocyte counts.

The necropsy findings, in general, agreed with those reported in the literature from field cases of colibacillosis in neonatal pigs.

Histologically the lesions were predominantly in the gastrointestinal tract and ranged from an acute hemorrhagonecrotic enteritis to a histological picture, in persistently scouring pigs, microscopically indistinguishable from that seen in clinically normal germfree animals. The most common lesion observed in the epithelium of the villi of infected pigs was hydropic degeneration. Basic changes in apparent function of the villi were also noted. It is hypothesized that the changes observed result in malabsorption.

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By

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For "Smitha"
Mrs. Sally Kleinschmidt.
1965 - 1967.

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INTRODUCTION

The distribution of various bacterial species in the intestine of the normal pig is influenced by the age, diet, level of intestine and the bacterial species themselves.

In recent years there has been a renewed interest in the association of Escherichia coli with neonatal diarrhea in human infants, calves and pigs. There have been periods in the study of this association when an appreciation of the commensal nature of the organism in the normal gut flora has led investigators away from serious consideration of the role of E. coli as a pathogen.

The ability to differentiate strains of E. coli by serological typing, initiated by Kauffman (1943) and developed by others, has enabled relationships between strains, host animals and pathogenicity to be established.

Pigs derived by hysterotomy or hysterectomy and maintained free of all detectable microorganisms, or specifically contaminated with known organisms, are called "gnotobiotic" pigs. The recent development of techniques that make such animals available has been of value to pathology and microbiology. There have been a number of attempts to characterize the etiology and pathogenesis of colibacillosis in neonatal pigs. Some of the most valuable work has been done

with hysterectomy-derived, colostrum-deprived and artificially reared pigs. There are, however, many concepts concerning pathogenesis of colibacillosis that have little basis in fact. It is likely that the basic problems of the elucidation of the syndrome of colibacillosis can most successfully be investigated in "germfree" animals.

The objectives of this study were:

1. To observe the route by which E. coli O138:K81:NM (Michigan origin) established an infection in gnotobiotic pigs following introduction of the organism into the subcutis of the umbilical stump without contamination of the external environment or oral contamination of the experimental animals.

2. To observe the route by which E. coli O138:K81 (Minnesota origin)--the same serotype referred to above, but from a different source and with a known different clinical expression--established itself in the gnotobiotic pigs, using the same experimental conditions as used with the above serotype in order to provide a basis for comparison of these organisms.

3. To observe the route by which E. coli O138:K81:NM established an infection in gnotobiotic pigs following oral inoculation of the organism.

4. To compare the clinical syndrome produced by oral inoculation of gnotobiotic pigs with E. coli O138:K81:NM, and the syndrome produced by injection of the same organism into the subcutis of the umbilical stump.

5. To observe and record the gross and microscopic lesions resulting from infection of germfree pigs with these two serotypes of E. coli.

LITERATURE REVIEW

Escherichia coli is an organism of many races, of great variety serologically and continually varying in virulence. It was Theobald Escherich, in 1885, who first found and described "*Bacterium coli commune*" from the feces of breast-fed babies. His detailed description leaves no doubt that this was the organism we now know as *E. coli* (Sojka, 1965). Escherich did, however, consider this organism to be a harmless saprophyte, and his published opinions might have had considerable bearing on the fact that it has been only in recent years that a renewed interest has been taken in the potential of *E. coli* as an important pathogen of both man and animals.

Reliable methods of classifying *E. coli* became available in the 1940s with the discovery of the "O" agglutination masking effect of the "L" antigen and subsequent publications concerning the antigenic structure of *E. coli* (Kauffman, 1944; Kauffman, 1947; Kauffman and Dupont, 1950). Since then classification of the organism according to serotype has been widely accepted, and the literature pertaining to serotyping of *E. coli* antigens has been reviewed and tabulated by Edwards and Ewing (1962). Some serotypes of *E. coli* are pathogenic in their own right. Most serotypes,

however, are of low virulence and cause disease because of either their overwhelming numbers or because the host has been weakened by other factors.

Certain strains of E. coli produce powerful endotoxins capable of evoking tissue reaction when the organism invades tissues outside those of its normal habitat (Ribi et al., 1964), but one assumes that the power to invade tissues is not necessarily associated with the pathogenicity of the endotoxin of the organism.

Colibacillosis in Human Medicine

It is of concern to note in important medical publications in this field statements such as that of Robbins (1962) that strains of E. coli are separable by serologic methods, but that these immunologic differences are "apparently not important in clinical medicine."

In human medicine certain serotypes of this organism are important etiologic agents of focal pyogenic skin infections, urinary tract infections, peritonitis, acute appendicitis, cholecystitis, cholangitis, and infectious biliary cirrhosis. In all of these sites E. coli evokes a nonspecific suppurative reaction with a degree of abscess formation that is often indistinguishable from reaction of tissues to invasion with Staphylococcus spp. (Robbins, 1962). In addition, there are specific strains of E. coli, identifiable by serotyping procedures, that produce severe forms

epidemic diarrhea of infants. These infections are often associated with pneumonia and other bacteremic complications.

The importance of the immunologic differences between strains of these organisms is that strains that are important in clinical medicine can now be identified and separated from those of less clinical significance.

Infections with E. coli can occur at any age but are particularly severe in infancy and in advanced life. The age distribution is probably due to lack of immunity in the young or general debility and increased susceptibility of the aged (Jubb and Kennedy, 1963). Escherichia coli sets up a symbiotic residence in the intestinal tract almost at birth, and it is thought that the organisms can enter the tissues of the host from this source via the lymphatic route or by contamination of the body surfaces or through breaks in these surfaces (Hopps, 1966).

Colibacillosis in Veterinary Medicine

In the veterinary field, Jubb and Kennedy (1963) summarized the prevailing concepts when they listed the three basic situations in which this organism behaves as a pathogen. Certain strains act as primary pathogens; others of lower virulence act as pathogens only when they are present in numbers sufficient to overwhelm the host tissue defenses; and other strains act as secondary or opportunist invaders

which enter and produce, or combine with other organisms to produce, disease in a host tissue weakened by a previous invader or stress situation. In the latter case it is considered that, once the strain of E. coli has become established, the predisposing factors may become unnecessary. As an example, young animals contract the disease and may die without the predisposing factors' operating. These authors (Jubb and Kennedy 1963) list the predisposing stress factors in young nonprimates: (1) inadequate housing with exposure to extremes of weather; (2) mistakes in dietary management (often overfeeding); (3) insufficiency of colostrum; (4) congenital weakness; and (5) specific vitamin deficiencies.

In a study of "calf scours" Smith and Crabb (1956) could not find evidence of a specific strain of E. coli associated with an individual outbreak of the disease. They found, in the healthy control group, 22 of the 23 phage types of E. coli that had been isolated from the diseased group. They also observed a change in the predominant phage type during the course of the disease.

It is not yet known what factors are associated with virulence in a particular strain, and there is no in vitro test for this property available at this time. Gay (1965) has recorded that, in a particular species of host animal, there is variation and overlapping in the lethal dose between serotypes considered to be pathogens and nonpathogenic

strains from healthy animals. Studies on the pathogenicity of these strains, using laboratory animals or chick embryos, are of little value.

In the experience of some workers (Saunders et al., 1960) a specific serotype may or may not be hemolytic, the conditions of incubation (particularly the carbon dioxide potential) being a determinant in this phenomenon.

In general there is no relationship between virulence and O and K antigens. With serotypes isolated from "gut edema" and coliform gastroenteritis of swine, however, Gay (1965) recorded a relationship between these two antigens and pathogenicity.

The most recent review of the identification and classification of the serotypes of the E. coli group of importance in veterinary medicine is well documented, brief and quite comprehensive (Gay, 1965).

Colibacillosis in Swine

Much confusion has resulted from the consideration by many workers that diarrhea of baby pigs is an entity rather than a clinical sign. The relationship between E. coli and porcine neonatal diarrhea has been accepted for a number of years.

It was Jensen (1899) who first recorded that an infection by E. coli could produce diarrhea in baby pigs.

In 1934, there was described an outbreak of diarrhea in 3-day-old pigs that was considered to be a manifestation of E. coli infection (McBryde, 1934). In his account McBryde described a duodenitis and a jejunitis with no obvious lesions in other organs. He cultured E. coli from internal organs and mesenteric lymph nodes of these pigs.

Glässer, Hupka and Wetzel (1950) described an acute outbreak of swine enteritis associated with E. coli infection as being characterized by diarrhea, anorexia and, at necropsy, catarrhal gastritis and enteritis with redding and swelling of the intestinal mucous membrane.

In their investigation of an acute diarrhea affecting pigs within 6 hours and up to a few days after farrowing, Gordon and Luke (1958) found that the pathogen involved was E. coli. The disease was characterized by high mortality in a litter that was apparently normal at the time of farrowing. They observed that shortly after birth the pigs became dull and were disinclined to suckle. A profuse diarrhea (with yellow to orange feces) resulted in dehydration, followed at the end of the first and second day by coma and death. Less acute cases were seen to have "scalded" hindquarters and, in some pigs, necrosis and sloughing of the tail. Gastroenteritis was a consistent sign at necropsy. Almost pure cultures of E. coli were grown from large intestine and stomach samples, and occasional isolations of the same organism were made from liver and spleen.

Saunders et al. (1960), while investigating 58 outbreaks of disease in pigs in which E. coli was considered significant, came to recognize a clinically well defined syndrome affecting piglets within an age range of 48 hours to 2 weeks. The syndrome that these workers attributed to infection with E. coli was characterized by purity, abundance and consistency of the serotype; absence of these features from other cases examined routinely during the same period was noteworthy. The finding of Saunders et al. (1960) that E. coli was not often recovered from viscera other than the intestines, even when there was a delay of several days between death and post-mortem examination, concurs with the finding of Stevens (1961).

In a recent review, Sojka (1965) listed 37 papers published since 1928 in which E. coli was considered to be involved in gastroenteritis and septicemia of young pigs.

In a recent study of 100 pigs submitted to a diagnostic laboratory, Gossling and Rhoades (1966) obtained isolates of E. coli O138:K81:NM from 6 pigs with "enteritis." These authors stated that "two serotypes, 08:K85:H19 and O138:K81:NM,. were identified as possible specific etiologic agents of enteritis in baby pigs."

In a comprehensive analysis of outbreaks of enteritis and diarrhea over a 5-year period, Stevens (1963) was able to discern 3 groups in which a characteristic syndrome was associated with a specific age group. These syndromes

seemed to be associated with certain serotypes of E. coli. These syndromes and the age groups were: (1) piglet enteritis, 1 to 4 days of age; (2) enteritis of unweaned piglets, 3 weeks of age; and (3) post-weaning enteritis of pigs, 10 to 12 weeks of age or shortly after weaning, manifested chiefly as a "catarrhal enteritis." Stevens included hemorrhagic enteritis, edema disease and necrotic enteritis in his "post-weaning enteritis" group.

The experimental reproduction of piglet enteritis with 2 serotypes frequently associated with field cases is recorded by Saunders et al. (1963). The disease resembled natural infections associated with these serotypes, diarrhea following the experimental exposure within 12 to 24 hours and some deaths occurring within 3 days. At necropsy the same serotypes were obtained in profuse pure culture from the intestines and, at times, from other viscera. Varying degrees of ileitis and colitis, commonly associated with slight edema of the colonic mesentery, were found in the dead pigs, and mild gastritis was seen in approximately 1/3 of these animals.

The Pathogenesis of Colibacillosis

In studies of experimental colibacillosis using a particular serotype, Saunders et al. (1960) found the organism to be present in all the viscera of some piglets but only in the intestines of others in the same group.

This phenomenon was observed when 2 other serotypes were used, and these workers concluded that bacteremia was a stage in the development of the infection.

Jubb and Kennedy (1963) listed the favored sites of localization of E. coli as the intestinal tract and described the infection as septicemic since it is possible to isolate the organism from all tissues. Localization of E. coli in the meninges, joints and lungs was described by these authors, who pointed out that, although these infections accompany the enteric phase, their manifestations appear later than does diarrhea. Moreover, umbilical infections might never show themselves as enteric infections but remain as tissue infections. The favored sites of localization of E. coli in the tissues are the meninges, joints, the lungs--especially if virus porcine pneumonia lesions are present--and probably the kidney, in which site it produces the interstitial nephritis known as "white spotted kidneys." The renal infection may produce a descending pyelonephritis from which the organism is recoverable.

It is evident from bacteriological examination of cases of colisepticemia that systemic invasion can occur from the umbilical stump, nasal or pharyngeal mucosae, or from the intestinal tract (Fey and Margadant, 1961; Fey, 1962; Jubb and Kennedy, 1963).

Fey et al. (1962) produced an E. coli septicemia by oral or intranasal infection of 3 colostrum-deprived neonatal

calves despite ligation and section of the esophagus. A fourth calf, with esophagus left intact and exposed intranasally to the same serotype, had an E. coli bacteremia 20 hours after challenge, but the organism was not obtained from the contents of the small intestine, collected by laparotomy, nor from bile. These workers concluded that, in calves, infection of the intestine was by the circulatory system rather than by the digestive system. A number of workers have reported that strains of E. coli from cases of infant epidemic diarrhea have the ability to cause diarrhea in adults as well as in infants when the organism is fed in sufficient numbers (Furguson, 1956; Neter and Shumway, 1950).

The so-called "three-week enteritis" of Stevens' classification (Stevens, 1963) is considered to be due to an increase in the number of E. coli organisms already existing in the alimentary tract and not to the introduction of another serotype.

Of the third of Stevens' clinical groups (Stevens, 1963), the only specific manifestation that need be mentioned here is of hemorrhagic enteritis. This acute disease is characterized by sudden death and is often associated with dietetic or environmental change. At necropsy the intestinal tract is partly filled with ingesta and blood, and one finds gross and microscopic lesions of severe hemorrhagic enteritis (Jennings, 1959; Philip and Shone, 1960; Thomlinson, 1963; Kinnaird, 1964; Bennett, 1964). Buxton and Thomlinson (1961)

and Thomlinson and Buxton (1962) proposed that the hemorrhagic enteritis syndrome was an anaphylactic reaction. Stevens (1963) supported this view.

Colibacillosis in "Specific Pathogen Free" and Gnotobiotic Pigs

Experimental reproduction of colibacillosis in "pathogen-free," "germfree" or gnotobiotic pigs is generally not well documented. Saunders, Stevens, Spence and Betts (1963) used hysterectomy-derived, colostrum-deprived and artificially reared pigs in a series of experiments. In one experiment, using a known pathogenic serotype of E. coli (E68 type I) and oral doses ranging from 1000 to 100 x 10^6 organisms, they were able to produce diarrhea within 15 to 36 hours and recovery or, in some instances, death within 30 to 33 hours. In those pigs which died, the test organism was isolated from some or all of the tissues--intestines, liver, brain and lung. Those pigs which recovered were killed 6 days after infection, and the organism was cultured at that time from some or all of the sites--intestines, brain, heart, blood and liver.

In a second experiment, using a "non-pathogenic" serotype (P50) in 3 pigs, some diarrhea was observed within 20 hours of oral inoculation with 1 x 10^6, 100 x 10^6 and 100 x 10^6 organisms, respectively. These pigs recovered quickly and were soon seen to be healthy with soft to normal feces.

Two of these pigs were subsequently inoculated orally with serotype E68, type 1, and died within 2 and 5 days, respectively. The test organism was recovered in profuse pure culture from the intestinal tract and in sparse but pure culture from the brain, heart blood and liver.

In the final experiment of the series Saunders et al. (1963) found that the pathogenic serotype used in the previous experiments in neonatal pigs was equally pathogenic when used in 6-day-old pigs. They considered that there was no correlation between the severity of effects and the number of organisms administered and that the use of "pathogen-free" pigs, which were shown to be free of cytopathogenic viruses, indicated that their pathogenic serotype of E. coli was a primary pathogen.

Using E. coli O8:K?:H21 in gnotobiotic pigs infected at 4 to 6 days of age, Kohler and Bohl (1966) detected a bacteremia as a constant finding for 3 days after infection. They attributed this bacteremia to the fact that the experimental animals were colostrum-deprived, since bacteremia was not detected in the clinically ill, conventionally reared pigs from which the strain of E. coli was originally isolated. These workers did not observe enteritis in gross examination of pigs which died in the acute phase of the disease.

In a comprehensive study of the pathogenicity of E. coli, serotype O83, Britt and Waxler (1964) used 7 litters

of gnotobiotic pigs and recorded characteristic lesions in 78% of the 64 piglets exposed at ages ranging from 2 to 12 days. The outstanding lesion reported by these workers was a serofibrinous or fibrinopurulent polyserositis, including polyarthritis.

Kenworthy and Allen (1966), in studies on germfree and gnotobiotic pigs, demonstrated a variation in intestinal villus and crypt structure associated with the degree of bacterial contamination of the intestional tract. The villi of pig intestine monocontaminated with E. coli O141:K85a, c,(B):H4 were generally similar to those in the germfree control animal. Pigs contaminated with E. coli serotypes O141:K85a,c,(B):H4 and O8:H had intestinal villi with considerable variation in shape, including some branching forms. Slight edema in the lamina propria of the intestines of these duocontaminates was also observed. In pigs maintained in a "normal" environment, leaf-shaped villi were observed, with evidence of stunting, clubbing and fusion of villi. Those villi most severely affected were at the crests of the plicae. Cellular infiltration into the lamina propria was heavy, and reticular tissue appeared coarser and more abundant than in the monocontaminated pigs. These workers concluded that a biochemical interaction between the intestinal flora and the diet might result in the appearance of an altered metabolic pathway which, in turn, depended on the symbiosis or antagonism of the

bacterial species present and the various substrates present in the intestinal tract. The morphology of the mucosa of the small intestine is dependent, in some measure, on these factors (Kenworthy and Allen, 1966).

MATERIALS AND METHODS

General Plan

In a study that involves following the progress of a specific organism through a sequence of body tissues or systems and the collection of tissues from the test animals so that the tissue changes can be correlated with the movement of the organism, it is necessary to make some assumptions regarding the behavior of organisms in host tissues. The validity of these assumptions can be increased by standardizing genetic and environmental factors in the experiment.

It was expected that litter mates held under the same environmental conditions and submitted to identical treatments, provided they were of similar physiological activity, would generally respond or react in a similar way. It was not possible to carry out the extensive site sampling called for in this experimental method without killing the individual pig; hence, it was assumed that observations made on each individual animal at a particular time interval following exposure to pathogens would constitute a general picture of the progress of infection through any particular pig of that litter when held under the same circumstances and when the disease was permitted to run its course. The gnotobiotic pigs were obtained according to the modified hysterotomy

method described by Waxler et al. (1966). This technique has been further modified by Dr. A. L. Britt* and Dr. G. L. Waxler,* in that a standard surgical skin preparation of detergent, alcohol, then chloroform, was used prior to the sealing of the skin of the sow to the plastic sheet that formed the floor of the fiberglass ring set in the base of the surgical isolator (Waxler, Schmidt and Whitehair, 1966). Other modifications have included the use of Allis tissue forceps to clamp the edge of the incised plastic sheet to the apposite edge of the incised skin of the sow at approximately 2-cm. intervals. This modification has helped to maintain the adhesion between these 2 surfaces and to prevent movement of fluid from the surgical field out between the skin of the sow and the wall of the isolator.

The use of umbilical cord clamps** to occlude the umbilical cord vessels prior to separation of the pig from the placenta has decreased the operation time and considerably facilitated manipulations within the surgical isolator.

Animals

An experimental unit consisted of 1 or more litters of gnotobiotic pigs, using some as control animals and some as test animals in an accepted experimental design. Between-litter variation was minimized so far as possible. The pigs

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*Department of Pathology, Michigan State University.

**Disposable Cord-Clamp, Hollister "Double Grip," Hollister, Inc., Chicago, Ill.

used in this series of experiments were from related sows and were the progeny of 1 boar; they were fed a standard volume of the same diet;* they were maintained in sterile plastic-film isolators at an environmental temperature of 85 to 90 F. Details of litter size, sex distribution, birth weights, bacterial serotype, numbers of organisms administered, route of exposure, and age of pigs at exposure are shown in Table 1.

Determination of Initial Sterility of Test Animals

Immediately prior to the introduction of the infective material into the sterile isolators, composite fecal, oral, nasal, and fecal tray samples were taken with sterile swabs and inoculated into 3 tubes of thioglycollate medium.** These tubes were incubated at 37 C, 50 C, and at room temperature. (In litters 3 and 5 the tubes were incubated only at 37 C.) These tubes were examined daily for 7 days. If there was no growth after 7 days, the isolators were considered to have been free of bacteria. Since these were very short-term experiments, the sterility of the isolators was determined immediately prior to the exposure of the pigs to the test organism, and generally this was done within 48 hours of the delivery of the pigs into the isolators.

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*SPF Lac, Borden Co., New York, N.Y.

**Bacto Fluid Thioglycollate Medium (Dehydrated), Difco Laboratories, Detroit 1, Michigan, U.S.A.

Table 1. Details of experimental animals, control animals, serotype of infective organisms, and number of organisms per inoculum.

Litter Number	Average Birth wt. (Kg.)	Number and Sex of Test Animals	Organisms			Age of Pig at Exposure (hr.)	Control Pigs		
			Serotype	Number x 10^6	Route of Exposure		Sex	Age at Necropsy (hr.)	Hours from inoculation of test pigs until control pig was killed
1	not recorded	6 M 7 F	O138:K81: NM(Mich.)	0.42	Umbilical stump	46	F M	70 198	24 152
2	1.0	6 M 6 F	O138:K81: NM(Mich.)	63.00	Umbilical stump	29	F M	65 101	36 72
3	1.1	6 M 3 F	O138:K81: NM(Mich.)	0.80	Umbilical stump	20	M M	23 44	3 24
4	0.8	8 M 7 F	O138:K81 (Minn.)	135.00	Umbilical stump	8	F F M	44 154 176	36 146 168
5	1.0	7 M 6 F	O138:K81: NM(Mich.)	4.80	per os	44	F M	52 68	8 24

Control Animals

Although it is essential that a sample of pigs from each litter should be kept as control animals in order to check the sterility of the intrauterine environment during gestation and the sterility of the hysterotomy and delivery procedures, only 2 or 3 pigs from each litter were retained for this purpose.

It was planned that control animals from each litter be killed on a time schedule so that, together, they would adequately cover the actual-age scale of the test animals in the experiments constituting the whole of this series. Since between-experiment variation was negligible in terms of diet and environment, and genetic and congenital variation was minimal (see birth weights recorded in Table 1), it was considered that control animals from any 1 litter in the series would provide adequate tissue controls for test animals of equal age from other litters in the series.

Infective Agents

The organism used in litter 2 was Escherichia coli O138:K81 (Minn.) and was obtained from Dr. D. K. Sorenson of the University of Minnesota. This organism was reported to be one of the strains associated with edema disease and gastroenteritis and which had been shown to cause an enteric mucoserous exudate. This organism did not produce marked clinical signs when inoculated into gnotobiotic pigs. In

all other litters in this series of experiments the same serotype, from a different source, was used. This organism was E. coli O138:K81:NM (Mich.) and was isolated in August, 1959, by Dr. G. L. Waxler from experimental pigs at Michigan State University. These organisms had been cultured, lyophilized in glass ampules, and stored at -20 C until required.

Twenty-four hours before the pigs were due to be infected, an ampule of the culture was broken open and its contents inoculated into liquid brain-heart infusion medium* and streaked onto a bovine blood agar plate and a MacConkey Agar plate.** These cultures were incubated at 37 C. After 24 hours' incubation, immediately before the experimental animals were due to be inoculated and when it was apparent that a pure culture was present, the tube culture was diluted 1 in 1000 in sterile saline (0.85% NaCl). This saline suspension of the organisms was the material used in the isolators to inoculate the test animals.

Serial tenfold dilutions of this inoculum were made in sterile saline and 0.1 ml. aliquots of these dilutions were pipetted into standard Petri dishes, to which was added melted Eugonagar.† Immediately, the contents were gently

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*Bacto Brain Heart Infusion (B 37); Difco Laboratories, Detroit 1, Michigan, U.S.A.

**Bacto MacKonkey Agar (B 75); Difco Laboratories, Detroit 1, Michigan, U.S.A.

†Eugonagar Vera, Baltimore Biological Laboratory, Baltimore, Maryland, U.S.A.

mixed, allowed to cool and solidify, and were then incubated overnight. The viable count of the inoculum was calculated from the colony count on plates growing clearly separable colonies after 24 hours' incubation at 37 C.

The inoculum was loaded into a sterile disposable tuberculin syringe* using strict aseptic technique. Once the syringe was loaded, the loading needle was removed, the tip of the syringe was flamed almost to the point of melting the plastic tip, and the tip of the syringe was capped with a covered sterile disposable 23 G needle.** The capped syringe was then thoroughly sprayed with 2% peracetic acid (with approximately 0.1% wetting agent added).† The syringe was then introduced into the port of the sterile isolator and the port resprayed and closed. After 30 minutes the syringe was introduced into the isolator through the internal cap, and the inoculum was then ready for use. A standard dose volume of 0.25 ml. per pig was used throughout the experiments.

Injection Technique within the Isolator

An assistant restrained the pig in lateral recumbency a few inches above and separated from a sterile towel on the floor of the isolator. Meanwhile the operator, having

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*Tomac Disposable Tuberculin Syringe, American Hospital Supply Co., Evanston, Ill., U.S.A.

**Discardit Sterile Disposable Yale 23G 1, Becton-Dickinson, N.Y., U.S.A.

†Nacconal, N.R.S.F., National Aniline Division, Allied Chemical Corporation, New York, N.Y., U.S.A.

donned sterile plastic gloves over the normal isolator gloves, removed the outer cap of the syringe, exposing the needle and thereby theoretically exposing the interior of the isolator to the organisms within the syringe. Holding the umbilical clamp in the left hand, it was possible for the operator to insert the tip of the needle into the umbilical stump distal to the clamp. The needle was then passed through the umbilical cord tissue held between the jaws of the clamp and then, with care, it was possible to place the tip of the needle in the subcutis of the umbilical stump immediately proximal to the clamp. The injection of 0.25 ml. of a suspension of the organisms was made at this stage.

As the needle was withdrawn from the stump, the clamp pressure prevented the flow of inoculum back along the track of the needle. This, combined with the fine gauge needle that was used, meant that an absolute minimum of the suspension of the organism remained at the site of withdrawal of the needle. This site was immediately swabbed with copious amounts of a 2% solution of peracetic acid. At no stage of the operation did the assistant or the pig touch the operator's hand which held the syringe. The only contact with the other hand of the operator was with the end of the umbilical clamp, and this in turn did not contact the needle, the inoculum or the hand working the syringe. After all pigs in the isolator had been inoculated in similar

fashion, materials used in the injection technique, including the outer plastic gloves, were immersed in a flask of 2% peracetic acid solution, and the flask was immediately removed from the isolator.

Although fraught with a number of hazards from the point of aseptic technique, especially since the operation was performed in an otherwise sterile environment, this technique was apparently successful in that, at no time during these experiments, was there detected contamination of the oral, skin, or isolator environment until the injected organisms had become established in the intestinal tract and these were being excreted in the feces.

In litter 5, in which the oral route was used, the syringes of inoculum were prepared as described (see above) and sprayed into the isolator. After each pig had been fed 4 fluid ounces of the standard milk formula diet, the 0.25 ml. dose was squirted onto the back of the tongue.

Hematology

Five-milliliter amounts of blood were collected from the anterior vena cava according to the method of Carle and Dewhirst (1942). An initial blood sample was taken within 24 hours of each pig having been delivered to the rearing isolator. A terminal sample was taken from each animal immediately prior to death. Total and differential leukocyte counts were carried out as described by Benjamin (1964).

Hemoglobin estimation was by the cyanmethemoglobin method and packed-cell volume by the micro-hematocrit method (Benjamin, 1964).

Necropsy and Microbiologic Procedures

Although Stevens (1961) has recorded that coliform bacteria do not usually invade the general viscera within 24 hours of death, even at the environmental temperature of the isolators that were used in these experiments, Dunne (1964) is emphatic in his opinion that E. coli is capable of invading the blood stream from the intestinal tract within an hour of death and states that ". . . isolation of the organisms from tissues under such conditions would be of little immediate value in establishing the presence of a septicemia."

In order to minimize post-mortem migration and/or multiplication of test organisms within tissues, necropsies were completed within 90 minutes of euthanasia. Immediately after the pigs were removed from the isolaters, they were killed by concussion and exsanguination (by severing the axillary vessels).

At necropsy the pig was placed in lateral recumbency on its right side and a midline skin incision was made from the symphysis pubis to the body of the mandible. The skin fold, including the foreleg, was reflected, the medial mass of the hind leg muscles was separated, and the hind leg was

laid back. Both the prescapular and prefemoral lymph nodes were exposed in this fashion.

Microbiologic technique. With a hot spatula it was possible to dry and singe the surface of the lymph nodes and with a flamed, tightly wound, sterile wire loop* (Figure 1) to penetrate the singed surface, sample the contents of the node, and streak the loop onto the marked portion of a MacConkey Agar plate (Figure 2).

The thoracic cavity was opened with heated scissors and a sample of pleural fluid collected with the sterile wire loop and streaked onto culture media. The pleural serosa of the parietal pericardium was in turn singed with the spatula, the pericardium was broken with a touch of the hot loop of wire, and a sample of pericardial fluid was collected and streaked onto the medium. In similar fashion, using the small heated spatula, the myocardial surface was dried and singed, and the sterile wire loop was forced through the myocardium into the right ventricle. A loop of heart blood was withdrawn and streaked onto the medium.

Using the hot spatula it was possible to first dry, then sterilize, the surface of any of the organs or tissues prior to using the sterile wire loop to collect material for culture. In sampling the humeroradial joint and the

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*Nichrome wire, 25 gauge; Scientific Products, Evanston, Illinois, U.S.A.

Figure 1. A coiled nickel-chromium alloy wire was used to penetrate and sample the selected organ and tissue sites. The spatula was used to sear tissue surfaces before sampling.

Figure 2. MacConkey Agar plate. A typical culture plate on which E. coli colonies are growing.

midbrain and medulla of the central nervous system a hot scalpel was also needed to clear away superficial tissues prior to sampling.

The MacConkey Agar plates were streaked centripetally, and an attempt was made to use a standard stroke while making the streak and to use a minimal amount of inoculum. This procedure resulted in adequate separation of the streaks from different organs; and, in lightly contaminated tissue, separation into relatively small groups or organisms, or even individualization of organisms was possibly achieved (Figure 2).

The MacConkey Agar plates were seeded as follows: the first plate was used for prefemoral lymph node, prescapular lymph node, pleura, pericardium, heart blood and lung samples; the second plate was inoculated with material from the peritoneum, liver, spleen, kidney, urocyst, iliac lymph node, humeroradial joint fluid and brain stem; the third plate was used for gastric mucosa, jejunal mucosa, mucosa of the ileocecal valve, rectal mucosa, the injection site (or the oral cavity in the experiment in which the infecting dose was given per os) and the subcutaneous tissue 2 cm. lateral to the inoculation site.

Histopathologic technique. Tissues taken for histologic examination were: prescapular lymph node, transverse section of the thyroid gland, thymus, transverse section of the trachea and esophagus at the mid-cervical level, cross

section of the right cardiac lobe of the lung, transverse section of the myocardium (including left and right ventricles with their serosal surfaces and portion of the interventricular septum), transverse section of the aorta in the mid-thoracic region, the skin and subcutis at the injection site, the skin and subcutis 2 cm. lateral to the injection site, cross section of the right adrenal gland, pancreas, midsection of the spleen, posterior section of the right central lobe of the liver, transverse section of the cholecyst in situ, transverse section of the right kidney at the level of the pelvis, section from the anterior dorsal wall of the urocyst, sections of cardiac, fundic and pyloric regrions of the stomach wall, duodenum, transverse sections of the jejunum at the 50 cm., 1m., and 2m. levels, transverse section of the ileum immediately cranial to the ileocecal valve, and sections of the cecum, spiral colon (including relationship with other coils and the mesocolon) and a transverse section of the rectum at the level of the pelvic brim.

Tissue sections or slices approximately 0.5 cm. thick were identified and fixed in 10% formalin and processed for examination by procedures described in the Manual of Histologic and Special Staining Techniques of the Armed Forces Institute of Pathology, Washington, D.C. (1957).

Reclamation of the Test Serotype

After the MacConkey Agar plate cultures had been examined and read, a sterile loop was used to sample a typical colony from each of the positive streaks, and this loop was used in turn for sampling the positive isolations from each of the pigs which had inhabited a particular isolator during the experiment.

This loop of organisms selected from different tissue isolations and different animals, but all from the one isolator, was inoculated into 2 tubes of boiled thioglycollate medium and incubated for 16 to 24 hours, one tube at 37 C and the other at 55 C. A composite of the growth from either or both of these tubes was inoculated onto a nutrient agar slant and onto 2 bovine blood agar plates, one to be incubated aerobically and the other anaerobically for 18 to 24 hours at 37 C. The nutrient agar slant was incubated for 24 hours at 37 C, sealed with a foam plug, and stored at room temperature until all samples were ready for dispatch to the serotyping service.

In this way it was possible not only to check the validity of the monocontamination but also to insure that the serotype isolated from the test animals was, in fact, the serotype that was used in the initial infecting exposure and that a mutant of altered serotype had not appeared during the course of the experiment.

RESULTS

Experiment 1

The Experimental Plan

In this experiment 3 litters of pigs were infected by injection of E. coli O138:K81:NM (Mich.) into the subcutis of the umbilical stump but without oral or envirnomental contamination. In this way it was hoped to simulate infection of the umbilical stump of neonatal pigs and to study the course of infection subsequent to this in colostrum-deprived animals.

Litter 1. These 13 pigs were 46 hours old when 0.42×10^6 E. coli O138:K81:NM (Mich.) organisms were injected into the subcutis of the umbilical stump of each of 11 of them.

The animal necropsy numbers, sex distribution, period of elapsed time between inoculation and necropsy, and sites of positive isolation from tissues are tabulated (Table 2).

Four hours after injection it was recorded that there was heavy contamination of the injection site and moderate contamination of the subcutis 2 cm. lateral to the injection site. One organism each was cultured from the heart blood and the lung, and 8 organisms were detected in the loop of fluid taken from the spleen.

Table 2. Bacteriologic findings from pigs in Litter 1.

Pig Number	J4662	J4663	J4664	J4666	J4669	J4668	J4667	J4672	J4673	J4680	J4681	Controls J4670	Controls J4671
Sex	M	F	F	M	F	F	M	M	F	F	M	F	M
Hours after injection	4	8	11	24	32	40	48	77	101	123	151	24	152
Isolation or organisms from the following sites*													
Prescapular lymph node	-	-	-	++	++	-	-	-	-	-	-	-	-
Prefemoral lymph node	-	1	-	+	++	-	-	2	-	-	9	-	-
Pleura	-	-	-	20	20	-	-	-	-	-	-	-	-
Pericardium	-	-	-	+	-	-	-	-	-	-	-	-	-
Heart blood	1	+	-	++	++	-	-	-	-	1	-	-	-
Lung	-	-	-	++	++	-	-	-	-	-	-	-	-
Peritoneum	1	-	-	20	50	-	-	-	-	-	-	-	-
Liver	-	8	-	+	++	-	-	-	-	6	-	-	-
Spleen	8	16	-	+	++	2	-	-	-	-	-	-	-
Kidney	-	1	-	+	++	-	-	-	-	1	-	-	-
Urocyst	-	-	-	20	+	-	-	-	-	-	-	-	-
Iliac lymph node	-	-	-	3	++	20	-	-	-	-	-	-	-
Brain	-	2	-	+	++	-	-	-	-	-	-	-	-
Humeroradial joint	-	3	-	+	50	-	-	-	-	-	-	-	-
Injection site	++	++	++	20	++	20	-	-	++	+	-	-	-
Subcutis 2 cm. lateral to the injection site	+	-	-	-	++	-	-	-	-	-	-	-	-
Stomach	-	-	-	10	20	++	++	++	+	+	++	-	-
Midjejunum	-	-	-	50	++	++	+	+	++	++	++	-	-
Ileocecal valve	-	1	-	3	20	++	++	++	++	++	++	-	-
Rectum	-	-	-	5	++	++	++	++	++	++	++	-	-

*Bacterial colony counts are expressed as follows: The number = less than 51 colonies; + = more than 50 colonies and less than 101 colonies; ++ = more than 100 colonies; - = no growth.

No gross lesions were observed in this pig.

After 8 hours, heavy growth was obtained from the injection site, and light to moderate growth was obtained from the heart blood. One isolation was made from the prefemoral lymph node, and a few organisms were cultured from liver, spleen, kidney, brain and humeroradial joint cavity. One organism was obtained from the mucosa of the ileocecal valve.

Gross abnormalities observed in this pig were ventral abdominal edema and micrognathia.

In the pig subjected to necropsy 11 hours after injection, organisms were isolated only from the injection site. Culture of this site indicated that a heavy contamination was present. The organisms were not present in the subcutis 2 cm. lateral to the injection site.

There were no gross lesions in this animal.

Twenty-four hours after injection very few organisms remained in the injection site, no organisms were detected in the subcutis 2 cm. lateral to that site, but there was a heavy systemic contamination of the pig. At this stage very light contamination of the mucosa of the intestinal tract at the test sites was recorded (Table 2). The gross lesions in this animal were hemorrhagic edema of the injection site and an area of erythema, 1 cm. in diameter, of the parietal peritoneum at the point of entry of the umbilical vessels.

At 32 hours after injection there was moderate to heavy systemic and gastrointestinal tract contamination, positive isolations being made from all 20 sites sampled. Grossly, the liver was soft and very dark. Throughout its length the ileum was distended with thick, intensely red fluid consisting mostly of blood lost from the raw, denuded surface of the ileum (Figure 3). The gross appearance was that of hemorrhagic enteritis. After 40 hours a very few organisms were isolated from the injection site, iliac lymph node and spleen. All other systemic sites were negative. Very heavy contamination was recorded from the 4 sites of the gastrointestinal tract.

Clinically, this animal was drinking very well and had a profuse diarrhea. The feces were watery, yellow, and flecked with fine white curds. Gross lesions included edema of the mesocolon, slight hemorrhagic edema of the injection site, and petechiae of the visceral peritoneum at the umbilical entrance (Figure 4).

The remaining pigs in this litter were killed at 48, 77, 101, 123, and 151 hours, respectively, post injection. In each case the gastrointestinal tract remained heavily contaminated, which suggests that this organ had become colonized by the test organism. Very few organisms were found in other sites (Table 2).

Gross lesions consistently found in these animals were enlargement and edema of the superficial lymph nodes, slight to moderate hyperemia of the injection site, and

Figure 3. Pig J4669. Hemorrhagic contents of the jejunum and ileum. E. coli was injected 32 hours before necropsy.

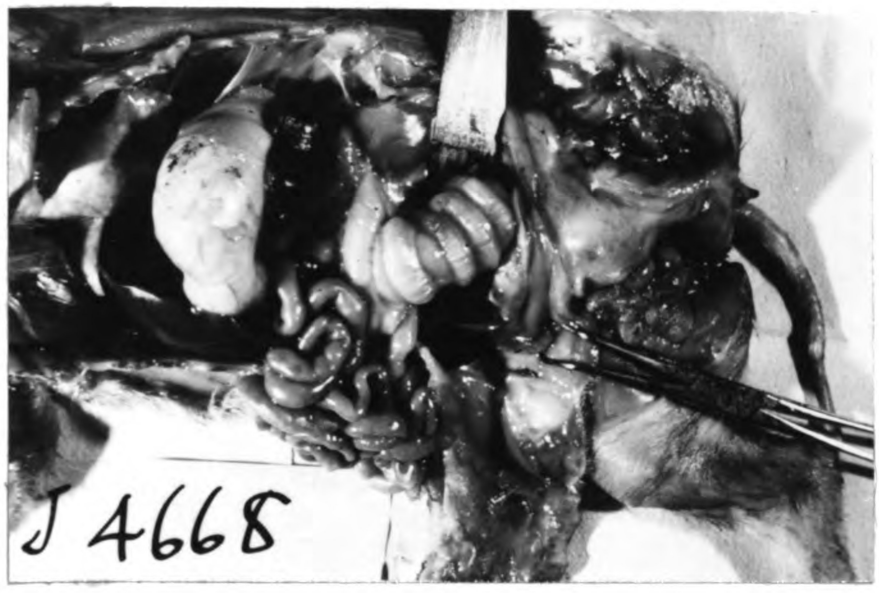

Figure 4. Pig J4668. E. coli was injected 40 hours before necropsy. Edema of the mesocolon (supported by the spatula) petechiation and marked hyperemia of the umbilical entrance to the peritoneal cavity (towel clamp).

slight to moderate localized edema of the ventral abdominal wall in the umbilical region. Ample food was present in the anterior gastrointestinal tract, and it was apparent from this and the feeding records that the appetites of the animals had not been impaired. Some hyperemia of the serosal surface of the stomach and the jejunum was recorded, and in some instances the jejunum appeared thicker-walled than normal. The contents of the cecum and colon were consistently watery, and moderate to marked edema of the mesocolon was observed in these pigs.

The 2 control pigs from this litter were free of detectable microorganisms at necropsy, and all attempts to culture bacteria from the standard test sites on MacConkey Agar plates failed. These animals were killed at 70 and 198 hours of age, respectively, and were used for clinical and histological controls for this and other litters in this experimental series.

Litter 2. This litter was 29 hours old when 63×10^6 E. coli O138:K81:NM (Mich.) organisms were injected into the subcutis of the umbilical stump of each of 10 of the 12 pigs in the litter.

The animal necropsy numbers, sex distribution, period of elapsed time between inoculation and necropsy, and sites of positive isolation from tissues are tabulated (Table 3).

At 1 1/2 hours after injection only 1 organism was isolated from the injection site. The subcutis 2 cm. lateral

Table 3. Bacteriologic findings from pigs in Litter 2.

Pig Number	J6208	J6209	J6200	J6201	J6202	J6203	J6204	J6205	J6206	J6207	Controls J6211	Controls J6210
Sex	F	M	F	F	M	M	M	F	M	M	F	F
Hours after injection	1.5	2.5	4	6.5	8	12	16	24	48	72	72	36
Isolation of organisms from the following sites*												
Prescapular lymph node	-	-	-	-	-	-	-	-	-	-	-	-
Prefemoral lymph node	-	-	-	-	-	-	-	-	-	-	-	-
Pleura	-	-	-	-	1	-	-	-	-	-	-	-
Pericardium	-	-	-	-	-	-	-	-	-	-	-	-
Heart blood	1	-	-	-	1	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-	41	-	-	-	-
Peritoneum	-	-	-	-	-	-	-	-	-	-	-	-
Liver	15	-	-	-	2	-	-	-	-	1	-	-
Spleen	22	-	-	-	+	-	-	9	-	-	-	-
Kidney	-	-	-	-	-	-	-	-	-	-	-	-
Urocyst	-	-	-	-	1	-	-	-	-	-	-	-
Iliac lymph node	-	-	-	-	-	-	-	10	-	-	-	-
Brain	-	-	-	-	-	-	-	-	-	-	-	-
Humeroradial joint	-	-	-	-	-	-	-	-	-	-	-	-
Injection site	1	++	-	++	-	2	4	++	++	-	-	-
Subcutis 2 cm. lateral to the injection site	-	-	-	-	-	++	-	2	-	-	-	-
Stomach	-	-	1	48	6	+	++	++	++	++	-	-
Midjejunum	-	-	12	1	6	++	++	++	++	++	-	-
Ileocecal valve	1	-	-	++	++	++	++	++	++	++	-	-
Rectum	-	-	-	-	++	++	-	++	++	++	-	-

*Bacterial colony counts are expressed as follows: The number = less than 51 colonies; + = more than 50 colonies and less than 101 colonies; ++ = more than 100 colonies; - = no growth.

to this site was sterile. Light contamination was recorded from the heart blood, liver and spleen cultures, and 1 organism was isolated from the ileocecal valve mucosa.

No gross abnormalities were observed in this pig.

Culture of organisms was successful only from the injection site in the pig submitted to necropsy 2 1/2 hours after injection. Gross abnormalities observed were slight edema of the superficial lymph nodes and the mesocolon and marked hemorrhagic edema of the umbilical stump.

At 4 hours after injection the organisms were not recovered from the injection site, and isolations indicating very light contamination were obtained only from the stomach and midjejunal mucosae. During this necropsy a few fibrin strands were seen on the surface of the liver and spleen, and there was slight excess of faintly cloudy peritoneal fluid.

After 6 1/2 hours, heavy growth was obtained from culture of the injection site, but it was apparent that the organisms had not spread laterally in the subcutis, as culture from the latter site was negative. Moderate growth from the gastric and midjejunal mucosae and very heavy growth from the mucosa of the ileocecal valve were recorded. The rectum was sterile at this stage.

At necropsy the iliac lymph nodes were enlarged and moderately edematous. The mesenteric lymph nodes were normal, but there was marked hyperemia of the vessels located

between these lymph nodes and the mesenteric attachment of the jejunum. The aortic lymph nodes were enlarged, edematous and hyperemic. The injection site was markedly edematous, and the skin 2 cm. lateral to the umbilical stump was hyperemic and edematous.

In the pig killed 8 hours after inoculation, light to moderate contamination was recorded from heart blood, pleura, liver, spleen and urocyst. There was light contamination of the gastric and midjejunal mucosae and very heavy contamination of the mucosae of the ileocecal valve and rectum. At gross examination of this pig the superficial, iliac and aortic lymph nodes were slightly enlarged, and there were 2 subcapsular ecchymoses and areas of subcapsular hyperemia of the liver. There was slight edema of the mesocolon, moderate edema of the injection site, and an associated ventral edema. The cecal contents were very copious and of watery consistency. The rectal contents were firm.

Twelve hours after injection very few organisms remained in the injection site, although the subcutis lateral to the umbilical stump was heavily contaminated. All sites in the mucosa of the gastrointestinal tract were heavily colonized; all other test sites were sterile. At necropsy the injection site was moderately edematous and there was an associated ventral abdominal edema.

After 16 hours, light contamination was recorded from culture of the injection site, and no growth was obtained from the subcutis lateral to that site. Very heavy growth was obtained from the mucosal samples of stomach, midjejunum and ileocecal valve. The rectal mucosa was sterile. All other sites were sterile. The only gross lesions in this animal were slight hyperemia of the injection site and slight edema of the prescapular lymph node.

The remaining infected pigs in this litter were killed at 24, 48 and 72 hours after injection. At 24 hours after injection, moderate contamination of the lung, spleen and iliac lymph node was recorded. The organisms were found in profuse numbers in the injection site and had spread in the subcutis to a slight degree. The entire gastrointestinal tract was heavily colonized at this time. All other sites were sterile.

Immediately prior to euthanasia a bright yellow, curdy and watery stool was observed.

This pig had an optimum food intake, and there was no evidence of anorexia. At necropsy the stomach was filled with clotted milk and the intestinal tract was filled with watery yellow fluid in which flakes of milk curd were suspended. There was an ecchymosis at the injection site; there was also hyperemia of the peritoneum at the point of entry of the umbilical vessels.

In each of the remaining 2 pigs the gastrointestinal tract was heavily colonized and was the only site of any significant isolation of organisms. In each case the hair coat was erect, the animal was scouring profusely before death, and it was dehydrated. The appetite was normal, the stomach was filled with clotted milk, and the intestinal tract was uniformly flaccid and filled with the watery contents as described for the previous pig.

The two control pigs from this litter were free from detectable microorganisms at necropsy, and all attempts at culture from the routine tissue sites on the MacConkey agar plates were negative. These animals were killed at 65 and 101 hours of age, respectively, and were used for clinical and histological controls for this and other litters in this experimental series.

In the older pig, moderately enlarged and edematous prescapular lymph nodes were observed. The intestinal tracts of both control pigs were of uniform color throughout.

Litter 3. This litter was 20 hours old when 0.8×10^6 *E. coli* O138:K81:NM (Mich.) organisms were injected into the subcutis of the umbilical stump of each of 9 of the 11 pigs in the litter.

The animal necropsy numbers, sex distribution, period of elapsed time between inoculation and necropsy, and sites of positive isolation from tissues are tabulated (Table 4).

Table 4. Bacteriologic findings from pigs in Litter 3.

Pig Number	J6785	J6784	J6782	J6783	J6789	J6790	J6791	Controls J6792	Controls J6793
Sex	M	M	M	F	F	M	F	M	M
Hours after injection	1.5	2.5	9	24	32	48	72	3	24
Isolation of organisms from the following sites*									
Prescapular lymph node	-	-	-	-	-	-	8	-	-
Prefemoral lymph node	-	-	-	-	-	-	-	-	-
Pleura	-	-	-	-	-	-	-	-	-
Pericardium	-	-	-	-	-	-	-	-	-
Heart blood	-	-	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-	-	-
Peritoneum	-	-	-	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-	-	-
Spleen	-	-	-	-	-	-	-	-	-
Kidney	-	-	-	-	-	-	-	-	-
Urocyst	-	-	-	-	1	-	++	-	-
Iliac lymph node	-	-	-	-	-	-	-	-	-
Brain	-	-	-	-	-	-	-	-	-
Humeroradial joint	-	-	-	-	-	-	-	-	-
Injection site	++	-	+	-	++	1	-	-	-
Subcutis 2 cm. lateral to the injection site	-	-	-	-	-	-	-	-	-
Stomach	-	-	-	-	1	+	++	-	-
Midjejunum	-	-	-	-	-	++	++	-	-
Ileocecal valve	-	-	++	-	-	++	++	-	-
Rectum	-	-	-	-	-	++	++	-	-

*Bacterial colony counts are expressed as follows: The number = less than 51 colonies; + = more than 50 colonies and less than 101 colonies; ++ = more than 100 colonies; - = no growth.

At 1 1/2 hours after injection only the injection site was positive for presence of the test organism. The only gross post-mortem lesion in this pig was a moderate hemorrhagoserous exudate at the injection site.

In the pig killed 2 1/2 hours after injection, the organisms could not be recovered from any of the routine sites, and it was considered that the antiseptic procedures used during the injection technique could have sterilized the inoculum in this instance.

There were no gross abnormalities observed in this animal at necropsy.

After 9 hours had elapsed between injection and euthanasia, moderate growth was obtained from the injection site and heavy growth was obtained from the mucosa of the ileocecal valve. All other sites were negative. At necropsy the superficial lymph nodes were slightly edematous, and there was apparent flaccidity of the ileum and large intestine. There was slight edema of the injection site and periumbilical edema.

The pig subjected to necropsy at 24 hours was sterile in all culture sites. There was no edema of the injection site. The midjejunum was distended and flatulent, and the rectum was partly filled with meconium.

Thirty-two hours after injection there was heavy contamination of the injection site. One colony each was isolated from the urocyst and from the gastric mucosa. All other sites were negative.

Gross lesions recorded for this pig were as follows. The terminal jejunum and ileum were moderately enlarged, with flaccid walls and very soft contents; there was slight edema of the mesocolon, and meconium was still present in the rectum. The injection site was slightly hemorrhagic and the periumbilical peritoneum was hyperemic in an area 1 cm. in radius. At the peritoneal attachment of the falciform ligament there was a 3-mm.-diameter vesicle in the subserosa. This vesicle contained a slightly cloudy serous fluid.

Forty-eight hours after injection, very light growth was obtained from culture of the injection site, and heavy growth was obtained from the 4 alimentary tract sites. All other sites were sterile. At necropsy there was moderate edema of the superficial lymph nodes and the mesenteric lymph nodes. The mediastinal lymph nodes were hyperemic, there was moderate edema of the mesocolon, the jejunum was uniformly flaccid, and the ileum and large intestine appeared to have normal intestinal tone. At the injection site there was a 5-mm.-diameter vesicle containing a seropurulent fluid. This vesicle was surrounded by a 1-cm.-wide hyperemic annulus. The subcutis lateral to this lesion was not noticeably edematous. Prior to euthanasia this pig had profuse watery stool and was the first animal in this litter to show this sign.

At 72 hours after injection, very light growth was obtained from the prescapular lymph node, and heavy growth was recorded from each of the alimentary tract sites. It was observed that the perineal epithelium of this female pig was inflamed and partly eroded and macerated as a result of the continual diarrhea. The culture of the mucosa of the urocyst was positive with a very heavy growth of organisms. Since all other systemic sites, including the kidney, were sterile in this pig it was presumed that this urocyst infection could have resulted from ascending contamination with organisms from the perineum. The intestinal tract was flaccid and filled with a watery fluid in which fine yellow curds were suspended. There was slight hemorrhagic edema of the injection site, and the superficial lymph nodes were slightly edematous.

The 2 control animals from this litter were free from detectable microorganisms at necropsy, and all attempts at culture from the standard test sites on MacConkey plates were negative. These animals were killed at 23 and 44 hours of age, respectively, and were used for clinical and histological controls for this and other litters in this experimental series. In the older pig the thymus was very small and was found only with difficulty.

Experiment 2

The Experimental Plan

In this experiment a litter of pigs was infected by injection of E. coli O138:K81 (Minn.) into the subcutis of the umbilical stump but without oral or environmental contamination. This serotype of E. coli, although possessing the same O and K antigens as the organism used in Experiment 1, was from a different source and was milder in its clinical expression in infected pigs. It was proposed to observe the pathogenicity of this organism and to compare these observations with those recorded in Experiment 1 in order to determine microbiologic and pathologic differences between organisms of the same broad serotype but with differing clinical manifestations in gnotobiotic pigs.

Litter 4. This litter was 8 hours old when 135×10^6 E. coli O138:K81 (Minn.) organisms were injected into the subcutis of the umbilical stump of each of 12 of the 15 pigs in the litter.

The animal numbers, sex distribution, period of elapsed time between inoculation and necropsy, and sites of positive isolation from tissues are tabulated (Table 5).

At 4 hours after injection there was moderately heavy contamination of the injection site, and light to moderate populations of the test organism were obtained from heart blood, liver, spleen and kidney. Both the adrenals in this

Table 5. Bacteriologic findings from pigs in Litter 4.

Pig Number	J5726	J5712	J5713	J5714	J5715	J5716	J5717	J5718	J5719	J5720	J5721	J5722	Controls J5725	J5724	J5723
Sex	M	F	M	F	F	M	M	F	F	M	M	M	M	F	F
Hours after injection	4	8	12	17	20	25	32	40	48	72	121	168	176	154	44
Isolation of organisms from the following sites*															
Prescapular lymph node	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
Prefemoral lymph node	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
Pleura	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pericardium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Heart blood	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Peritoneum	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
Liver	40	10	4	1	-	-	-	-	-	-	-	-	-	-	-
Spleen	12	+	10	10	5	-	1	-	-	-	-	-	-	-	-
Kidney	5	-	-	3	-	-	-	-	-	-	++	-	-	-	-
Urocyst	-	-	-	-	-	-	-	-	4	-	++	-	-	-	-
Iliac lymph node	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Brain	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-
Humeroradial joint	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Injection site	+	-	++	-	+	23	++	4	++	++	++	20	-	-	-
Skin 2 cm. lateral to the injection site	-	-	-	-	1	+	-	-	+	-	1	9	-	-	-
Stomach	-	10	-	12	12	+	++	++	++	++	18	11	-	-	-
Midjejunum	-	10	-	++	++	++	++	++	++	++	++	18	-	-	-
Ileocecal valve	-	-	++	++	++	++	++	++	++	++	++	++	-	-	-
Rectum	-	-	+	-	+	++	++	++	++	++	++	++	-	-	-

*Bacterial colony counts are expressed as follows: The number = less than 51 colonies; + = more than 50 colonies and less than 101 colonies; ++ = more than 100 colonies; - = no growth.

pig were enlarged and pale golden in color. The ventricular endocardium was dull in appearance; the liver was noticeably enlarged and of a bright golden color. The thymus was not found, despite close inspection during gross examination of the region.

Eight hours after injection, the organisms were not recovered from either the injection site or from the subcutis 2 cm. lateral to that site. Culture of the spleen indicated heavy contamination of that organ. A few coliforms were isolated from brain and liver. Ten colonies each were cultured from the sites in the mucosae of the stomach and mid-jejunum.

Grossly, the injection site was hemorrhagic and swollen, the liver was pale golden in color, the kidneys were pale, and the thymus was almost nonexistent.

Twelve hours after injection there was observed very heavy contamination of the injection site and light contamination of the liver and spleen. Heavy contamination of the ileocecal valve and moderately heavy contamination of the rectal mucosa were recorded from this pig. All other routine sampling sites were negative.

At gross examination the liver was pale, there were edema and hemorrhage of the ventral abdominal wall at the umbilical entrance and moderate edema of the mesocolon.

After 17 hours, no organisms were detected from the injection site or from the subcutis nearby. A few organisms were detected in the liver, spleen and kidney. The

stomach mucosa was lightly contaminated, and heavy growth was obtained from the mucosae of the midjejunum and the ileocecal valve. The rectum was sterile.

Upon gross examination, all superficial lymph nodes were enlarged and soft, and a clear fluid flowed freely from the cut surface. There were some areas of atelectasis in the apical and cardiac lobes of the lungs and a slight excess of pericardial fluid. The jejunal contents were much more fluid than the normal. The cecum and colon still contained meconium, and there was slight edema of the mesocolon. There was hemorrhagic edema of the injection site and edema of the abdominal wall in the vicinity of the umbilical stump. Again, the thymus was almost nonexistent.

After 20 hours, moderately heavy growth was obtained from culture of the injection site, and very light contamination of the subcutis lateral to the injection site was recorded. Light contamination of the spleen and of the gastric mucosa was found. The midjejunum, ileocecal valve and rectum were heavily colonized. All other sites were negative.

The superficial lymph nodes were enlarged, soft and edematous. There was a moderate excess of fluid in the pericardial sac, a moderate edema of the mesocolon, hemorrhagic edema of the subcutis at the injection site, and a localized area of edema less than 2 cm. in radius at the umbilical stump. The thymus was very small.

In the pigs killed 25 hours or more after injection, organisms were consistently cultured in large numbers from the stomach, midjejunum, ileocecal valve and rectum. Generally, the organisms were cultured from the inoculation site and sometimes from the subcutis 2 cm. lateral to that site. Occasional isolations, indicating very light contamination, were made from sites such as peritoneum, spleen and superficial lymph nodes.

One very significant isolation was made from the male pig killed 120 hours after inoculation. Heavy growth was obtained from each of the samples taken from the kidney and urocyst. All other sites, except the 2 skin sites and the gastrointestinal tract sites, were negative for presence of the organisms. It is apparent that in this pig a resident infection of the renal parenchyma had been established and that a descending infection of the urinary tract had occurred. It is of note that neither kidney nor urocyst was macroscopically abnormal.

Significant gross lesions in the 7 pigs killed between 25 hours and 168 hours after injection were as follows. The superficial lymph nodes were consistently enlarged and edematous, there was slight to moderate excess of pericardial fluid, and the livers were generally large, somewhat paler and with a more golden hue than were the livers of the control pigs. These livers did not appear to be excessively fatty, nor were they more friable than those obtained from

the noninfected controls. There was slight to moderate edema of the mesocolon and consistent hemorrhagic edema of the injection site with a moderate to marked ventral edema in the region of the umbilical stump.

In the 2 pigs killed at 121 and 168 hours after inoculation, the intestinal tract was darker in appearance from the midjejunum to the large intestine. The intestinal loops of the pigs infected for a shorter period of time before necropsy, and those of the control pigs, were uniformly light in appearance (Figure 5).

The 3 control pigs remained free of microorganisms throughout the experiment, and all cultures taken from the routine sites were negative. On gross examination the following was consistently found: slight to moderate edema of the superficial lymph nodes, slight to moderate excess of pericardial fluid, and slight edema of the mesocolon. In 1 control pig killed 44 hours after delivery there was mild ventral edema, and in the pig killed 154 hours after delivery there was a bright red ecchymosis of the parietal subperitoneum at the umbilical inlet. In all 3 control pigs the small intestine was uniform in color throughout its length.

Experiment 3

The Experimental Plan

In this experiment a litter of gnotobiotic pigs were given, *per os*, a suspension of *E. coli* O138:K81:NM (Mich.) to enable observation of the route by which this organism

Figure 5. The appearance of the external surface of the caudal half of the small intestine (C) of the monocontaminated pigs was characteristically much darker than that of the control pigs' small intestines. The latter were of uniform color throughout.

established an infection. These observations were compared with those made in Experiment 1, in which the same organism was introduced by injection into the subcutis of the umbilical stump.

Litter 5. This litter was 44 hours old when 4.8×10^6 E. coli O138:K81:NM (Mich.) organisms were given, per os, to each of 11 of the 13 pigs in the litter.

The animal necropsy numbers, sex distribution, period of elapsed time between inoculation and necropsy, and sites of positive isolation from tissues are tabulated (Table 6).

At 1 1/2 hours after exposure the organisms were cultured in large numbers from the oropharynx, stomach, duodenum, and midjejunum of 1 pig. The remainder of the gastrointestinal tract and all other sites cultured were sterile. There were no abnormal clinical signs in this pig nor gross abnormalities at necropsy.

In a second pig submitted to necropsy 1 1/2 hours after inoculation, organisms were found to have progressed through the gastrointestinal tract as far as the ileocecal valve. The rectum was still sterile at this time, as were all other sites cultured. There were no gross lesions in this animal, and it was clinically normal immediately prior to death.

In 1 pig, 4 hours after inoculation, organisms were found throughout the gastrointestinal tract and on the skin surface. Moderately heavy contamination of the urocyst

Table 6. Bacteriologic findings from pigs in Litter 5.

Pig Number	K52	K53	K57	K58	K62	K54	K55	K56	K59	K60	K61	Controls K63	Controls K64
Sex	F	M	F	M	F	M	M	M	M	F	F	F	M
Hours after oral exposure	1.5	1.5	4	4	8	16	22	24	32	40	123	8	24
Tissue--isolation of organisms from the following sites*													
Prescapular lymph node	-	-	-	1	-	-	-	-	-	-	-	-	-
Prefemoral lymph node	-	-	-	-	-	1	-	-	-	-	-	-	-
Pleura	-	-	-	-	-	-	-	-	-	-	-	-	-
Pericardium	-	-	-	-	-	-	-	-	-	-	-	-	-
Heart blood	-	-	-	4	-	-	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-	-	-	-	-	-	-
Peritoneum	-	-	-	-	-	-	-	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-	-	-	-	-	-	-
Spleen	-	-	-	-	-	-	-	-	-	-	-	-	-
Kidney	-	-	-	-	-	-	-	-	-	-	-	-	-
Urocyst	-	-	34	-	-	-	-	-	-	-	-	-	-
Iliac lymph node	-	-	1	-	-	-	-	1	-	-	-	-	-
Brain	-	-	-	-	-	-	-	-	-	-	-	-	-
Humeroradial joint	-	-	-	-	-	-	-	-	-	-	-	-	-
Oropharynx	++	++	++	++	++	++	++	++	++	+	-	-	-
Stomach	++	++	++	++	++	16	++	+	++	10	-	-	-
Duodenum	++	++	++	++	++	+	+	+	++	-	-	-	-
Midjejunum	++	++	++	++	4	+	+	+	++	16	-	-	-
Ileocecal valve	-	++	++	++	++	++	++	++	++	++	-	-	-
Rectum	-	-	++	++	-	++	++	8	++	++	-	-	-
Umbilical stump	-	-	+	-	-	++	+	+	++	++	-	-	-

*Bacterial colony counts are expressed as follows: The number = less than 51 colonies; + = more than 50 colonies and less than 101 colonies; ++ = more than 100 colonies; - = no growth.

epithelium was recorded, and 1 bacterial colony was cultured from the material taken from the lymph node draining the urocyst. All other routine sites were negative on culture, and it is possible that in this female pig the colonization of the urocyst was a result of perineal contamination and an ascending infection. The early involvement of the iliac lymph node indicates that systemic infection could have developed from contamination of the urocyst.

At necropsy the stomach was seen to be filled with clotted milk, and there was a marked flaccidity of the intestinal tract. The tract was filled throughout with watery yellow fluid, and this pig had just started to scour prior to euthanasia.

Another pig was killed 4 hours after inoculation, and the organism was cultured from all levels of the gastrointestinal tract. Some systemic contamination had occurred, with positive cultures of E. coli being obtained from prescapular lymph node and heart blood. All other systemic sites were sterile. This pig did not have diarrhea, and the intestinal tract contents were not markedly fluid.

At 8 hours after inoculation, 1 pig was killed, and heavy growth of organisms was obtained from the mucosae of the oropharynx, stomach, duodenum, and ileocecal value. Only 4 colonies were cultured from the midjejunal mucosa, and the rectal sample was sterile. The skin surface and all systemic sites in this animal were sterile. This pig

was clinically normal when killed and, at necropsy, the intestinal contents were very fluid within the small intestine, cecum and colon. There was still some meconium retained within the rectum.

The remaining inoculated pigs in this litter were killed at 16, 22, 24, 32, 40 and 123 hours, respectively, after oral exposure with the bacterial suspension. Except for the last pig, these animals were uniformly contaminated to a moderate or marked degree in each of the routine sites sampled within the gastrointestinal tract. In 1 pig, 1 organism was detected in a prefemoral lymph node. In a second pig, 1 organism was detected in an iliac lymph node. In the pig killed 40 hours after inoculation the stomach and mid-jejunum were found to be very lightly contaminated, and the duodenum was sterile. The remainder of the intestinal tract in this pig was heavily contaminated. Each of these pigs had profuse watery stool up to the time of euthanasia. Generally the intestinal tract was markedly flaccid and filled with watery fluid at necropsy.

The pig killed 16 hours after inoculation had scoured for several hours, and the skin was markedly inflamed in the perineal region. However, the intestine was of normal tone in this animal, even though the intestinal contents were characteristically fluid.

Edema of the mesocolon was not observed in any of the test or control animals in this litter.

In the pig killed 123 hours after inoculation, all cultures from all 21 routine sampling sites were negative. This animal had all the signs of baby pig enteritis; it had prolific diarrhea with watery stool and was markedly dehydrated. It had shared a plastic isolator with 2 other infected pigs used in this experiment and from which positive cultures had been obtained. As this was the final pig in the series, the isolator had been dismantled, and tissues remaining after necropsy had been incinerated or preserved in formalin before the cultures were found to be negative. No explanation was found for this phenomenon.

The 2 control pigs from this litter were free from detectable microorganisms at the time of necropsy. These animals were killed at 8 and 24 hours of age, respectively, and were used for clinical and histological controls for this and other litters in this experimental series. There were no gross abnormalities observed in either animal at necropsy.

Recovery and Serotyping of the Organisms

Seventeen cultures were grown on nutrient agar slants. Each individual culture represented a composite of organisms harvested from the positive culture plates of the experimental pigs of a particular litter that were housed in the same plastic isolator. They were forwarded to a serotyping

service* at the conclusion of the experiments. This service center reported that all cultures belonged to the E. coli O group 138 and had the K81 antigen, but no check was made for the presence of H antigen nor for hemolytic activity.

Clinical and Pathologic Findings

Observations were made during the preceding experiments on the clinical effect of oral inoculation and subcutaneous injection of neonatal gnotobiotic colostrum-deprived pigs with E. coli serotypes O138:K81:NM (Mich.) and O138:K81 (Minn.). A record and study of the lesions associated with colibacillosis due to these specific serotypes was made.

Clinical Findings

There was a distinct difference between the clinical expression of the infections with E. coli O138:K81:NM (Mich.) and E. coli O138:K81 (Minn.). Also, a difference was found in the time taken for clinical signs of disease to be observed when 1 serotype was used but introduced by a different route. Results were as follows:

Experiment 1

[E. coli O138:K81:NM (Mich.) injected into the subcutis of the umbilical stump]

Details of the time of development of systemic contamination and enteric infection and the time of disappearance

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of the organisms from the specific testing sites are given in Tables 2, 3 and 4.

Litter 1. Although pigs killed at 4, 8 and 24 hours after injection had varying degrees of systemic and enteric involvement with the organism (Table 2), the first clinical sign of disease was seen in a pig when it was killed 32 hours after injection. This pig had marked hemorrhagic panenteritis (found at necropsy), and prior to euthanasia the soft pasty feces of this pig were seen to be very dark and blood-stained.

After 32 hours other pigs in this litter began to pass copious amounts of soft or watery feces. By 40 hours after injection the remaining pigs in the litter were scouring. Pigs were killed at 40, 48, 77, 101, 123 and 151 hours after injection. The fecal material from those killed early in this series was of watery consistency, pale yellow, and with suspended fine whitish curds. As time passed the color of the watery material varied between pigs, and the suspended curds were less commonly seen, even though the pigs continued to drink well throughout the experiment.

At about 100 hours after injection the pigs were arching their backs as though discomforted by the diarrhea. The first signs of raised hair coat and "scalding" (erythema venenatum) of the perineum were seen in the pig killed 123 hours after injection. At 151 hours after injection the last pig in this experiment had a raised hair coat, was

apparently losing weight, had a "scalded" perineum, and had a very watery stool in which there were thin white curds. This animal did not have anorexia at any stage of the experiment.

The germfree control animals continued to pass soft or typically pasty feces, gained weight, and maintained apparent good health until the time of euthanasia.

Temperatures of control and test pigs were taken daily in the morning, and all readings tended to be on the low side of an accepted normal (102.0 F.) for pigs (Blood and Henderson, 1963). There were no significant changes in temperature recorded during the experiment.

Litter 2. Although pigs killed at 1 1/2, 2 1/2, 4, 6 1/2, 8, 12 and 16 hours after injection had varying degrees of systemic and enteric involvement with E. coli O138:K81:NM (Mich.) (Table 3), the first clinical sign of disease was seen in a pig when it was killed 24 hours after injection. This pig had bright yellow watery feces in which find white curds were suspended. At 48 hours after inoculation the watery feces were light yellow and there was a loss of "bloom" in the hair coat. In the pig killed 72 hours after injection, diarrhea was recorded. The pig had an arched back, was thin, was apparently dehydrated, and had an erect hair coat.

Anorexia was not seen in any pig in this litter during the experiment.

Temperatures were taken under the same conditions as for Litter 1, and no significant differences were noted.

The control pigs remained germfree and healthy throughout the experiment.

Litter 3. Although pigs killed 1 1/2, 2 1/2, 9, 24, and 32 hours after injection had varying degrees of systemic and enteric involvement with E. coli O138:K81:NM (Mich.) (Table 4), the first clinical sign of disease in this litter was seen in a pig when it was killed 48 hours after injection. Both this pig and the animal killed at 72 hours after injection had profuse watery diarrhea at the time of euthanasia.

No instances of anorexia were recorded in this litter during the experiment.

Temperature readings were not taken in this experimental group.

The control animals remained germfree and in good health throughout the experiment.

Experiment 2

[E. coli O138:K81 (Minn.) injected into the subcutis of the umbilical stump]

Litter 4. Although pigs killed at 4, 8, 12, 17, 20, 25, 32 and 40 hours after injection had varying degrees of systemic and enteric involvement with the test organism (Table 5), the first clinical sign of colibacillosis in this litter was seen in a pig when it was killed 48 hours

after injection. This animal had very soft feces and marked subcutaneous edema of the axilla and hind legs. In the pig killed 72 hours after injection, ventral abdominal edema was observed and the feces were very sloppy in consistency. At 121 hours, the feces were excessively soft, and there was moderate ventral edema. The pig killed at 168 hours after injection had excessively soft feces, but no ventral edema was recorded.

Temperatures were taken daily in the morning from this litter, but there were no significant changes in the readings recorded during the course of the experiment.

Control animals remained germfree and in good health throughout the experiment.

Experiment 3

[*E. coli* O138:K81:NM (Mich.) inoculated *per os*]

Litter 5. Details of organ and tissue isolations with respect to time after inoculation are recorded in Table 6.

The first sign of diarrhea was observed in 1 of the pigs submitted to necropsy 4 hours after inoculation. The entire gastrointestinal tract of this pig was heavily contaminated by that time. A second pig killed 4 hours post-inoculation did not have clinical evidence of disease and was found to have its gastrointestinal tract heavily contaminated only so far as the ileocecal valve. The rectum of this pig was still bacteriologically sterile at the time of necropsy (Table 6).

One pig killed 8 hours after inoculation did not have a fully colonized gastrointestinal tract (Table 6) and did not have clinical signs of disease. The single pig killed 16 hours after inoculation had profuse, clear, watery, yellow feces in which fine yellow curds were suspended. This pig had a "scalded" perineum and had been scouring for an unrecorded period of time prior to euthanasia.

Profuse watery yellow feces and "scalding" of the perineum were characteristic of the pigs killed at 22, 24, 32, 40 or 123 hours postinoculation. The pig killed at 123 hours postinoculation was extremely thin and apparently dehydrated. Anorexia was not reported regarding any pig in this litter during the experiment.

Temperatures of the pigs in this litter were taken 3 times daily (8 a.m., noon, and 5 p.m.). During the first 2 postnatal days the temperatures were on the low side of normal for all pigs in the experiment, but by the 3rd day of life (first day postinoculation) the temperatures of the remaining pigs were within the range of 100.4 to 103.2 F and remained at approximately this level throughout the experiment.

Control pigs remained germfree and in good health throughout the experiment.

Hematology

In only 3 litters was it possible to get satisfactory blood samples from the experimental animals both immediately

after delivery and again immediately prior to euthanasia. These were Litters 3, 4 and 5.

Litter 3. In this litter both control pigs had a marked fall in total leukocyte counts to 1/2 and 1/3, respectively, of the numerical values recorded from samples taken on the day of delivery. The depletion of lymphocytes was relatively greater in both pigs than was the neutrophil depletion. At the time of necropsy the age of these 2 pigs was 23 hours and 44 hours, respectively.

The fall in leukocyte counts was not uniform throughout the injected pigs in this litter, and no trend could be seen either in total leukocyte count or in the absolute differential leukocyte counts of these animals.

Litter 4. In 2 of the 3 control pigs complete hemograms were recorded. In 1 pig, killed at an age of 44 hours, there was a slight drop in the leukocyte count to a value still within the normal range. In the second pig, killed at an age of 176 hours, there was a marked drop in the leukocyte count to a value 2/3 of that accepted as a normal minimum (Calhoun and Smith, 1964). In this case, however, the decrease was due to a loss of neutrophils. The lymphocytes were actually increased in total numbers in this animal.

The general trend in the hemograms recorded for day-of-birth and terminal blood samples was a moderate to marked fall in the leukocyte count, usually from initial values at

the lower level of the accepted normal for conventional pigs of this age (as cited by Calhoun and Smith, 1964) to values far below the acceptable normal minima. This decrease was due either to an equal decrease in both neutrophils and lymphocytes or to a marked decrease in the lymphocyte count which in 2 instances was associated with a concomitant absolute increase in the neutrophilic fraction.

Litter 5. The hemograms from the germfree animals in this litter were insufficient in that the terminal hemogram from 1 of the animals was not recorded because the blood sample clotted while being removed from the isolator. The second control animal was killed 24 hours after inoculation. It had a ninefold increase in total leukocytes, due almost entirely to an increase in the neutrophilic fraction of the leukocytes.

The function of these hemograms as control hemograms was discounted.

In the hemograms of the 2 pigs killed 1 1/2 hours postinoculation, there was an approximate 2/3 reduction in the circulating leukocytes, the major reduction being in the circulating neutrophils. A similar change was recorded in the hemograms of the 2 pigs killed 4 hours after inoculation.

In the pig killed 16 hours postinoculation the leukocyte count was approximately half of that recorded at birth (60 hours before), and the fall in the count was apparently

due to approximately equal decreases in both the lymphocyte and neutrophil counts.

This trend persisted in the hemograms of the pigs killed 22, 24, 32 or 40 hours after inoculation. At 40 hours postinoculation, the total hemoglobin content and leukocyte counts were still below the normally acceptable limits for conventional pigs of this age range (Calhoun and Smith, 1964).

Histopathologic Findings

The histopathologic lesions found in this study were predominantly in the gastrointestinal tract. The lesions ranged from those of an extensive acute hemorrhagonecrotic enteritis to a histologic picture, in persistently scouring pigs, microscopically indistinguishable from that seen in clinically normal germfree control animals.

Studies were made of a number of systems, and lesions were recorded as follows:

Skin and umbilical stump. Within 1 1/2 hours of injection of E. coli O138:K81:NM (Mich.) there was mild hemorrhagic edema and perivascular fibrinous exudate in the subcutis of the umbilical stump. By 2 1/2 hours neutrophils had entered but were still concentrated in perivascular locations (Figures 6 and 7). Neutrophils, eosinophils and lymphocytes increased in numbers in the site of inflammatory edema for the next 12 to 24 hours. At 12 hours postinjection, necrosis of inflammatory cells was evident and, at

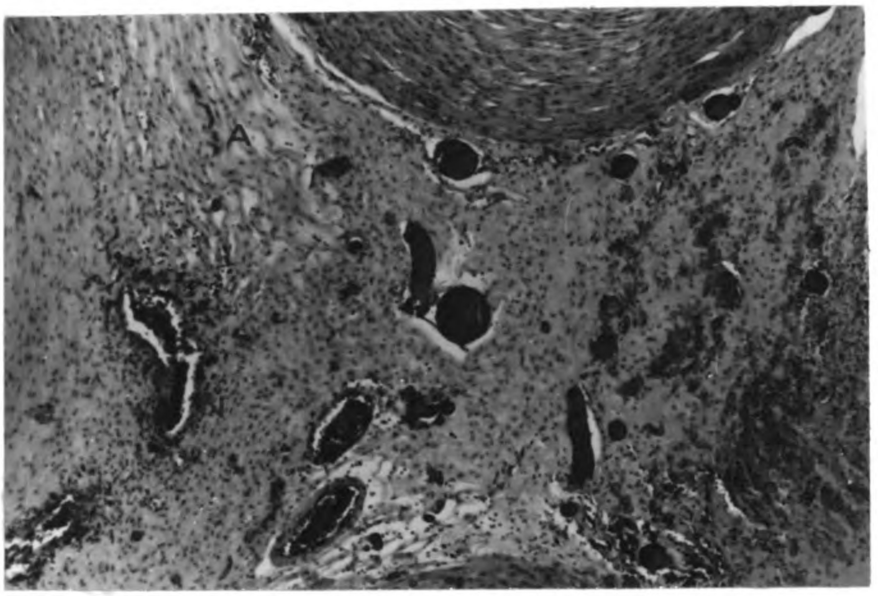

Figure 6. Pig J4662. Injection site 4 hours after injection of E. coli. Connective tissue edema (A) and perivascular collection of neutrophils (N). Hematoxylin and eosin. x 190.

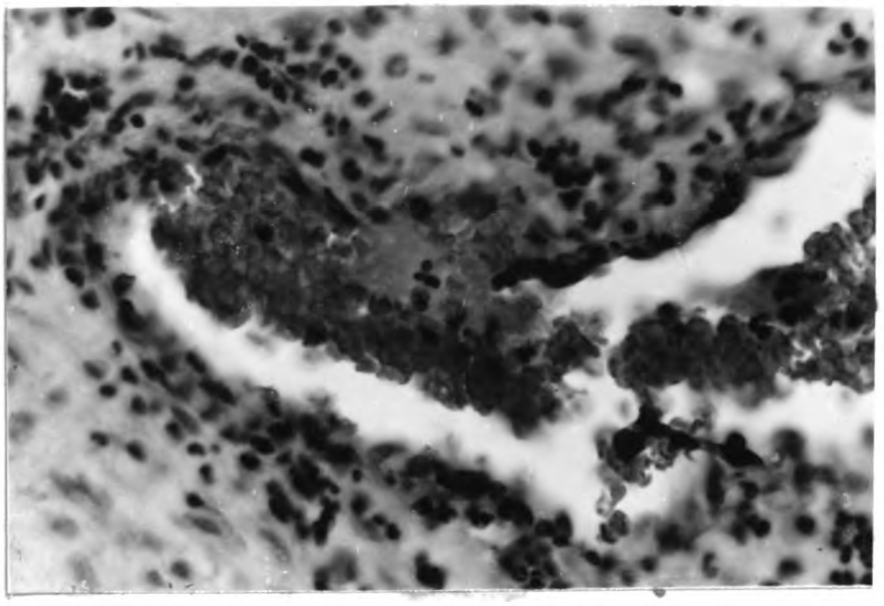

Figure 7. Pig J4662. Same field as Figure 6. Perivascular neutrophils are spreading into the injection site from neighboring vessels. Hematoxylin and eosin. x 470.

14 hours, macrophages were observed in the area. At 72 hours, attempts to enclose a necrotic mass of neutrophils with fibroblasts were observed. By 123 and 168 hours there was abscess formation with necrotic cells walled off by foamy macrophages and granulation tissue (Figure 8).

In the first 40 hours following injection of E. coli O138:K81 (Minn.) (as in Experiment 2) the tissue response was essentially the same as for E. coli O138:K81:NM (Mich.). A significant observation was that in each of the 4 pigs killed after longer periods of time (48, 72, 121 or 168 hours postinjection) there was abscess formation at the site with the increasing development of a wall of macrophages and fibroblasts. Peripheral to the developing abscess there was slight to moderate hemorrhage and infiltration with inflammatory cellular (primarily neutrophilic) exudate.

Superficial lymph nodes. Within 4 to 8 hours after injection of the test organisms, there was reduction in the number of mature lymphocytes present in the superficial lymph nodes (Figure 9). At time intervals beyond 8 hours there was an increase in the numbers of neutrophils and eosinophils in the nodes and an associated peritrabecular and perinodal edema. Eosinophils were markedly increased in the lymph nodes by 100 hours postinjection. Neutrophilic lymphadenitis was observed in 1 pig killed 101 hours after

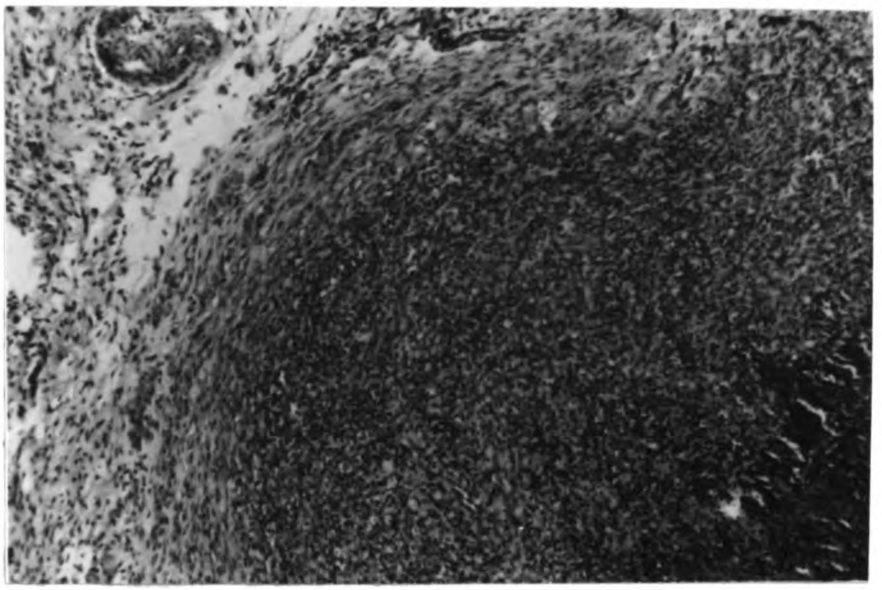

Figure 8. Pig J5722. The injection site. E. coli was injected 168 hours before necropsy. An abscess surrounded by macrophages, fibroblasts, other inflammatory cells and edema. Hematoxylin and eosin. x 75.

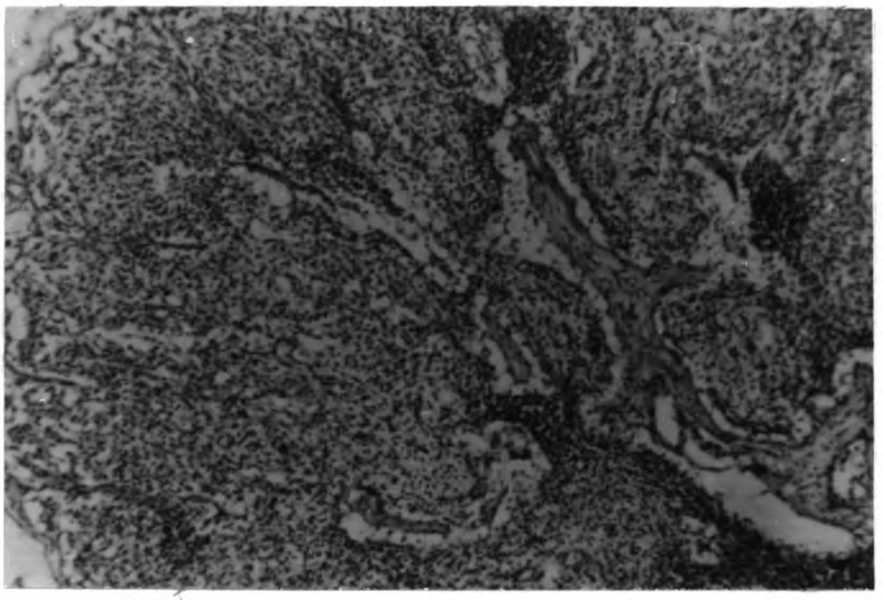

Figure 9. Pig J6205. Prescapular lymph node. E. coli was injected 24 hours before necropsy. There is edema and an apparent lack of mature lymphocytes in the germinal centers. Hematoxylin and eosin. x 75.

injection (Figure 10). Superficial lymph nodes from the germfree controls were also edematous and depleted of mature lymphocytes, but these changes were not nearly so marked in degree as were those of the infected pigs.

Trachea and lungs. Consistent findings in histopathological examination of the lungs were atelectasis and emphysema. There were, in addition, many instances of alveoli being filled with homogeneous eosinophilic material. It is possible that these phenomena, equally present in control animals, are a result of the hysterotomy procedures at delivery.

In 2 instances, hydropic degeneration of the terminal bronchiolar epithelium was observed--both pigs were undergoing early systemic involvement and early establishment of the test organism in the intestinal tract.

In 2 pigs heavily contaminated in all routine sites (including lung) and in which gross intestinal lesions were observed, there were significant changes in the lungs. In the first pig, killed 24 hours postinjection, there were hyperemia of the alveolar capillaries and fibrinous exudate partly or completely filling some of the alveoli. In other alveoli an exudate of erythrocytes, fibrin, proteinaceous fluid, neutrophils, lymphocytes and macrophages with desquamated alveolar cells was observed. These were also vacuolation and desquamation of the mucous epithelium of the associated bronchioles.

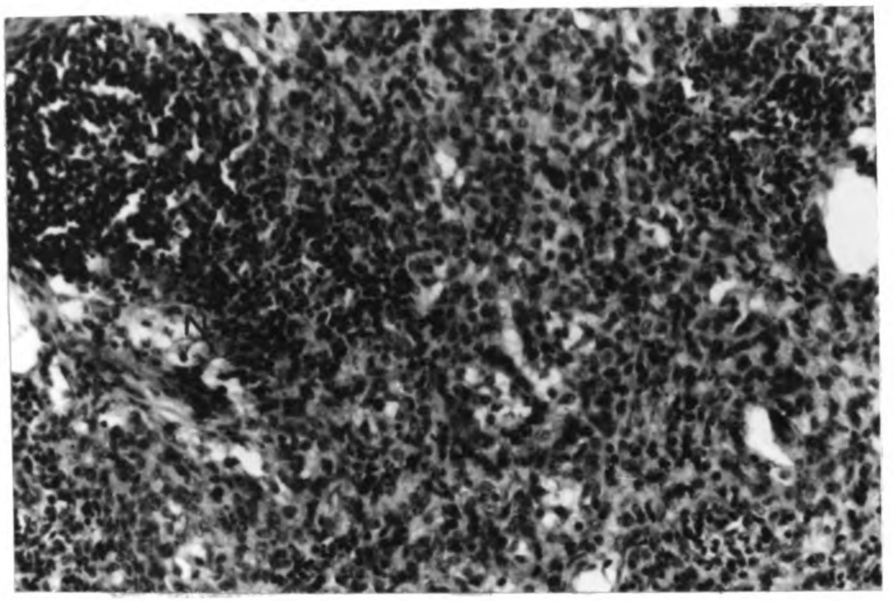

Figure 10. Pig J4673. Prescapular lymph node. E. coli was injected 101 hours before necropsy. Acute lymphadenitis. The lymph node is infiltrated with neutrophils (N). Hematoxylin and eosin. x 190.

In the second pig, killed 32 hours after injection, there was marked hyperemia of the alveolar capillaries. No other changes were observed.

There were no apparent changes of significance in the trachea.

Heart and aorta. No consistent changes were observed in the heart or the aorta. In almost all instances cells with the configuration of nuclear chromatin characteristic of the Anitschkow myocyte were extremely common in the sections of myocardium of both infected and control animals; in some instances they were so common as to suggest that the Anitschkow myocyte is not a myocardial histiocyte but an immature normal myocardial cell and of little or no significance when observed in sections of the myocardium of a germ-free pig (Figure 11).

Myocardial edema and diffuse intramyocardial hemorrhages were observed in 1 pig with systemic bacterial contamination and established enteric infection. This pig was killed 20 hours after injection of the test organism.

In another pig with systemic and early enteric contamination resulting from injection with the test organism 8 hours earlier, the aortic endothelium was distended, swollen and vacuolate, with the nuclei consistently displaced toward the lumen of the aorta (Figure 12). Almost every muscle cell in the section of the myocardium of this pig was an Anitschkow myocyte.

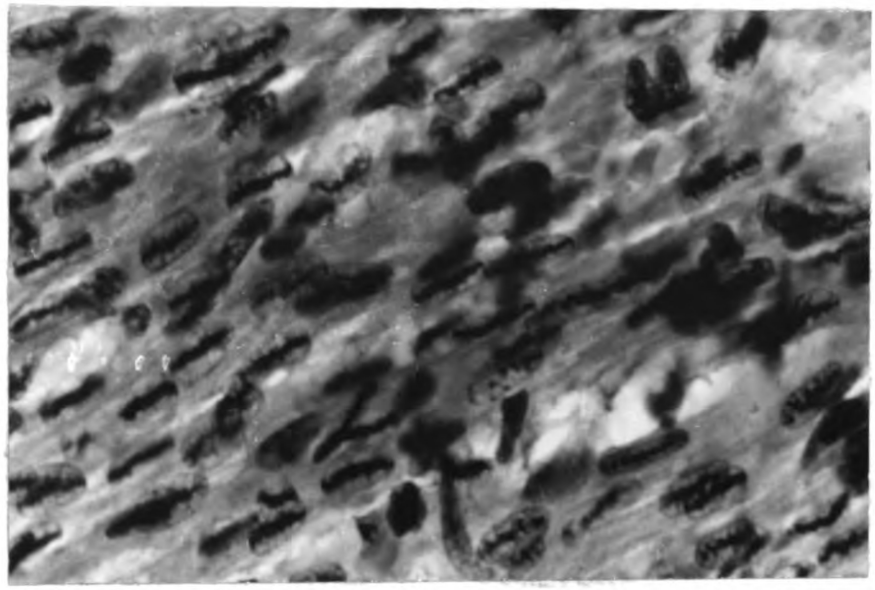

Figure 11. Pig J5716. Myocardium. A majority of the myocardial nuclei have the chromatin configuration characteristic of Anitschkow myocytes. Hematoxylin and eosin. x 750.

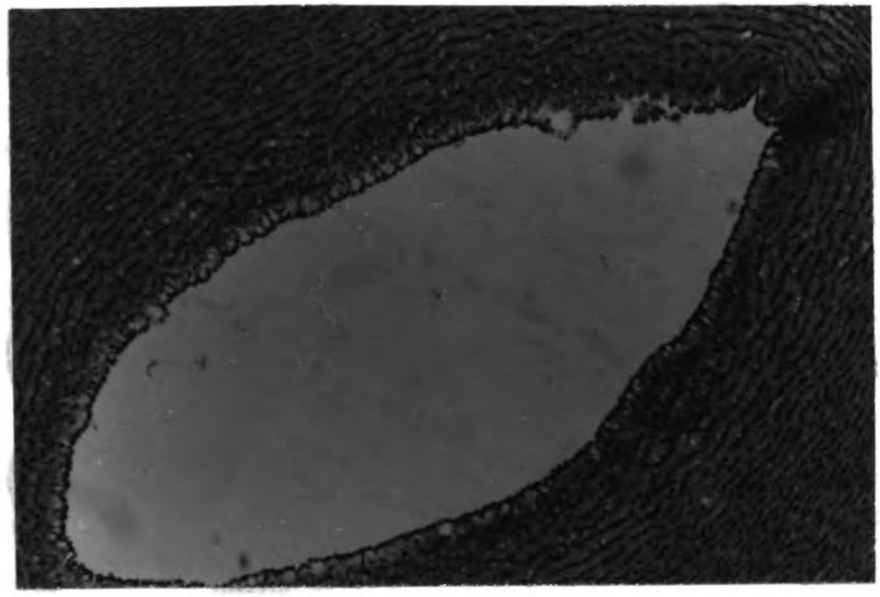

Figure 12. Pig J5712. Aorta. E. coli was injected 5 hours before necropsy. Vacuolization of endothelial cells. Hematoxylin and eosin. x 75.

Spleen. It was observed that there was an apparent slight to moderate depletion of mature lymphocytes from the spleens of some of the infected animals when compared with those of the germfree controls.

Thymus. No consistent changes were observed in the thymus. In 1 pig (J4663) killed 8 hours after injection, from which there was isolation of the organism at 7 systemic sites, the interlobular septa of the thymus were infiltrated with edema fluid containing many mature lymphocytes.

In another pig with systemic and early enteric contamination with E. coli (J5712), a discrete mass of neutrophils was observed in the medulla of 1 lobule.

In the same experimental group, a pig (J5717) with slight systemic contamination and heavy colonization of the entire intestinal tract had a thymus markedly infiltrated with eosinophils.

Thyroid and adrenals. There were no consistent changes observed in the thyroid. In 1 pig killed 20 hours after injection (J5715), in which there was systemic contamination and enteric colonization, edema of the interstices of the thyroid was observed.

A generally consistent finding in the adrenals of both the monocontaminated and the germfree pigs was hyperemia of the medulla and the zona reticularis. This hyperemia was more pronounced in those pigs in which there was

only systemic bacterial involvement than in those killed 72 hours or more after injection. The hyperemic response in those pigs killed 72 hours or more after injection was more apparent than in the germfree control group.

Foci of eosinophils were observed in the adrenal cortex of 1 pig killed 25 hours after injection with the test organism (J5716).

Alimentary tract

Esophagus. No significant changes were observed. The test organism when given by mouth was seen to colonize in the debris characteristically attached to the esophageal epithelial surface in gnotobiotic pigs on liquid diets. In some instances there was parakeratosis of the epithelium (Figure 13) and infiltration of the epithelium with neutrophils was observed (Figure 14).

Stomach. Sections were cut from the cardiac, fundic and pyloric areas of the stomach of each pig. There was wide variation in the lesions observed in these tissues, and at the end of the scale of decreasing severity of lesions there were no apparent differences between the tissues of the monocontaminated pigs and the germfree pigs. In these pigs the simple columnar mucous epithelium was blocky and arranged in orderly fashion in a single layer of cells, the lamina propria and submucosa being infiltrated with occasional lymphocytes and mononuclear cells (Figures 15, 16 and 17).

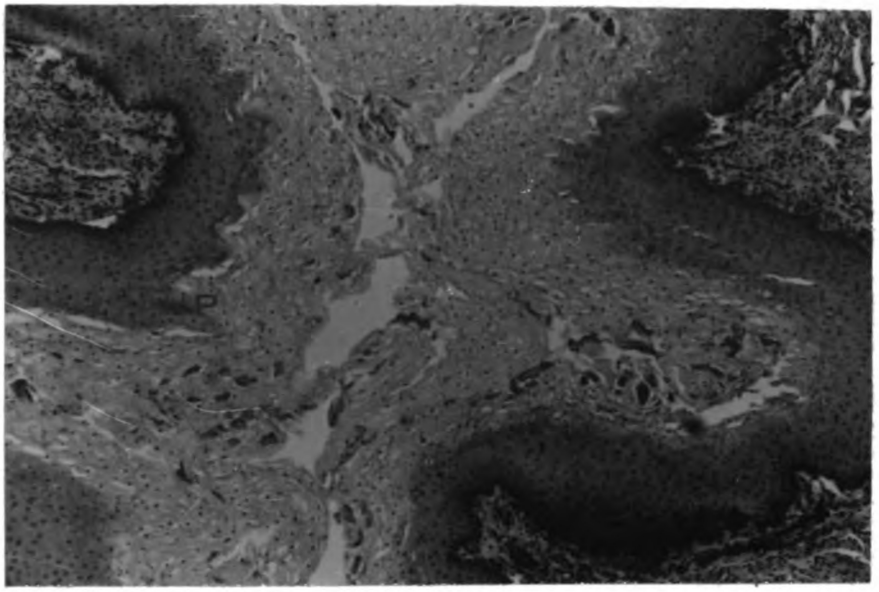

Figure 13. Pig J5716. Esophagus. Parakeratosis of the esophageal epithelium (P). Hematoxylin and eosin. x 75.

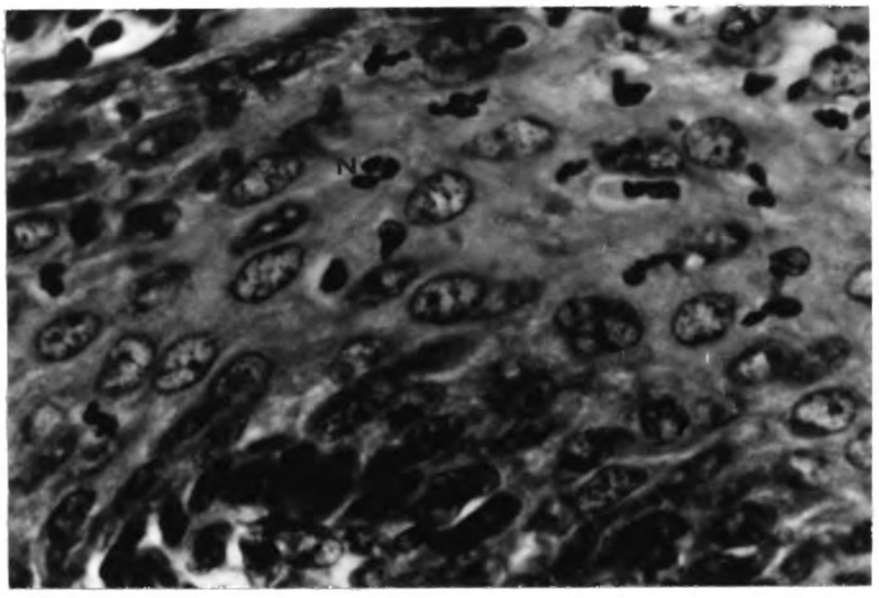

Figure 14. Pig J6207. Esophagus. E. coli was injected 72 hours before necropsy. Neutrophils (N) infiltrating the squamous epithelium of the esophagus near the cardia. Hematoxylin and eosin. x 750.

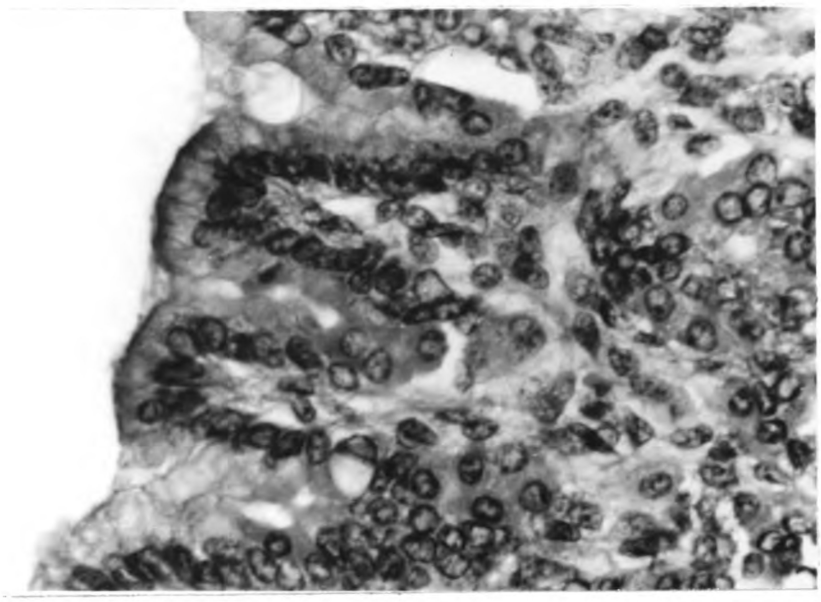

Figure 15. Pig K63. Mucosa of the cardiac region of the stomach from a germfree control, illustrating minimal epithelial activity to be seen in control animals. Hematoxylin and eosin. x 470.

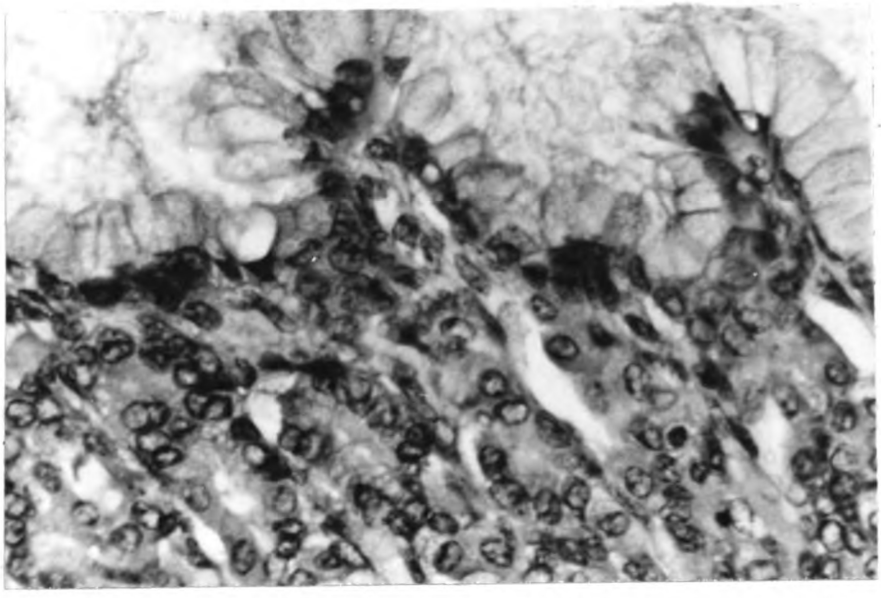

Figure 16. Pig K63. Mucosa of the fundic region of the stomach from a germfree control illustrating maximal epithelial activity seen in control animals. Hematoxylin and eosin. x 470.

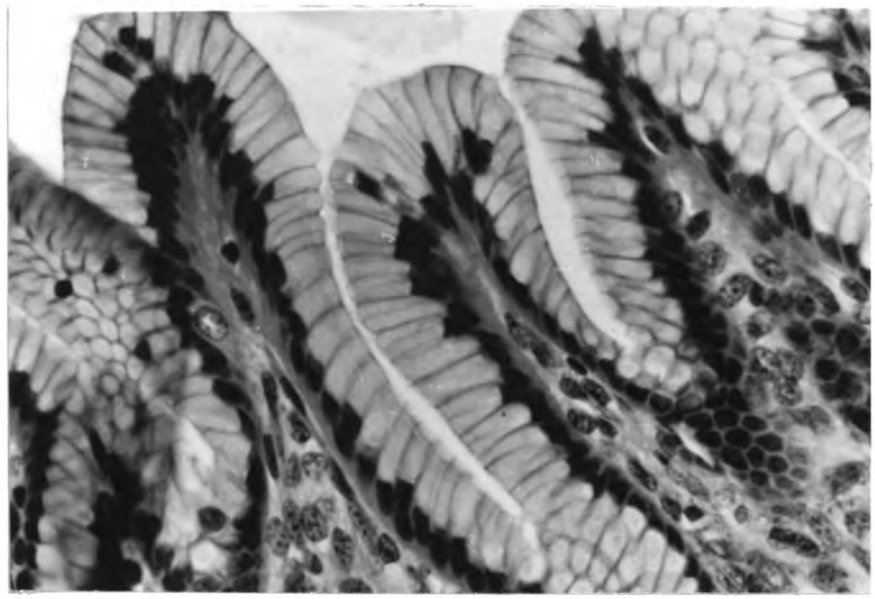

Figure 17. Pig J5718. Fundic region of the stomach. E. coli was injected 40 hours before necropsy. Moderate epithelial activity of the heavily colonized gastric mucosa of a monocontaminated pig. Hematoxylin and eosin. x 470.

In the mucosa of most of the pigs from which the test organism was cultured, the mucous activity of the epithelium was increased. The individual cells were larger, more densely packed, and elongated, and in some instances the cytoplasm was so pale staining and increased in amount as to give the mucous cells a vacuolate appearance.

In each instance where distinct microscopic lesions were observed, the cultures were found to be positive, indicating heavy contamination of the stomach by the test organism. Changes observed in the mucosa were distention and vacuolation of individual epithelial cells. Instances of massive simultaneous discharge of the epithelial cell contents with outpouring of mucus onto the gastric surface were seen. Necrosis, dissolution of the epithelial surface and some epithelial sloughs were recorded from some infected pigs (Figures 18 and 19). In the fundic mucosa of 1 pig there were foci of neutrophils (Figure 20). Hyperemia of the gastric mucosa of infected pigs was common, as were edema and hypercellularity of the lamina propria and areolar coat of the submucosa. Major cell types present to varying degrees in these tissues were primarily lymphocytes, plasma cells and neutrophils, with some macrophages and an unexpectedly high proportion of eosinophils.

The small intestine. Sections were routinely made from duodenum, jejunum at the 50-cm., 100-cm. and 200-cm. levels, and ileum close to the ileocecal valve. It is

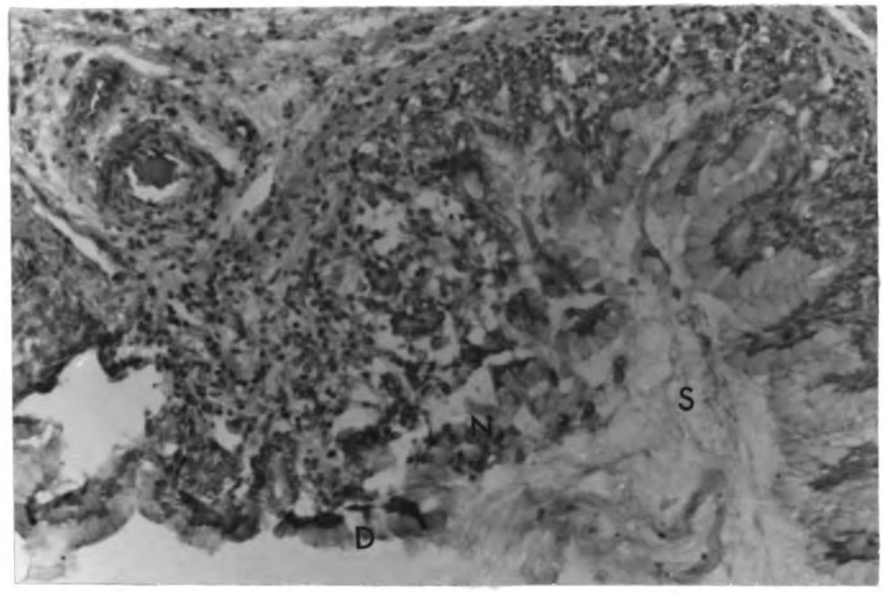

Figure 18. Pig J4669. Pyloric region of the gastric mucosa. E. coli was injected 32 hours before necropsy. There is necrosis (N) and dissolution (D) of portions of the epithelial surface and the lamina propria. Seromucus (S) is seen to be streaming from some epithelial elements. Hematoxylin and eosin. x 190.

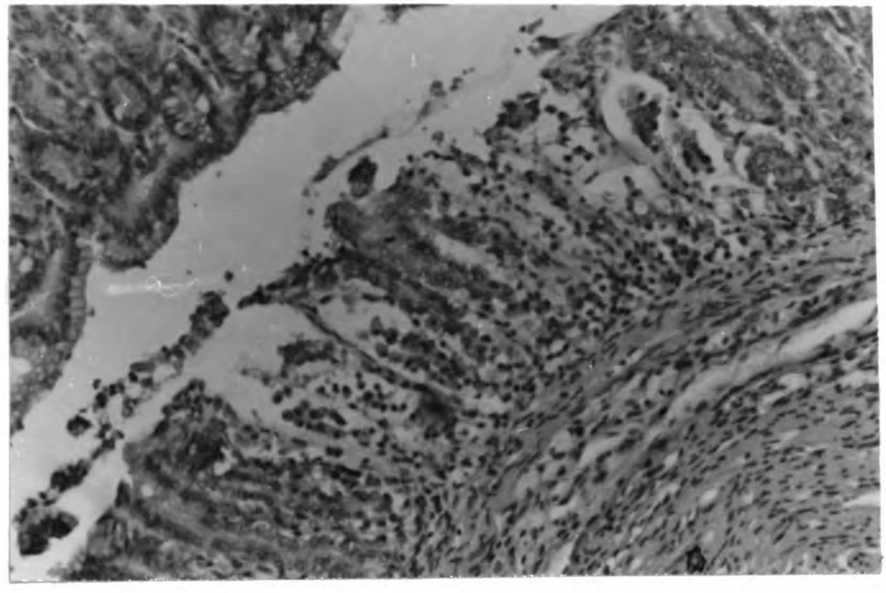

Figure 19. Pig J4669. Pyloric region of the stomach. E. coli was injected 32 hours before necropsy. There is a focus of necrosis and early ulceration of the mucosa. Hematoxylin and eosin. x 190.

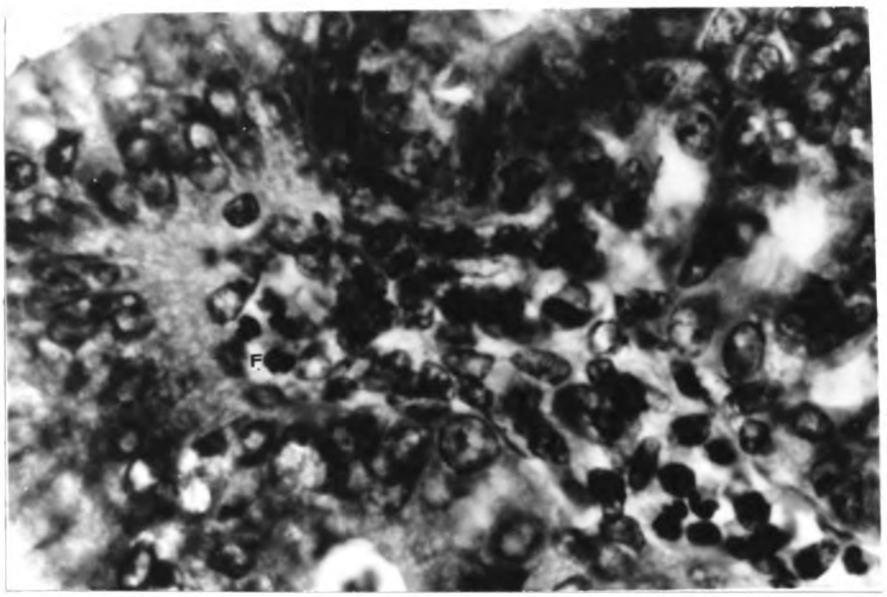

Figure 20. Pig J6207. Fundic region of the stomach. E. coli was injected 72 hours before necropsy. A few scattered neutrophils (F) are in the lamina propria. Hematoxylin and eosin. x 750.

apparent that marked variations from the normal can occur as a result of infection with these 2 serotypes of E. coli. It is also apparent that much variation from the normal to abnormal can occur as one examines tissues taken from neighboring sites in a heavily colonized intestinal tract.

(i) The epithelium and lamina propria. In some infected pigs and some germfree control animals the surface epithelium was ordered and regular with little or no mucous activity and with occasional active goblet cells filled with mucin and becoming more frequent in more caudal sites (Figure 21). According to the level of the section, lymphocytes were present singly or in aggregates in the submucosa and in increasing numbers caudad.

Some slight but distinct changes from the above were also seen in the control and monocontaminated pigs. The volume of the cytoplasm of the individual columnar cells of the mucous epithelium was uniformly greater and less intensely stained. In some instances the epithelial nuclei were uniformly centrifugally placed in relation to the cell cytoplasm and the axis of the villus (Figure 22); in other sections the epithelial cell nuclei were uniformly axially placed, close to the basement membrane of the epithelium. The cytoplasm of these cells was negative or very faintly positive to Mayer's mucicarmine stain.

A more advanced degenerative change very frequently observed was that in both control and infected animals columnar epithelial cells of the villi were markedly

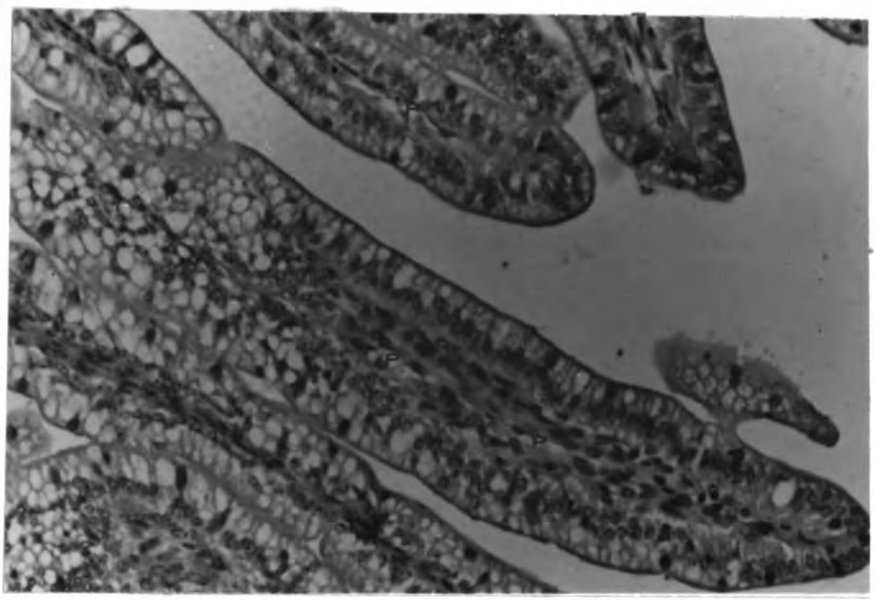

Figure 21. Pig J5723. Terminal ileum. Germfree control. Moderate vacuolization of the epithelium of the villus has little or no apparent effect on the ability of the villus to absorb some fluid from the lumen of the intestine. Lymphatic vessels and lacteals are visible (P), indicating a degree of function. Hematoxylin and eosin. x 190.

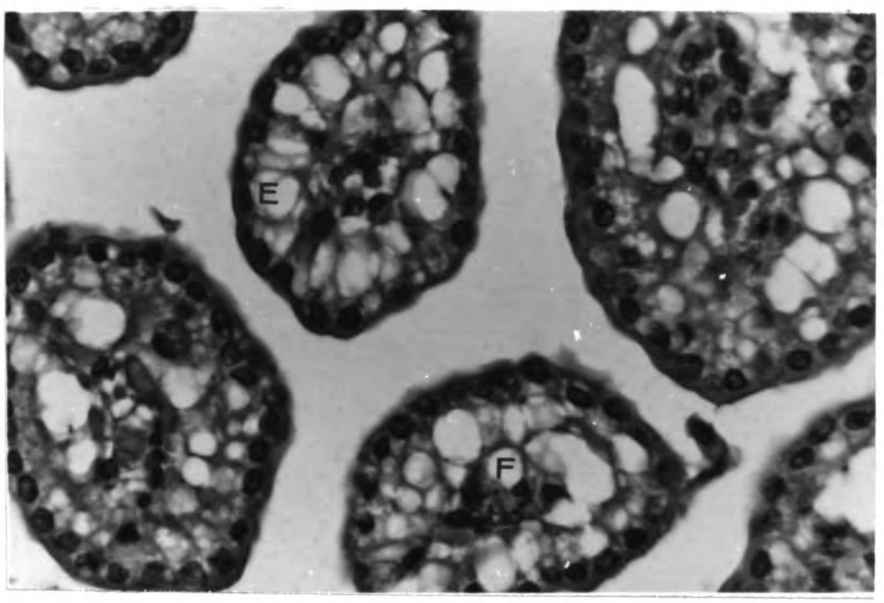

Figure 22. Pig K57. Terminal jejunum. E. coli was introduced per os 4 hours before necropsy. Transverse section of the villi. Some slight lymphatic and lacteal function is apparent (F); the epithelial cells are enlarged and vacuolated (E). Epithelial cell nuclei are abaxially situated. Hematoxylin and eosin. x 470.

distended and/or vacuolated either singly or en masse (Figures 23, 24, 25 and 26).

Varying degrees of subepithelial edema of the villi were seen to be associated with 1 or more of the epithelial changes described above (Figure 27). Commonly observed was edema of the tips of the villi, forming subepithelial bullae (Figures 28 and 29). In some instances this edema was restricted to the tip of the villus, but it was also seen to extend in some cases the entire length of the villus. In other cases the subepithelial edema was observed at the base or midvillus region leaving the tip relatively unchanged (Figure 30).

In a few instances distinct separation of the basement membrane from the lamina propria indicated that the subepithelial space was a fixation artefact. Generally, the condition of subepithelial edema was indicated by subepithelial vacuolation bounded by fibrous stroma (Figures 30 and 31) and sometimes containing pale-staining homogeneous or granular eosinophilic material and/or fibrin strands (Figures 27, 28, 29 and 32). At times, edema of the axial tissues of the lamina propria of the villus was seen.

Other changes commonly observed in the villi and not seen in the villi of the intestines of control pigs were hyperemia with or without hemorrhage and/or necrosis. In some instances there was surface necrosis with sloughing of the tips of the villi and shortening of these structures to produce a short, stumpy villus.

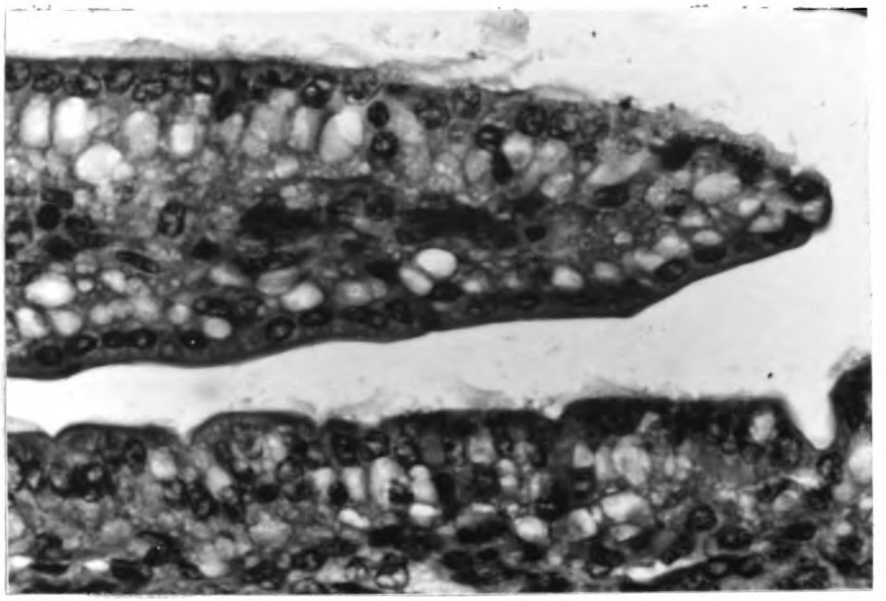

Figure 23. Pig J5721. Midjejunum. E. coli was injected 121 hours before necrospy. Epithelial cells are enlarged and vacuolated. Nuclei are abaxially situated. Hematoxylin and eosin. x 470.

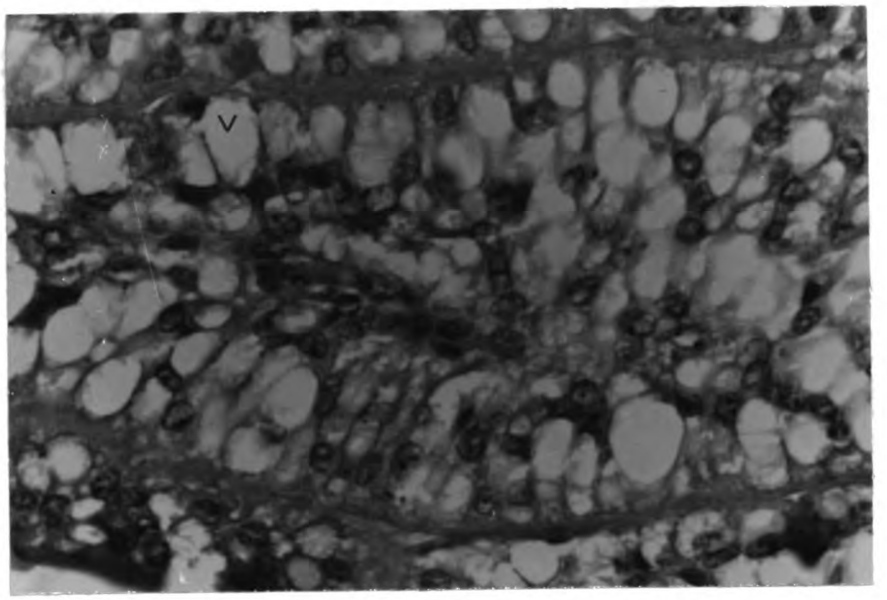

Figure 24. Pig J4672. Midjejunum. E. coli was injected 77 hours before necropsy. Marked vacuolization of the epithelial cells (V). Neighboring villi are very closely apposed and the laminae propriae of individual villi are compressed. Hematoxylin and eosin. x 470.

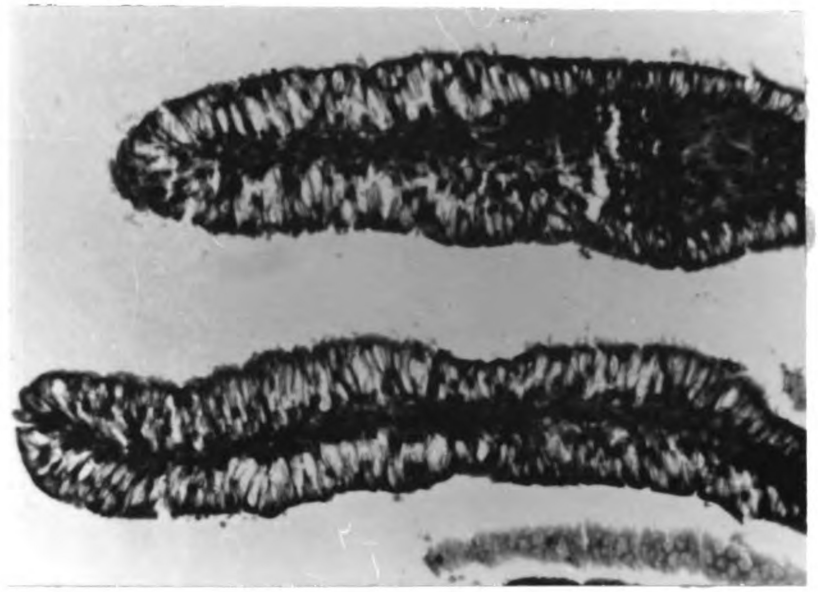

Figure 25. Pig J5721. Terminal ileum. E. coli was injected 121 hours before necropsy. Very pronounced vacuolization of the epithelial cells. Compression and apparent inactivity of elements of the lamina propria. Hematoxylin and eosin. x 190.

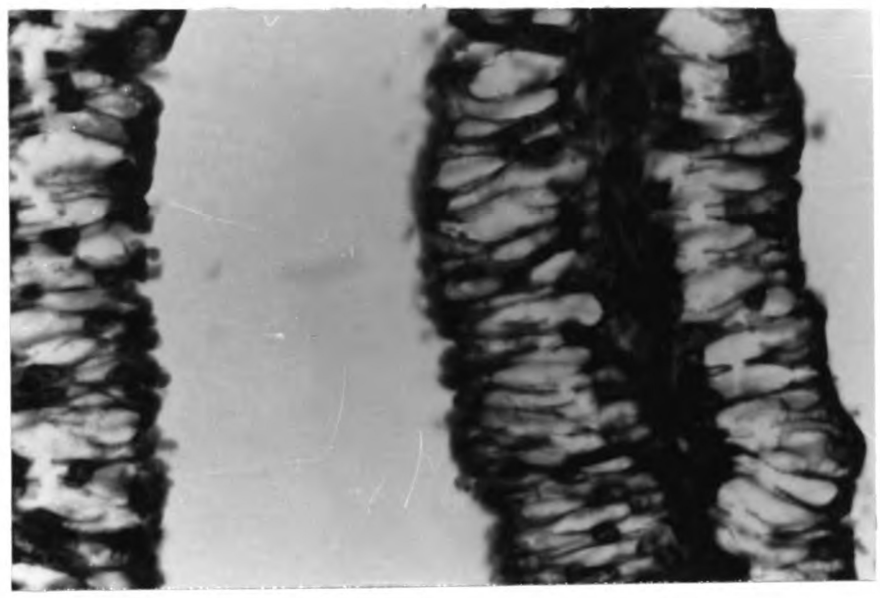

Figure 26. Pig J5721. Terminal ileum. From the same field as Figure 25. Marked vacuolization of epithelial cells. Hematoxylin and eosin. x 470.

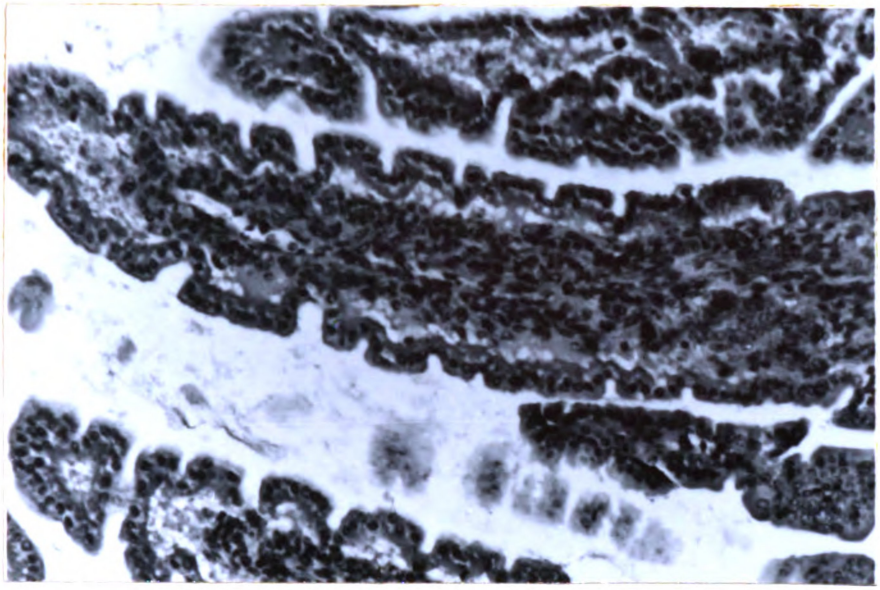

Figure 27. Pig J6206. Jejunum. E. coli was injected 48 hours before necropsy. There is early vacuolization of the epithelium, but it is within the range of normality of jejunal epithelium of germfree control pigs. There is evidence of fluid transfer from the jejunal lumen into the subepithelial spaces and the vessels of the lamina propria. The lamina propria is infiltrated with some neutrophils. The villus has undergone partial longitudinal contraction. Hematoxylin and eosin. x 190.

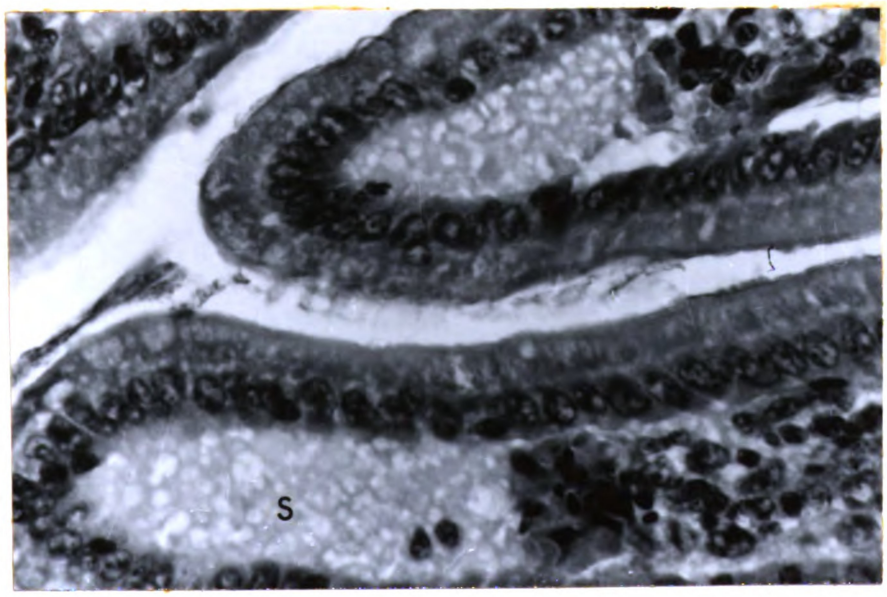

Figure 28. Pig J5721. Duodenum. E. coli was injected 121 hours before necropsy. Bullae at the tips of duodenal villi. There are some neutrophils in the lamina propria, and fluid absorbed from the lumen of the duodenum has been transported into the subepithelial spaces (S). Hematoxylin and eosin. x 470.

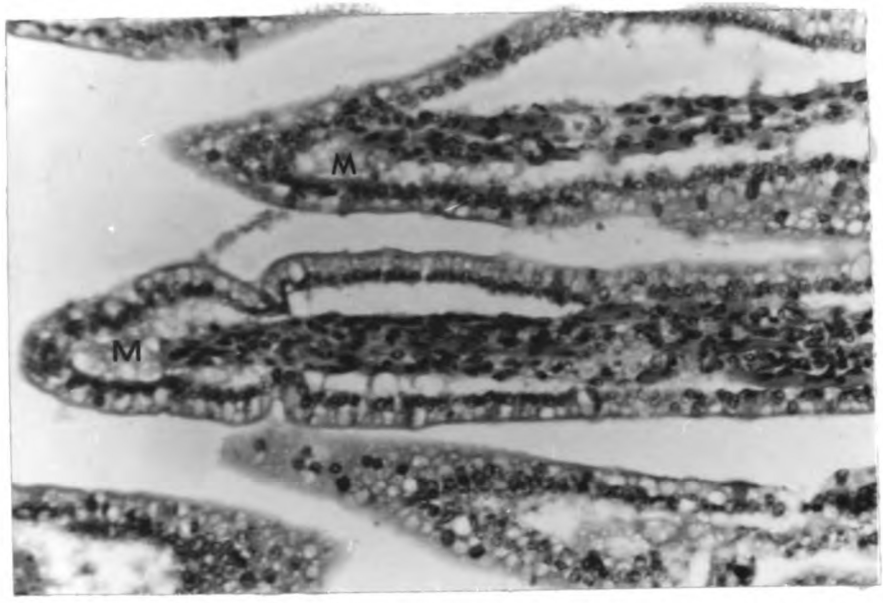

Figure 29. Pig K60. Midjejunum. E. coli was introduced per os 40 hours before necropsy. A villus undergoing an "inadequate" longitudinal contraction. There is some vacuolization of the epithelium and the villi are distended with eosinophilic granular material (M). Hematoxylin and eosin. x 190.

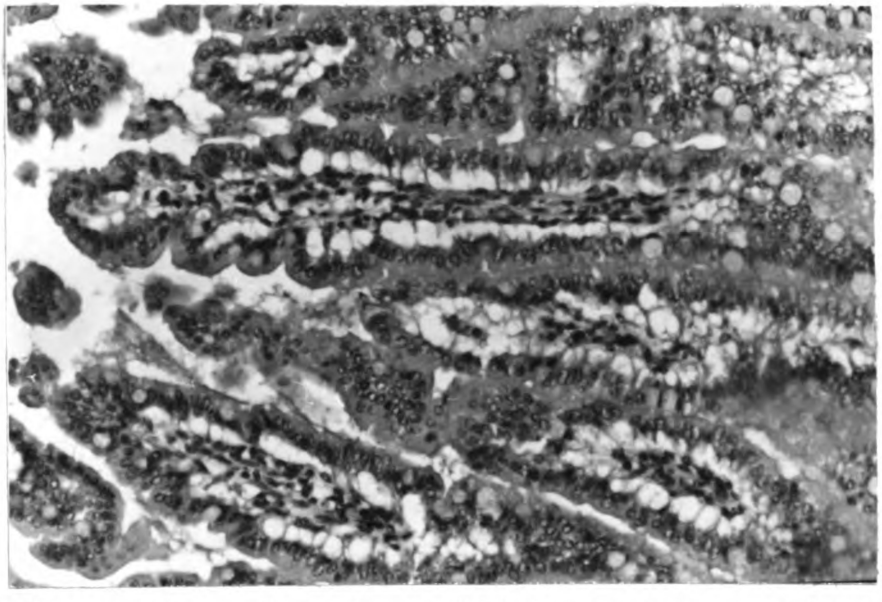

Figure 30. Pig K62. Midjejunum. E. coli was introduced per os 8 hours before necropsy. There is evidence that active longitudinal contraction has occurred. The epithelium is minimally vacuolated and apparently there is fluid transfer from the intestinal lumen into the subepithelial spaces, lacteals and lymphatic vessels. Hematoxylin and eosin x 190.

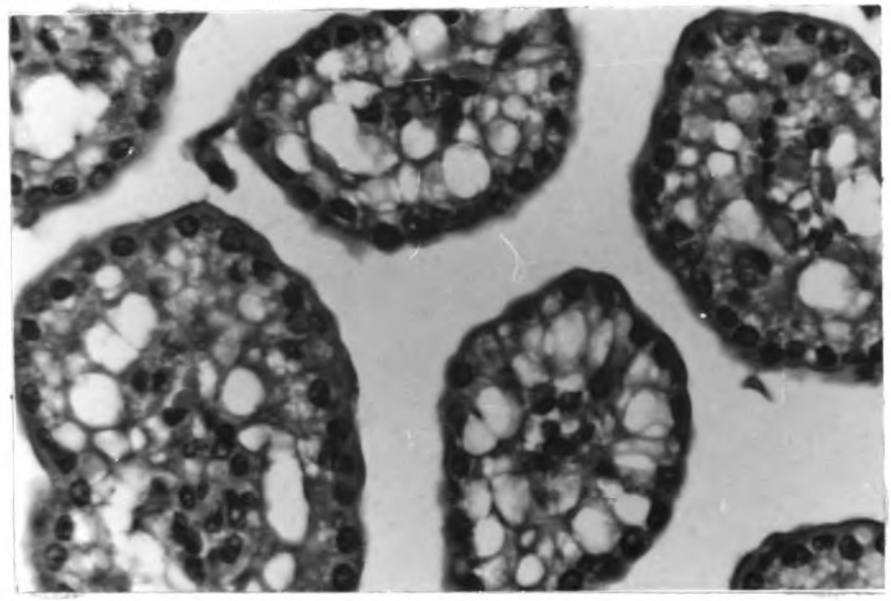

Figure 31. Pig K57. Terminal jejunum. E. coli was introduced per os 4 hours before necropsy. Transverse section of the villi of the terminal jejunum. There are varying degrees of vacuolization of the villus epithelium. Where vacuolization is marked, villus function indicated by subepithelial collection of transmitted fluid, and activity of lymphatic vessels and lacteals, is minimal. Hematoxylin and eosin. x 470.

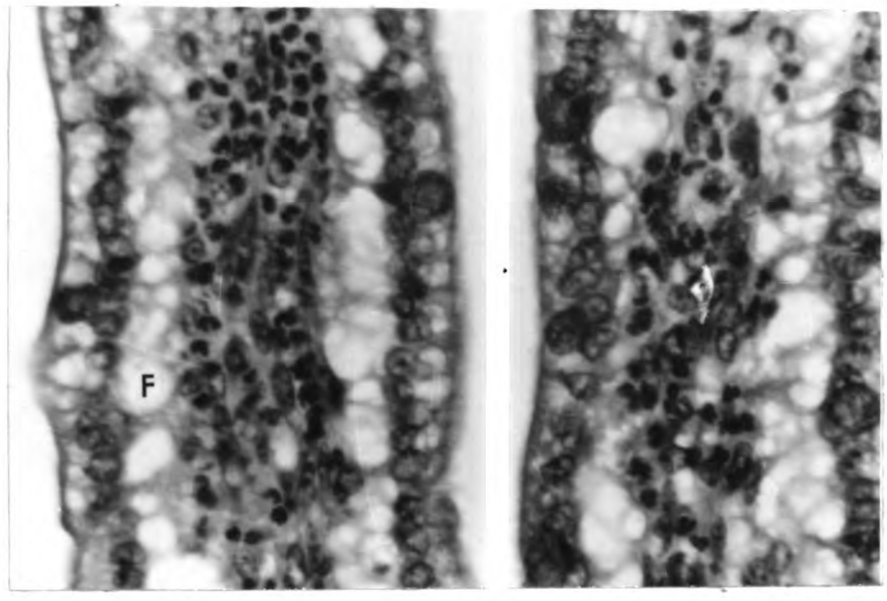

Figure 32. Pig K54. Terminal ileum. E. coli was introduced per os 16 hours before necropsy. There is accumulation of fluid in the epithelium and the subepithelial spaces of the villi (F). The laminae propriae are infiltrated with neutrophils. Hematoxylin and eosin. x 470.

A further lesion seen in the infected intestine, and generally not associated with many of the changes described above, was that in which the villi were markedly enlarged due to vacuolization of the epithelial surface or due to subepithelial edema, so that the villi were in very close apposition and appeared adherent to each other, the microvilli forming a dense eosinophilic boundary common to both villi (Figures 33 and 34). The gross appearance of this intestine is of a thicker-walled intestine that is firm to the touch compared to the flaccid intestine that is commonly seen in colibacillosis.

At times in the infected intestine the lamina propria of some of the villi were infiltrated with inflammatory cells, primarily neutrophils (Figures 32 and 35), and in some instances these cellular aggregates formed microabscesses that distended the shaft of the villus. Other inflammatory cells commonly associated with a general edema of the villi were lymphocytes, eosinophils, plasma cells and occasional macrophages.

In some instances, the jejunal villi were seen to be very long and slender. There was almost uniform, advanced hydropic change in the epithelium of the villi in these sections, there was little or no subepithelial vacuolation, and Breucke fibers were not contracted (Figure 36). Frequently the laminae propriae of these villi were infiltrated with foci of neutrophils.

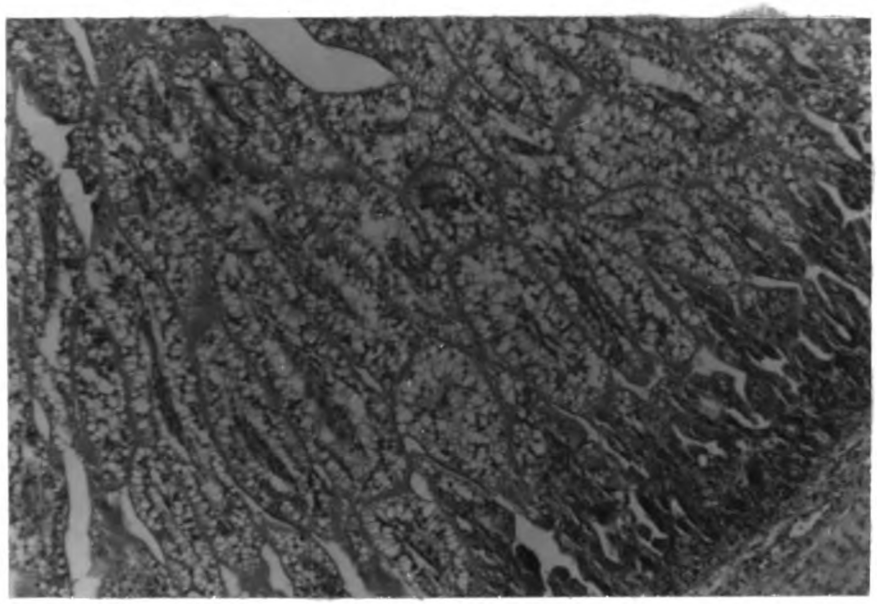

Figure 33. Pig J4672. Midjejunum. E. coli was injected 77 hours before necropsy. The villi are markedly enlarged and closely apposed. The intervillous spaces are closed, effectively reducing the surface available for absorption of fluids from the intestinal lumen. Hematoxylin and eosin. x 190.

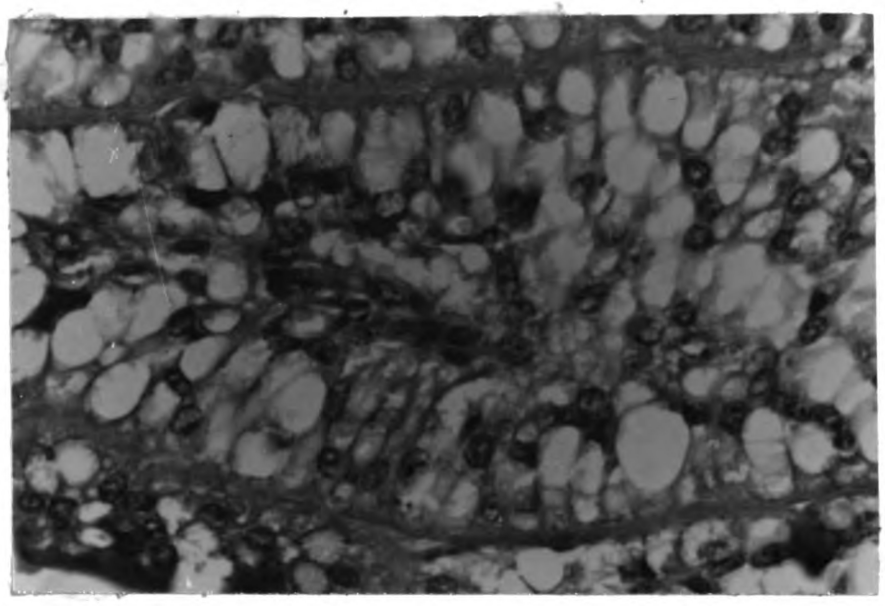

Figure 34. Pig J4672. Midjejunum. From the same field as Figure 33. The mucous membrane is markedly vacuolated and there is minimal functional activity in the lamina propria. Hematoxylin and eosin. x 470.

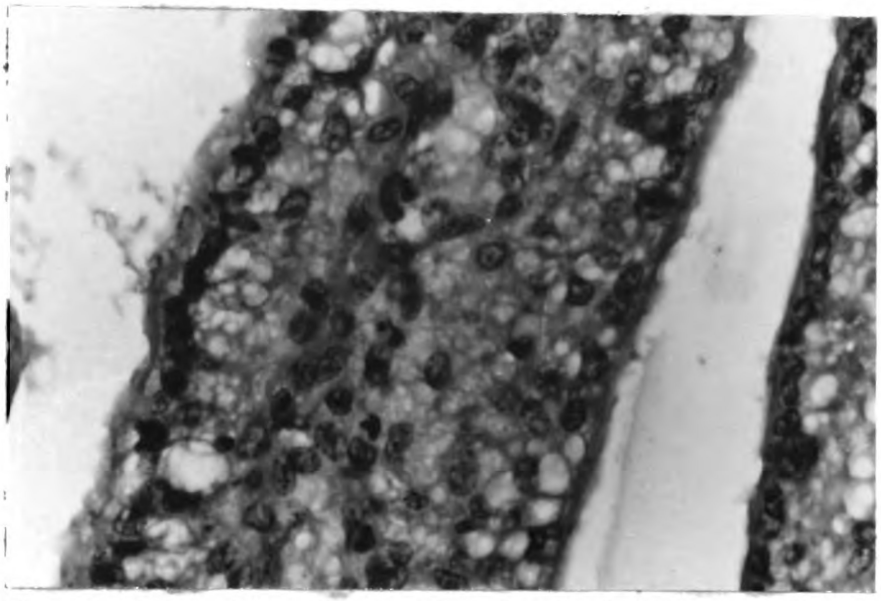

Figure 35. Pig K55. Terminal jejunum. E. coli was introduced per os 22 hours before necropsy. There are few changes. The lamina propria is infiltrated with some neutrophils. Hematoxylin and eosin. x 470.

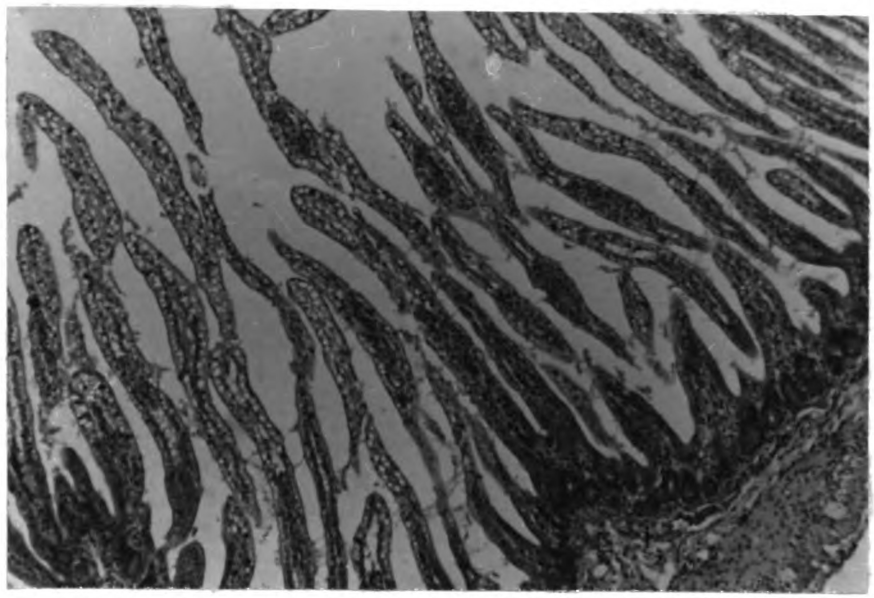

Figure 36. Pig K55. Midjejunum. E. coli was introduced per os 22 hours before necropsy. The villi are elongated and the Breucke fibers are not contracted. There is little evidence of absorption of fluids from the lumen of the jejunum. Hematoxylin and eosin. x 75.

(ii) The submucosa and other structures of the small intestine. The changes observed in the submucosa and the muscular and serosal layers of the small intestine of the monocontaminated pigs were primarily inflammatory edema and infiltration with lymphocytes, neutrophils, eosinophils and macrophages. Hyperemia and distended lymphatic vessels of the submucosa and occasionally intramuscular edema were observed in the infected pigs. Mature lymphocytes were present in the submucosa of the terminal jejunum and ileum of both the infected and the germfree control pigs, but there were increased numbers of lymphocytes in these tissues in the pigs heavily contaminated with the test organisms.

Mesenteric lymph nodes. Changes recorded in the mesenteric lymph nodes were inconsistent. In one instance there was a marked increase in the numbers of neutrophils present in foci in the interstices of the lymph node. Edema was common, and in many instances there was an apparent depletion of mature lymphocytes.

Large intestine. Sections of cecum and colon from the infected group were characterized by submucosal edema and hypercellularity of the lamina propria. In some instances there were hyperemia and excessive goblet cell activity in the mucosa. Edema of the mesocolon was a feature in both the infected and control pigs. The edema of the infected pigs, however, was inflammatory (Figure 37), and in one instance the edema of the mesocolon was hemorrhagic.

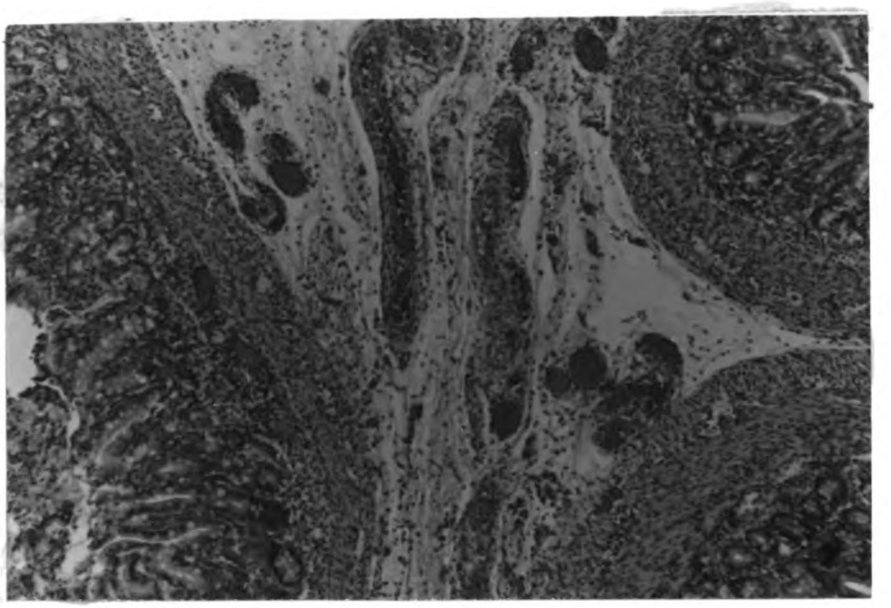

Figure 37. Pig J4669. Mesocolon. E. coli was injected 32 hours before necropsy. Edema and hyperemia of the mesocolon; lymphocytes predominate, with some neutrophils and histiocytes also present in the exudate. Hematoxylin and eosin. x 75.

Microabscesses and ulceration of the mucosa, and edema, fibrin and hemorrhage in the submucosa were observed in the colon of 1 pig killed 48 hours after injection with the test organism (Figures 38 and 39).

In 1 pig (J4669) classical lesions of hemorrhagic enteritis were noted, the lesions involving stomach, small intestine and large intestine. These lesions were characterized by necrosis, hemorrhage and sloughing of extensive areas of the mucosa. There were also hyperemia and edema of the muscular coats of the intestine (Figures 40, 41, 42, 43 and 44). In some areas of the jejunum, total mucosal slough, including portions of the underlying muscle coats, was observed (Figures 41 and 42). In the large intestine there were hyperemia, hemorrhage, and necrosis of the mucous epithelial surface (Figure 44) and catarrhal exudate with some pseudomembrane formation in the colon. The rectal mucosa of this pig was relatively undamaged.

Liver and pancreas. No consistent changes in the liver and pancreas were associated with infection.

In 1 liver section from which the organism had been cultured there were marked hyperemia and increased numbers of neutrophils scattered throughout the liver parenchyma and in the vicinity of larger blood vessels (Figure 45). In the livers of some pigs with an established enteric infection there was mild centrolobular vacuolization of the hepatic parenchymal cells (Figure 46). Edema of the submucosa of the cholecyst was observed in some infected pigs (Figure 47).

Figure 38. Pig J6206. Colon. E. coli was injected 48 hours before necropsy. Focal ulceration and suppuration (S) of the mucosa of the colon. Hematoxylin and eosin. x 75.

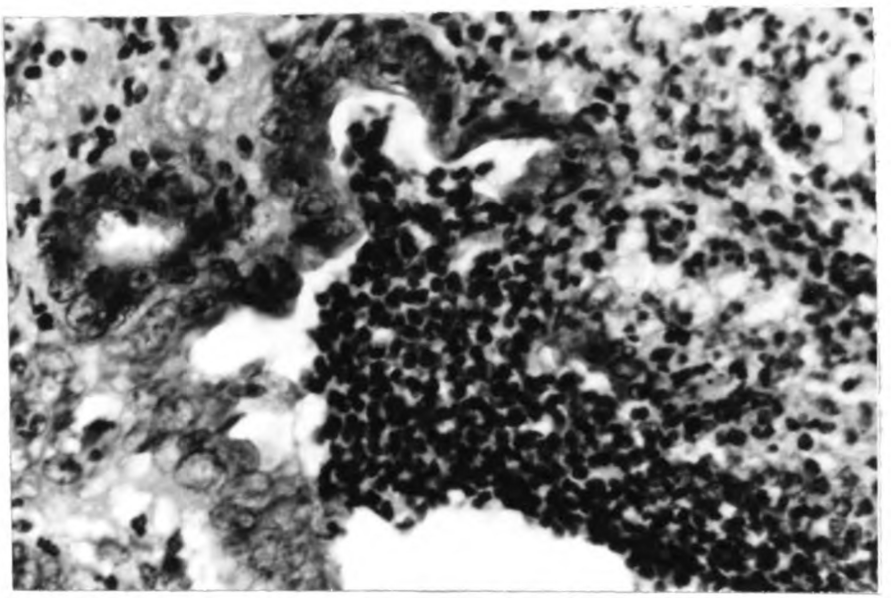

Figure 39. Pig J6206. Colon. From the same field as Figure 38. Neutrophilic cellular exudate from the ulcerated mucosa. Hematoxylin and eosin. x 470.

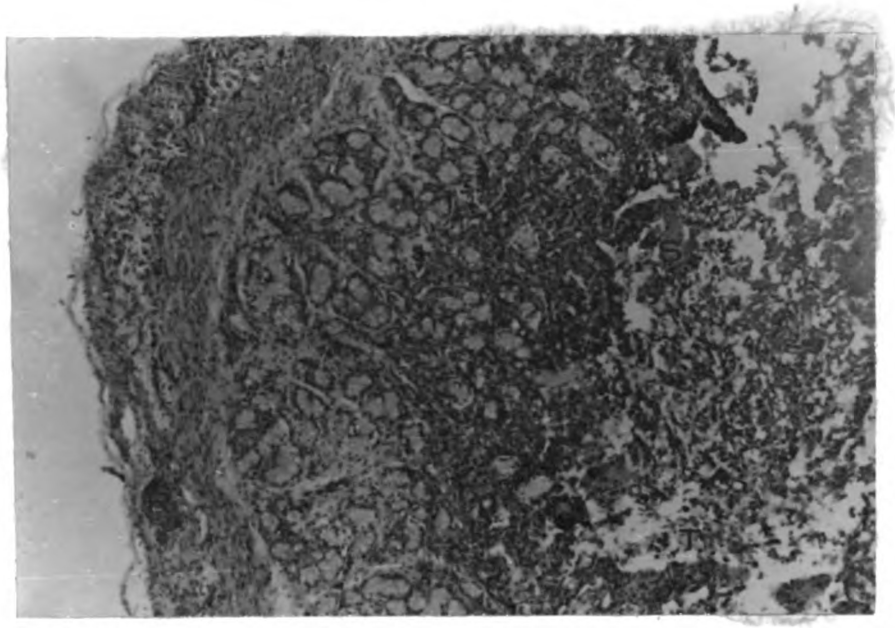

Figure 40. Pig J4669. Duodenum. E. coli was injected 32 hours before necropsy. Hyperemia (R), hemorrhage (S), necrosis and sloughing of the villi (T). Hematoxylin and eosin. x 75.

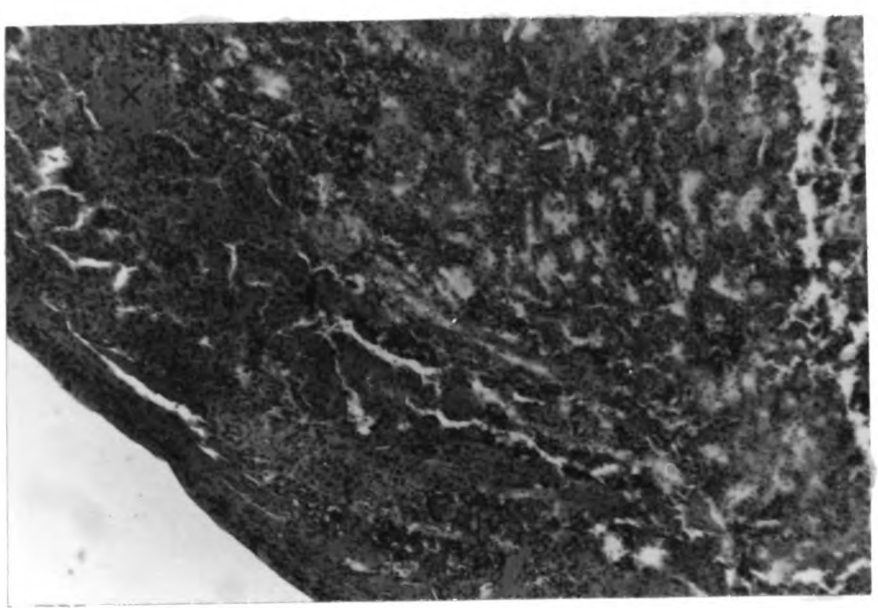

Figure 41. Pig J4669. Terminal jejunum. E. coli was injected 32 hours before necropsy. Hemorrhage and necrosis of the mucosa. In this instance the entire mucous membrane, lamina propria and muscularis mucosa are in the process of sloughing. Hyperemia and hemorrhage of the submucosa (X). Hematoxylin and eosin. x 75.

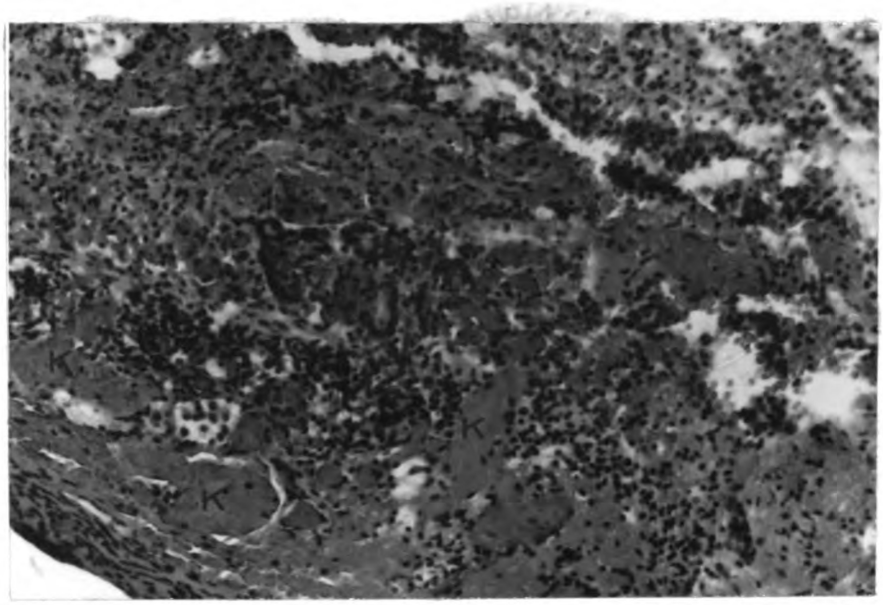

Figure 42. Pig J4669. Terminal jejunum. From the same field as Figure 41. Hyperemia (K), hemorrhage, necrosis and epithelial sloughs. Hematoxylin and eosin. x 190.

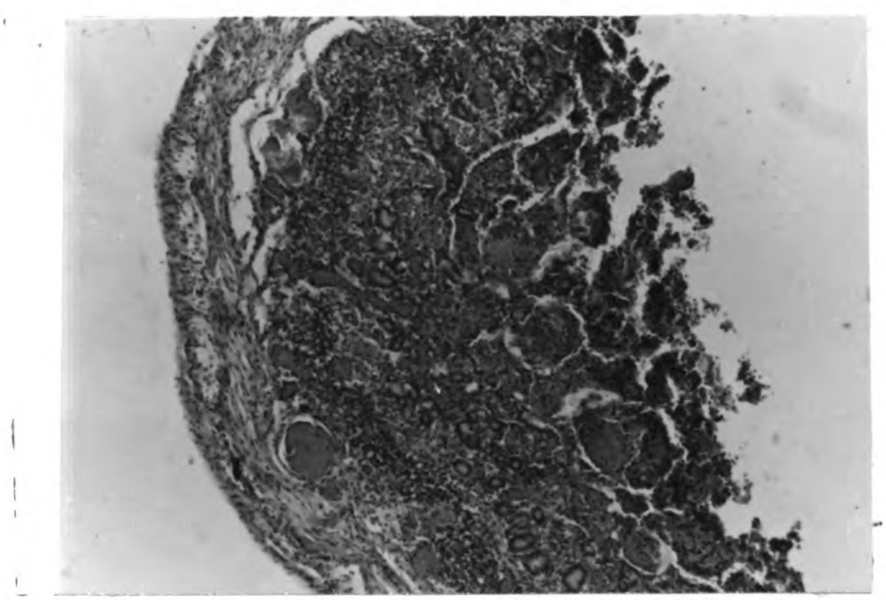

Figure 43. Pig J4669. Terminal ileum. E. coli was injected 32 hours before necropsy. Hyperemia, hemorrhage and necrosis. Hematoxylin and eosin. x 75.

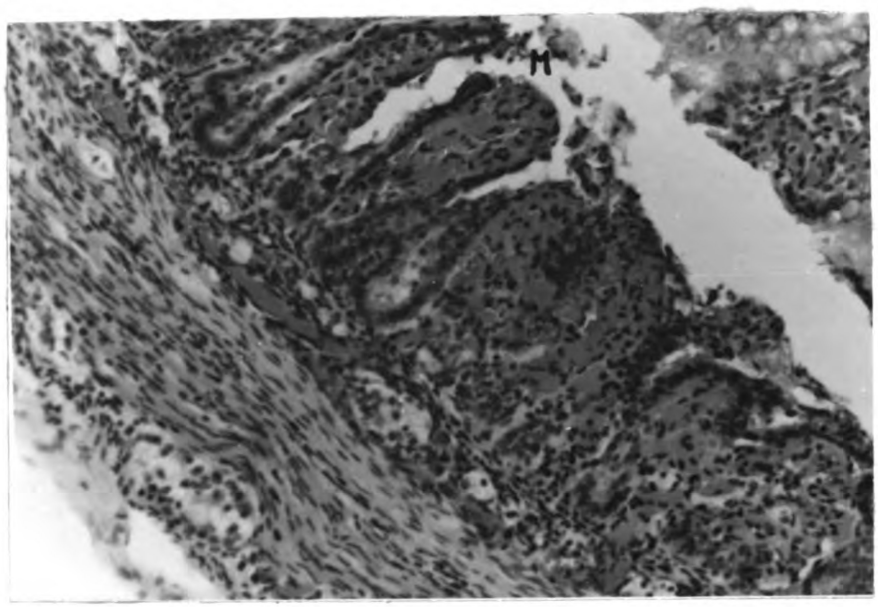

Figure 44. Pig J4669. Colon. E. coli was injected 32 hours before necropsy. Hyperemia and hemorrhage of the lamina propria of the mucous membrane. There is some necrosis of the epithelium (M). Hematoxylin and eosin. x 190.

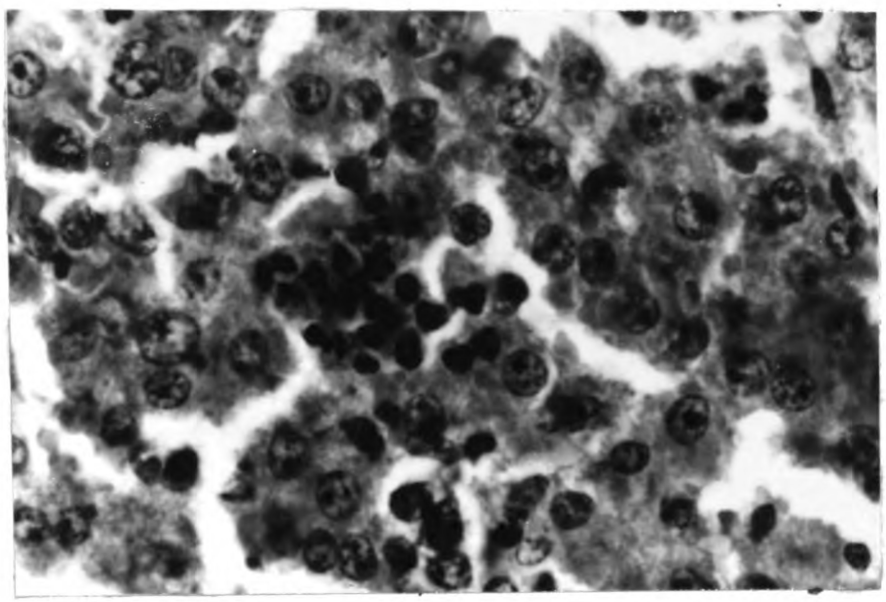

Figure 45. Pig J 6206. Liver. E. coli was injected 48 hours before necropsy. Foci of neutrophils infiltrating the parenchyma of the liver. E. coli was cultured from this liver. Hematoxylin and eosin. x 750.

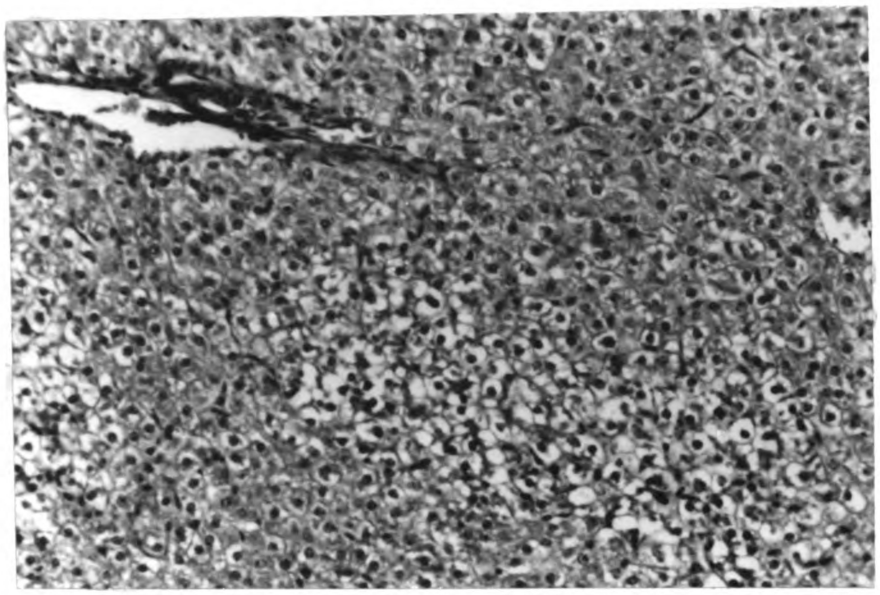

Figure 46. Pig J5714. Liver. E. coli was injected 17 hours before necropsy. Centrolobular vacuolization of hepatic cells was seen in a number of the pigs with enteric infections. Hematoxylin and eosin. x 190.

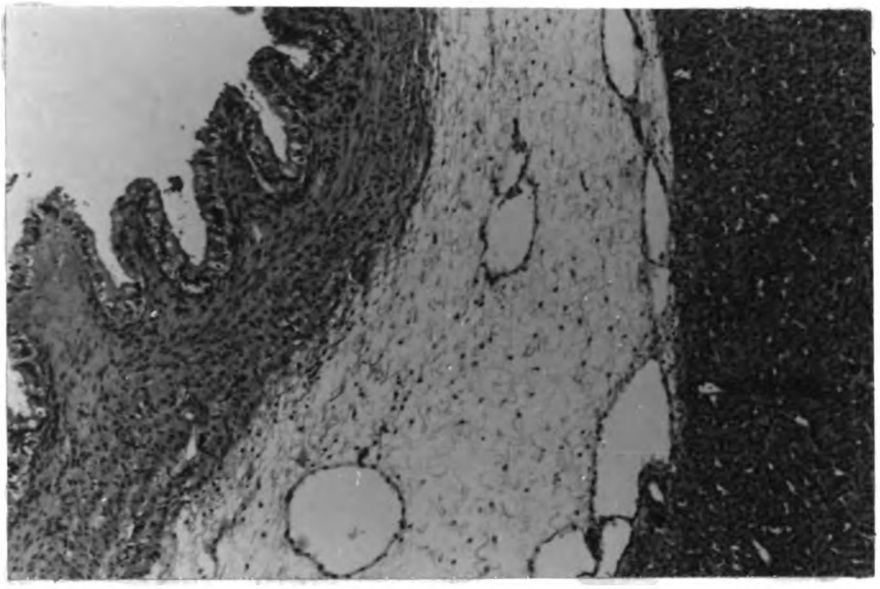

Figure 47. Pig K62. Liver and cholecyst. E. coli was introduced per os 8 hours before necropsy. Edema of the submucosa of the cholecyst. The lymph vessels are distended. Hematoxylin and eosin. x 75.

Urinary system. Positive isolations of a few organisms were common from the kidney during the early systemic phase of the infection.

Lesions noted in these instances were edema and hemorrhage of the medulla, hyperemia of the arcuate and interlobular vessels and the glomerular tuft, mild infiltration of the interstices of the cortex with mature lymphocytes, vacuolization of the epithelium of the excretory tubules and infiltration with mononuclear cells.

In a few instances, isolations from the epithelium of the urocyst indicated heavy contamination and the possibility of establishment of an infection at that site.

In only 4 pigs did the lesions observed suggest significance. These lesions were hyperemia, hemorrhage, edema and perivascular lymphocytosis of the lamina propria, mononuclear cell infiltration into the subepithelial tissues; and vacuolation, hydropic degeneration and necrosis of the transitional epithelium (Figures 48 and 49).

Joints. Bacteriological and gross examination of the humeroradial joint of each pig was undertaken as a routine necropsy procedure.

Positive isolations of the organism were obtained from this site from 3 pigs with systemic contamination in the 8- to 32-hour postinjection period.

There were no clinical signs or gross lesions of arthritis in these pigs.

Histopathologic examination of the joints was not done.

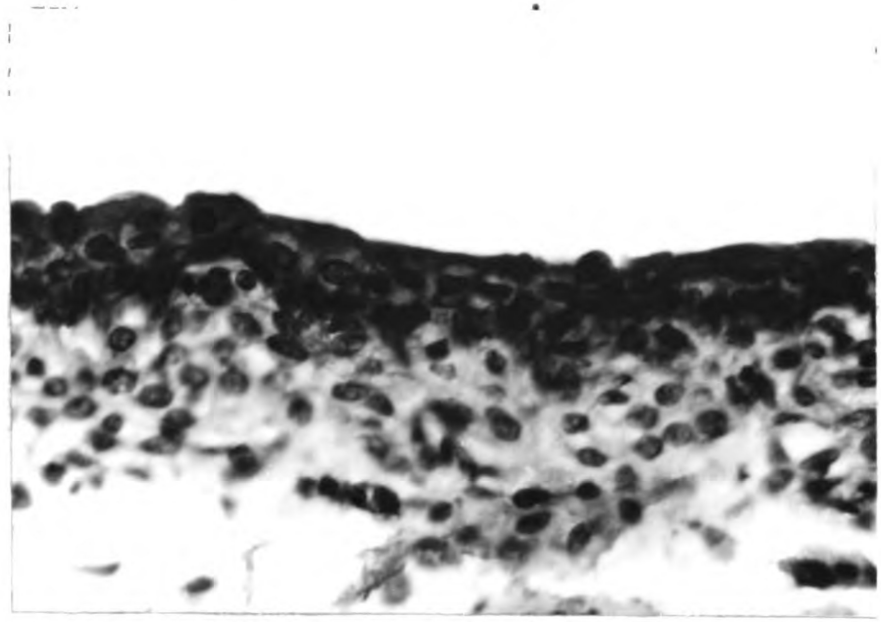

Figure 48. Pig J6211. Urocyst epithelium from a germfree control pig. Hematoxylin and eosin. x 470.

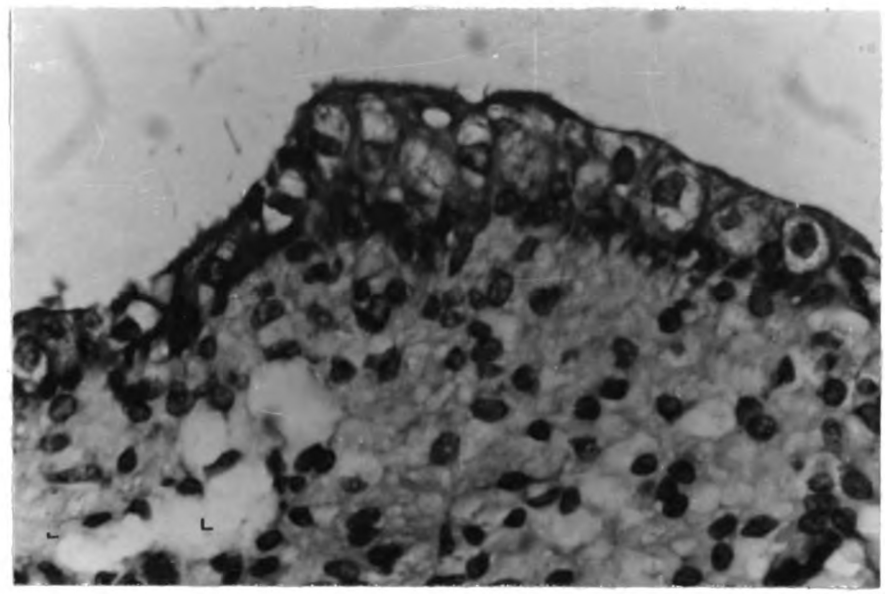

Figure 49. Pig J6791. Urocyst epithelium. E. coli was injected 72 hours before necropsy. Hydropic degeneration and distended lymphatics (L). Cultures from this epithelial surface indicated that a large population of E. coli O138:K81:NM (Mich.) was present. Hematoxylin and eosin. x 470.

DISCUSSION

Microbiologic Aspects of Experiment 1

The organisms did not actively spread from the injection site along tissue planes in the subcutis. There was some degree of spread from the injection site but this was minimal and the result probably of mechanical transfer with tissue fluid and inflammatory exudate. The organism was non-motile, and this might explain the lack of active movement from the injection site.

It was demonstrated in Experiment 1 that pathogenic serotypes of E. coli could systemically invade colostrum-deprived pigs within 1 1/2 hours of bacterial contamination of the umbilical stump. It is common, particularly in gilts, to find instances of lactations being delayed for some hours after farrowing, and it is apparent that a neonatal pig could be infected immediately after birth and develop bacteremia and early enteric infection before receiving its first colostrum, even if lactation was delayed for only 4 hours after parturition (Table 3). Therefore, contamination of the exposed tissues of the umbilical stump with coliforms during the neonatal period is possibly of greater significance than has previously been considered.

In the 3 litters used in Experiment 1 it took from 6 to 24 hours for the organism to pass through a systemic or bacteremic phase and to colonize the intestinal tract in significant numbers. The consistency with which this course of infection was followed indicates that the serotype under test will very readily colonize the intestinal tract from systemic or bacteremic contamination and that this may in fact be a common route of infection in slightly older pigs which have active gastric secretion and low pH of gastric contents. The reduction in numbers of organisms isolated from the stomach mucosa, as pigs approached an age of approximately 150 hours, indicates that developing gastric acidity might have had an adverse effect on the colonization of the stomach with this serotype.

It is of interest to note that, 24 to 48 hours after injection, systemic contamination was not common (Tables 3, 4 and 5). There was occasional localization in specific sites such as the kidney and urocyst, and isolations could be made from the lymph nodes draining these organs, indicating the possibility of transient or persistent bacteremias derived from such localization of infection. It was also apparent that occasional reentry of the organism into the circulation could occur from heavily populated enteric sites. Local damage from microabscesses and trauma of the mucosa by the ingesta could readily expose vessels to invasion by organisms resident in the vicinity.

Microbiologic Aspects of Experiment 2

Escherichia coli O138:K81 (Minn.) produced a milder clinical disease in gnotobiotic pigs than did the serotype used in Experiment 1. It did, however, behave in an essentially similar way--there was early bacteremia in the 4 to 12-hour postinjection period, and by 12 hours there was heavy colonization of the gastrointestinal tract. Systemic contamination was uncommon 20 hours after exposure, and only occasional systemic isolations were recorded after that time.

Microbiologic Aspects Common to Both Experiments 1 and 2

Contrary to the classically held concept that encapsulated virulent bacteria require sensitization with specific antibody before being susceptible to phagocytosis, the work of Wood *et al.* (1946) on the phenomenon called "surface phagocytosis" is partly borne out by observations made in these experiments. In independent experiments Brooks (1966) has shown that the pigs used in this series of experiments were agammaglobulinemic. It is apparent that bacteremia and localization of organisms in various systemic sites of these moncontaminated pigs was a transient phenomenon, and that a mechanism was operating to remove organisms from these tissue sites. Again it is clear from the histologic study of the injection sites and adjacent skin sections that there is a cellular response on the part of the baby pig in the infiltration of these sites with neutrophils, eosinophils

and macrophages. One can postulate, therefore, that despite the lack of gamma globulin, these animals were able to promptly phagocytose and/or inactivate and remove contaminant microorganisms from certain tissue sites, provided that the organisms were not present in excessive numbers.

The differences between specific and nonspecific opsonization seem to be quantitative rather than qualitative (Suter, 1956). It is probable that the numbers of pathogens introduced at the injection site far exceeded the number that the mobilized phagocytes could remove by "spontaneous phagocytosis" (phagocytosis unassisted by specific serum components). As a consequence, the attempted repair process at the injection site involved necrosis, abcess formation and fibrosis.

Wood et al. (1946) observed, however, that phagocytosis in the absence of serum components did not occur upon "smooth" surfaces or substances, such as when bacteria and phagocytes were placed together on a thin layer of mucus on a glass slide. It may be that the reason for the persistence of the organisms in the intestinal tract is the inability of the phagocyte to trap coliforms in the mucous menstruum such as is found in the intervillous lumen of the intestine of the agammaglobulinemic monocontaminated pig. Surface phenomena in the tissues assist phagocytes in their activities in clearing systemic sites such as liver, kidney, spleen and lung of contaminating E. coli.

Microbiologic Aspects of Experiment 3

The site consistently infected by this organism was the gastrointestinal tract. Following oral dosing the infection was immediately established in this site and did not necessarily pass through a pre-enteric bacteremic phase as recorded in the pigs from Experiments 1 and 2.

It was evident from the results of this experiment that a neonatal colostrum-deprived pig is very readily infected by oral contamination. It is apparent that even a brief period of agalactia in a parturient sow would permit the gastrointestinal tract of neonatal pigs exposed to pathogenic serotypes to become heavily contaminated, thereby constituting a serious threat to the survival of the litter.

Stevens (1963) states that conventionally-reared pigs undergo an invasion of the viscera with E. coli in the terminal stages of colibacillosis and that the organism can be more frequently recovered from the brain than from "other sites." He considered a possible reason for this phenomenon to be that there are fewer substances inhibitory to growth of the organisms in the brain. There were occasional instances of transient bacteremia recorded in Litter 5, and in 1 pig with an apparent ascending infection of the urinary tract there was a positive isolation from the iliac lymph node draining the urocyst (Table 6).

It is likely that the profuse diarrhea characterizing colibacillosis in gnotobiotic pigs due to E. coli O138:K81:NM

(Mich.) is dependent on the presence either in the mucosa of the intestinal tract or the lumen of this organ, of sufficient numbers of the organisms, and a latent period of 4 to 18 hours. Presumably, the shorter period of time related to the contaminating dose having reached this site in the gastrointestinal tract by a direct route. It is apparent that the oral dose of approximately 5×10^6 organisms of this serotype is sufficient to produce diarrhea in a gnotobiotic pig within 4 hours of the exposure.

In gnotobiotic pigs systemically infected with this serotype of E. coli, sufficient time must elapse for the organism to colonize the gastrointestinal tract, and in sufficient numbers, before the physiological activity of the intestine is so altered as to produce diarrhea.

Clinical and Pathologic Findings from Experiments 1, 2 and 3

Clinical aspects

There was a significant difference in the clinical expression of the disease associated with each of the 2 serotypes.

The serotype, E. coli O138:K81:NM (Mich.), produced very pronounced diarrhea and dehydration and, under field conditions, one would anticipate infection with this serotype to result in a high mortality rate.

Systemic infection with E. coli O138:K81 (Minn.) was associated with a higher incidence of localized ventral

edema. The diarrhea and dehydration produced in these animals, under the environmental conditions of the experiment, was relatively mild.

With both serotypes, in those instances where the perineum was grossly and persistently contaminated with feces containing the organism, erythema venenatum was observed. In these circumstances it is not surprising that an occasional urocystitis, probably from an ascending infection of the female urethra, was recorded.

The clinical signs of the disease produced by both oral exposure and subcutaneous injection of gnotobiotic neonatal pigs with E. coli O138:K81:NM (Mich.) closely resembled the clinical descriptions given elsewhere, for other pathogenic serotypes of E. coli (Gordon and Luke, 1958; Saunders et al., 1960; Stevens, 1963; Dunne, 1964). Anorexia may or may not be a part of the clinical syndrome as it is described in the literature. With more pathogenic serotypes of E. coli, anorexia is more apparent as the pigs become dehydrated and lethargic, and it is likely that anorexia is a sign of a deteriorating clinical state rather than a specific manifestation of colibacillosis. Anorexia was not a significant clinical finding in the series of experiments reported here.

Gross lesions.

The gross pathological changes observed in the gnotobiotic pigs in this experimental series resemble in part

those descriptions given by previous workers and recorded in highly regarded veterinary texts (Jubb and Kennedy, 1963; Dunne, 1964); but it is apparent that textbook descriptions should now take into account that different serotypes, readily identifiable, produce basically different syndromes in neonatal pigs. The enteric syndrome produced in this series of experiments is clinically and pathologically separate from the polyserositis syndrome described by Britt and Waxler (1964). Because these syndromes are produced by serotypically different organisms and are clinically distinct, it is misleading to combine the 2 clinicopathological syndromes in textbook descriptions of "colibacillosis."

Histopathologic lesions

Smith and Jones (1963) have reported that there were no histologic indications of any inflammatory response in the intestinal tract of pigs with colibacillosis. From the histologic studies made with E. coli serotypes O138:K81 (Minn.) and O138:K81:NM (Mich.) and described above, it is clear that there are inflammatory changes associated with clinical colibacillosis but that these changes might be insufficiently marked to be readily apparent in tissues taken from field cases of the disease. There are apparently no lesions pathognomonic of the infection. Occasional foci of neutrophils were seen in the parenchymatous organs of these experimentally infected pigs, and inflammatory exudates were observed in sections from the lamina propria and

submucosa of the intestinal tract. Endotoxins have long been known to induce neutrophilia (Carpenter, 1965) and it may be that, as organisms multiply and "spill over" from various sites of phagocytosis, they or their endotoxins can produce transient microabscesses in these tissues.

Bacterial exotoxins are potent, relatively unstable compounds with highly specific toxic effects (often sufficiently characteristic to identify the toxin) and are generally highly antigenic. Endotoxins derived from the Enterobacteriaceae are weakly toxic, relatively stable products of bacterial destruction. They are weakly antigenic and produce nonspecific toxic effects such as pyrexia, prostration, diarrhea, vascular malfunction (vasomotor shock, edema and hemorrhage) and at first hyperglycemia, then later, hypoglycemia. These so-called classical responses of host animals to endotoxins include altered response to epinephrine, and tissue necrosis at sites of endotoxin concentration (Smith, Conant and Overman, 1964).

(i) Histopathologic lesions in tissues other than the alimentary tract. The basic general histopathologic findings were specific epithelial degenerations in various organs and vascular changes indicated by edema.

The most common epithelial change was hydropic degeneration, and this change was observed in bronchiolar epithelium, aortic endothelium, the gastrointestinal tract, and the transitional epithelium of the urocyst. These

changes were inconsistent and were possibly associated with incidental local concentrations of endotoxic, or possibly exotoxic activity of the test organism.

There is no evidence that nonlethal levels of bacterial endotoxins have a generalized effect on the integrity of the capillary walls, but Zweifach et al. (1956) and Zweifach (1958) have demonstrated vasoconstriction at the venular end of the capillary bed of laboratory mammals following the application of endotoxin extracted from E. coli. "Nontoxic" doses of this endotoxin produced a slowing of blood flow through the microcirculation, but there was no change in the systemic blood pressure during this reaction. With increased levels of endotoxin there was a brief hyperreactive vascular phase as before, then a hyporeactive phase in which damage to the capillary and venular barrier per se was present. A continuing exposure to endotoxin led to protracted derangement of vascular reactivity, then capillary hemorrhage and stasis (Zweifach, 1964).

In this experiment, instances of these vascular changes were seen in some of the injection site sections, and the necrosis and hemorrhage observed in the intestine of 1 infected pig (J4669) might have been the result of either a massive release of endotoxin or high levels of endotoxin production from prolific growth of the coliforms in the intestine.

It is possible that the general signs of edema so commonly observed in tissues from these monocontaminants were a less severe manifestation of E. coli endotoxic activity on the vasculature. Dunne (1964) has associated the edema of the mesocolon seen in baby pigs with colibacillosis. It is apparent that edema of the colon may in fact be an indication of the disease, but mild edema of this tissue is nonsignificant under the conditions of these experiments in that it was commonly found in the control animals.

(ii) Histopathology of the alimentary tract. The changes in the epithelium and the lamina propria of the villi recorded as being common to both infected and germfree pigs are described in detail in the results because the difference between the 2 groups is distinctly one of degree.

It is apparent that in the process of absorption of fluids from the lumen of the intestine some of the histologic changes observed in the experimental pigs are likely to be found regardless of the presence or absence of E. coli (Figures 21 and 22). It may be that the presence of either endotoxin and/or exotoxin of E. coli so modifies these changes that they become significant in a pathologic sense (Figure 27).

It is suggested that the distinct hyperemic, hemorrhagic, necrotic and inflammatory changes found only in the infected pigs are manifestations of E. coli endotoxins

whereas the less marked pathologic changes which probably account for the major clinical sign of diarrhea are possibly due to the combined effects of exotoxin and endotoxin and may even resemble to a degree some of the physiologic changes that one can see in the digestive tract of germfree pigs (Figure 27).

In absorption from the intestinal tract the columnar epithelium actively transports fluid and metabolites from the lumen of the intestine into the lymphatics, lacteals and vessels of the lamina propria. A number of mechanisms can act to remove these intravillous fluids from the mucosa. One mechanism is the periodic rhythmic vertical contraction and horizontal swaying motion of the villi due to the contractions of the Breucke fibers. The vertical contraction has a pumping effect and occurs possibly at a level of villous distention that stretches the Breucke fibers and stimulates them to contract. Evidence of this vertical contraction activity of the villus can be readily seen in tissues from both the control pigs and those infected pigs which are not yet heavily colonized in the intestinal tract with E. coli. These contracted villi are seen as being shortened with localized thickening of the lamina propria and an "oak-leaf" outline to the profile of the villus (Figures 27 and 29).

With these possibilities in mind a number of hypothetical explanations for the intestinal tract changes observed in these experiments present themselves.

The presence in a crypt or on the surface of a villus or within the lamina propria of even a small colony of E. coli elaborating both endotoxin and exotoxin, could produce sufficient toxin to inhibit or disrupt the cellular metabolism of the columnar epithelium of the villus, and perhaps neighboring villi. The expression of this toxic effect as hydropic degeneration or as marked distention and vacuolation of the columnar epithelium may and probably will result in malfunction or complete cessation of the active transport system of fluids from the lumen of the intestine into the villus. One could postulate this happening in a very restricted locale, or in certain segments of the small intestine, or even throughout the entire heavily colonized intestinal tract. This might serve to explain the presence of an obviously degenerating inactive villus together with an actively contracting villus in the same tissue section. It might also explain the presence of signs of both physiologic activity (expressed in active vertical villus contractions and active hyperemic vessels in the lamina propria) and pathologic changes such as distention and vacuolation of some or all of the epithelial cells in a single villus.

Should the toxin be of sufficient concentration in the intestinal tract to produce necrosis of the epithelial surface, the sloughing of the necrotic epithelium, combined with hemorrhage and body fluid loss from exposure of the lamina propria to the hypertonic lumen contents, will produce

a clinical syndrome in the pig that will vary according to the extent of the lesions.

One could postulate first a stimulatory then an inhibitory effect of E. coli endotoxin on the function of the Breucke fibers. According to the relative concentrations of the E. coli in certain regions of the lumen, it is possible to visualize the presence in a tissue section of villi showing marked vertical contraction activity whereas elsewhere in the section one might see villi distended with fluids and unable to contract and eject this intravillous fluid because of toxic inactivation of the Breucke fibers. It is reasonable to assume that the lamina propria of such villi will absorb fluid to the point where the distention of the villus will prevent any further absorption of fluid.

Indeed, it was observed in sections from the intestines of some of the infected pigs that distention of the villi reached a point at which the epithelial surfaces of neighboring villi were closely apposed thereby removing from function an extremely high proportion of a potentially active absorptive surface (Figures 33 and 34).

Occasionally, tissue sections were seen in which there was both widespread hydropic change in the epithelium, and in which the villi were elongated and the Breucke fibers not contracted (Figure 36). There was little or no evidence of absorption of fluids in these tissue sections, and presumably the hydropic change in the epithelium had preceded

the opportunity for the villi to absorb fluid from the intestinal lumen. The lack of contraction of the Breucke fibers could be due either to the lack of stimulus for smooth muscle contraction because of a lack of distention of the villus with absorbed fluids, or the flaccid paralysis of the smooth muscle fibers due the the presence of high levels of bacterial endotoxin in the locale.

Zweifach (1964), reviewing E. coli endotoxin activity, records that it does not appear to have a direct cytotoxic action, but he does present evidence of a direct effect on the smooth muscle of the vasculature. The pattern of the effect of increasing levels of endotoxin is first, an increased rate, then at higher levels of endotoxin, a decreased rate of sequential reaction of the vasculature due to endotoxic sensitization of the muscular vessels to exogenous and endogenous stimuli. There is no overt evidence of damage to the endothelium of the capillaries and venules. There is increased capillary permeability, and E. coli endotoxin--a lipopolysaccharide--does produce stasis of the capillary bed by a mechanism of narrowing of the lumina of the draining venules (Zweifach, 1964). This evidence can be applied to the hypothesis that the hyperemia, edema, hemorrhage, necrosis and desquamation observed in the intestine of the infected pigs are direct and/or indirect results of vascular responses to the presence of E. coli endotoxin and its absorption either from the lumen or from the site of colonization.

It is possible, therefore, with a hypothesis such as this in mind, to reconcile some of the mass of confusing impressions gained from examination of the tissue sections from a number of sites of many pigs killed at various stages of the progress of colibacillosis.

It is suggested that colibacillosis, as it was observed in these experiments, is an expression of a malabsorption syndrome. The intestinal mucosa has become ineffective as an absorbing organ because of a number of anatomic and functional changes--change in the epithelium rendering it incapable of the passage of fluid materials through its walls; malfunction of the villus-draining mechanisms preventing movement of fluid from the intravillous site in the mucosa; reduction of effective absorptive surface area by swelling and compression of the villi; and vascular malfunction leading to hypofunction or anoxia and necrosis of the intestinal mucosa.

The small intestine is a highly differentiated organ and has only a limited number of ways to react to stress. It is likely that malabsorption syndromes will result from a number of mechanisms, and we can safely assume that a complex organism like E. coli will affect at least a number of these mechanisms.

SUMMARY

Research was conducted using a total of 62 gnotobiotic pigs in 3 experiments to determine the clinical effects, pathogenesis and lesions produced by 2 closely related serotypes of Escherichia coli--O138:K81:NM (Mich.) and O138:K81 (Minn.).

It was determined that both serotypes readily colonized the intestinal tract from experimental infection simulating neonatal contamination of the umbilical stump. Oral exposure was precluded.

A bacteremia was produced within 1 1/2 hours of injection, and by 24 hours the infection was generally established in the mucosa of the gastrointestinal tract. By 48 hours postinjection the bacteremia had subsided so that only an occasional isolation from organs other than the gastrointestinal tract was made.

The clinical signs of profuse watery diarrhea, dehydration and erect hair coat characteristic of colibacillosis in the neonatal pig were not apparent until the organism became well established in the gastrointestinal tract. The clinical signs of infection with E. coli O138:K81 (Minn.) were significantly milder but edema of the subcutis was a constant feature of infection with this serotype.

Oral exposure of 1 germfree litter to E. coli O138: K81:NM (Mich.) produced both heavy colonization of the entire intestinal tract and diarrhea within 4 hours. Intermittent bacteremia was evident in this litter. No definite trends in body temperature or hematologic findings were observed, although in some animals there was a marked reduction in leukocyte counts.

The gross necropsy findings, in general, agreed with those reported in the literature from field cases of colibacillosis in neonatal pigs.

Histologically the lesions were predominantly in the gastrointestinal tract and ranged from a acute hemorrhagonecrotic enteritis to a histological picture, in persistently scouring pigs, microscopically indistinguishable from that seen in clinically normal germfree animals.

An hypothesis of the pathogenesis of colibacillosis based on the histologic evidence is presented.

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