BRUCE ROBERT CHRISTIE

Ph. D.

THE PATHOGENESIS OF COLIBACILLARY DIARRHEA OF THE NEWBORN

An Electron Microscopic and Histochemical Study of the Disease in Neonatal Gnotobiotic Pigs

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
BRUCE ROBERT CHRISTIE
1969

This is to certify that the

thesis entitled

THE PATHOGENESIS OF COLIBACILLARY DIARRHEA OF THE NEWBORN
An Electron Microscopic and Histochemical Study of the Disease in Neonatal Gnotobiotic Pigs

presented by

Bruce Robert Christie

has been accepted towards fulfillment of the requirements for

Ph. D. degree in Pathology

Henry L. Klopfer
Major professor

Date May 29, 1969

O-169

ABSTRACT

THE PATHOGENESIS OF COLIBACILLARY DIARRHEA OF THE NEWBORN
An Electron Microscopic and Histochemical Study
of the Disease in Neonatal Gnotobiotic Pigs

by Bruce Robert Christie

A major world problem in human and animal health is colibacillary diarrhea of the newborn. Little is known of the pathogenesis of this disease in neonatal domestic animals, and there have been few attempts to determine the pathogenesis of infantile colibacillary diarrhea.

Pigs are considered suitable experimental animals for certain of the diseases of man, including that of infantile diarrhea and neonatal diarrheal disease of domestic animals.

Forty-eight gnotobiotic pigs were used in experiments in which 34 animals were fed between 1.8 and 3.0 x 10^6 *Escherichia coli* 0138:K81:NM organisms. Observations were made of the clinical effects of the infection. Pigs were killed from 4 to 96 hours after monocontamination. Observations were made of the macroscopic signs of the disease. Tissues were taken at 5 levels of the small intestine and submitted to optical microscopic and electron microscopic examination, and histochemical staining for cell inclusions and enzymes.

Diarrhea occurred between 8 and 12 hours after monocontamination with *E. coli*. Raised hair coat, projectile watery diarrhea and dehydration were also recorded within 18 hours of monocontamination.

Optical microscopic lesions ranged from those of an acute enteritis to a histologic picture, in persistently scouring pigs, similar to that seen in clinically normal germfree control animals. Vacuolization and hydropic degeneration, microscopically indistinguishable from the vacuolization associated with absorption, were seen in both monocontaminated and germfree neonates.

Electron microscopic examination revealed the basic difference between these two types of vacuolization. Absorption vacuoles were membrane enclosed, and were associated with normal function, normal cytologic enzyme activity and absence of diarrhea in germfree animals. Vacuolization of the mucosa of infected neonates was at first similar to that seen in the control animals, but within 12 to 20 hours of monocontamination there were diminution in form and histochemical function of the glycocalyx, loss of alkaline phosphatase and leucine aminopeptidase activity from the brush border, and degeneration of the microvilli. There were vacuolization of the cristae mitochondriales leading to swelling and rupture of the mitochondria, vacuolization then lysis of the endoplasmic reticulum and Golgi complex, and eventual complete disruption of the internal cytoplasmic structure. Associated with these changes was a loss of activity of alkaline phosphatase, acid phosphatase, adenosine triphosphatase, leucine aminopeptidase, succinate dehydrogenase and lactate dehydrogenase.

Effete cells generally were retained on the villal stalk by the persistence of the external cell membrane and the preservation of the mural interdigitations with neighboring cells. It is speculated

that this retention could explain the histologic difference between colibacillary diarrhea and enteric diseases involving villal atrophy in man and animals.

In the monocontaminated neonates evidence of functional inactivity of the vasculature was associated with edema and infiltration of the interstices of the lamina propria with collagen fibrils. It is considered that the endotoxic fraction of the *E. coli* enterotoxin is responsible for the inflammatory responses observed in the villi.

It is speculated (1) that the enterotoxin of *E. coli* 0138:K81:NM is readily absorbed by villal epithelial cells and is toxic for membrane-bound enzymes, (2) that it exerts its effects primarily on those energy producing systems involving adenosine triphosphatase -- the mitochondria, the endoplasmic reticulum and the Golgi complex, (3) that it is primarily toxic for the glycocalyx and secondarily toxic for this entity by its effect on the Golgi complex, (4) and that it is secondarily disruptive to the laminar organelles of the intestinal absorptive cell.

It is considered that neonatal colibacillary diarrhea is primarily a disease of malabsorption.

THE PATHOGENESIS OF COLIBACILLARY DIARRHEA OF THE NEWBORN

An Electron Microscopic and Histochemical Study of the Disease in Neonatal Gnotobiotic Pigs

By

Bruce Robert Christie

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

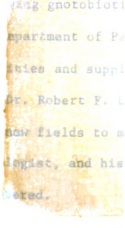
Department of Pathology

1969

For

Martha Bush Ashton

See PhD Thesis.
Martha Bush ASHTON
1972.



ACKNOWLEDGEMENTS

I wish to express my appreciation to my major professor, Dr. Glenn L. Waxler, for his guidance, consideration, encouragement, understanding and practical help throughout the course of these studies. His energy and skill in helping to secure and raise the experimental animals was invaluable; his ready assistance at any hour during the course of the experiments was very much appreciated; and his patience with my enthusiasms and weaknesses, I will remember with gratitude.

My sincere thanks to my academic committee: Dr. R. A. Fennell for his friendly help and advice in the histochemical aspects of this work; Dr. C. K. Whitehair, who first helped me to enter research involving gnotobiotic animals; and Dr. C. C. Morrill, Chairman of the Department of Pathology, Michigan State University, for providing facilities and supplies for this research.

Dr. Robert F. Langham with his teaching of histopathology opened many new fields to me. His dedication as a teacher, his skill as a pathologist, and his great warmth as a human being will long be remembered.

The preserved and embedded tissues for electron microscopy were sectioned and stained by Mrs. June P. Mack, who with much patience and forbearance operated the EM-100 whilst I hesitatingly entered the amazing world of electron microscopy. I wish to record here my

indebtedness and thanks to her, and to Dr. Gordon C. Spink of the Electron Microscopy Laboratory, Biology Research Center, Michigan State University.

I am extremely appreciative of the secretarial assistance given by Mrs. Gretchen King throughout the five years of my graduate studies; of the technical help given by Mesdames Sunderlin, Miller, and Whipple, and Miss Patricia Lamb, of the histopathology laboratory; and particularly the conscientious and careful work of Mr. James Southern, gnotobiotic animal caretaker at the Veterinary Research Farm, Hagadorn Road, East Lansing.

The kindness and help of Dr. E. Murray Pullar, Dr. A. K. Sutherland, Dr. Rodger Watson, and Dr. William A. Snowdon in supporting my application for graduate study in the United States of America will always be remembered by me. Again, the personal efforts on my behalf, of Dr. D. S. Wishart and Dr. D. M. Flynn of the Department of Agriculture, Victoria, when I sought study leave from the department is gratefully acknowledged.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
LITERATURE REVIEW	5
Colibacillosis in Human Medicine	9
Experimental Animal Models for Diarrheal Disease	12
Colibacillosis in Veterinary Medicine	13
Colibacillosis in Swine	14
Colibacillosis in "Specific Pathogen Free" and Gnotobiotic Pigs	17
Extrinsic Factors Complicating Colibacillary Diarrhea	21
The Mucosa of the Small Intestine	22
The Villus	29
Permeability of the Intestinal Mucosa	32
Macromolecular Absorption	32
Entry of Microorganisms to the Villal Mucosa	34
Absorption and Malabsorption	37
Absorption of Water and Salts	38
Absorption of Carbohydrates, Proteins and Fats	43
Histochemistry of the Intestinal Mucosa	47
Hydrolytic Enzymes	48
The Phosphatases	48
Alkaline Phosphatase	48
Acid Phosphatase	49
Adenosine Triphosphatase	49

Page

Proteolytic Enzymes 50

Leucine Aminopeptidase 50

Oxidative Enzymes. 51

The Dehydrogenases. 51

Succinate Dehydrogenase. 51

Lactate Dehydrogenase. 52

Cytochemical Stains for Cell Vacuoles and Inclusions and the Brush Border 52

The Pathology of Enteric Colibacillosis 53

In Human Infants and Calves. 53

In Conventional Pigs 55

In Gnotobiotic Pigs. 56

The Pathogenesis of Enteric Colibacillosis. 57

MATERIALS AND METHODS. 64

General Plan. 64

Animals 65

Age of Experimental Animals. 66

Determination of Initial Sterility of Test Animals 68

Control Animals. 69

Infective Agent 69

Infective Dose of Organisms. 71

Necropsy and Laboratory Procedures. 72

Histopathologic Technique. 74

Histochemical Methods. 76

Alkaline Phosphatase. 76

Acid Phosphatase. 78

Adenosine Triphosphatase. 78

Page

Leucine Aminopeptidase. 79

Succinate Dehydrogenase 79

Lactate Dehydrogenase 79

Electron Microscopy Methods. 80

Reclamation of the Test Serotype 81

RESULTS. 82

The Experimental Plan 82

Clinical and Gross Pathologic Findings. 82

Recovery and Serotyping of the Organism 87

Histopathologic Findings. 87

The Small Intestine. 87

The Mucosa and Lamina Propria 90

The Villal Epithelial Cell of the Small Intestine. 105

The Submucosa and Other Structures of the Small Intestine. 117

Enzyme Histochemistry 117

Histochemistry of the Villus and Villal Epithelial Cells 117

The Phosphatases. 117

Alkaline Phosphatase 117

Acid Phosphatase 124

Adenosine Triphosphatase 136

Proteolytic Enzymes 141

Leucine Aminopeptidase 141

The Dehydrogenases. 145

Succinate Dehydrogenase. 145

Lactate Dehydrogenase. 154

Page

Special Stain Techniques for Intracytoplasmic Contents and the Brush Border 163

Oil Red O Stain. 163

Periodic Acid-Schiff Reaction. 165

Best's Carmine Stain 165

Mayer's Mucicarmine Stain. 165

Alcian Blue Stain. 165

Brown and Brenn Stain. 166

Electron Microscopy 166

DISCUSSION 255

Clinical Findings 256

Histopathologic Findings. 258

Histopathologic Lesions in Tissues Other Than the Alimentary Tract. 258

Histopathologic Lesions in the Alimentary Tract 258

Villal Morphologic Change 266

Vacuolization of the Villal Epithelial Cell 269

Microbiologic Aspects 270

Ligated Intestinal-loop Preparations. 279

Histochemistry. 281

Alkaline Phosphatase 282

Acid Phosphatase 286

Adenosine Triphosphatase 288

Leucine Aminopeptidase 289

Succinate Dehydrogenase. 290

Lactate Dehydrogenase. 292

Page

Electron Microscopy 295

The Brush Border 295

The Glycocalyx. 296

The Apical Trilaminar Membrane. 300

The Terminal Web. 302

The Subapical Zone 303

The Apical Tubule System. 303

Endoplasmic Reticulum and the Apical Vacuoles. . 305

The Nucleus. 310

The Mitochondria 312

The Golgi Complex. 314

Maturation and Necrobiosis of Villal Epithelial Cells 315

Mural Interdigitations 319

The Lamina Propria 322

Absorption and Malabsorption. 325

CONCLUSION 330

SUMMARY. 333

LIST OF REFERENCES 337

VITA 365

LIST OF TABLES

Table		Page
1	Details of experimental animals	67
2	Time of exposure and necropsy of pigs	73
3	Preservation and fixation of tissues.	75
4	Clinical appearance of pigs monocontaminated with *E. coli* 0138:K81:NM	84

LIST OF FIGURES

Figure		Page
1	Pig 2258. Age 3 days. Terminal ileum. *E. coli* was introduced *per os* 18 hours before necropsy	88
2	Pig 2257. Age 3 days. Duodenum. *E. coli* was introduced *per os* 18 hours before necropsy.	88
3	Pig 2257. Age 3 days. Duodenum. *E. coli* was introduced *per os* 18 hours before necropsy.	89
4	Pig 9047. Age 2-1/2 days. Duodenum. Germfree control.	91
5	Pig 2253. Age 2 days. Terminal ileum. Germfree control.	92
6	Pig 9046. Age 2 days. Terminal jejunum. Germfree control.	93
7	Pig 9047. Age 2-1/2 days. Terminal ileum. Germfree control.	94
8	Pig 1006. Age 4 days. Terminal ileum. *E. coli* was introduced *per os* 32 hours before necropsy	95
9	Pig 9047. Age 2-1/2 days. Terminal ileum. Germfree control.	96
10	Pig 1010. Age 6 days. Terminal ileum. Germfree control.	96
11	Pig 9046. Age 2 days. Terminal ileum. Germfree control.	97
12	Pig 1005. Age 3 days. Midjejunum. Germfree control	98
13	Pig 1306. Age 4-1/2 days. Terminal jejunum. *E. coli* was introduced *per os* 8 hours before necropsy.	98

Figure		Page
14	Pig 1004. Age 3-1/2 days. Anterior jejunum. *E. coli* was introduced *per os* 28 hours before necropsy.	100
15	Pig 0657. Age 30 days. Anterior jejunum. *E. coli* was introduced *per os* 48 hours before necropsy.	101
16	Pig 1306. Age 4-1/2 days. Anterior jejunum. *E. coli* was introduced *per os* 8 hours before necropsy.	102
17	Pig 2255. Age 2-1/2 days. Terminal ileum. *E. coli* was introduced *per os* 16 hours before necropsy.	103
18	Pig 2255. From the same field as Figure 17	104
19	Pig 9043. Age 3 days. Terminal jejunum. *E. coli* was introduced *per os* 18 hours before necropsy.	106
20	Pig 9043. Age 3 days. Midjejunum. *E. coli* was introduced *per os* 18 hours before necropsy. . .	107
21	Pig 9038. Age 2-1/2 days. Duodenum. *E. coli* was introduced *per os* 8 hours before necropsy . . .	108
22	Pig 0657. Age 30 days. Terminal jejunum. *E. coli* was introduced *per os* 48 hours before necropsy.	109
23	Pig 9046. Age 2 days. Duodenum. Germfree control	111
24	Pig 2262. Age 4 days. Duodenum. Germfree control	112
25	Pig 1305. Age 4-1/2 days. Duodenum. *E. coli* was introduced *per os* 8 hours before necropsy . . .	113
26	Pig 9047. Age 2-1/2 days. Midjejunum. Germ-free control.	114
27	Pig 9043. Age 3 days. Terminal ileum. *E. coli* was introduced *per os* 18 hours before necropsy. . .	115
28	Pig 1306. Age 4-1/2 days. Anterior jejunum. *E. coli* was introduced *per os* 8 hours before necropsy.	118
29	Pig 0661. Age 29 days. Terminal ileum. Germ-free control.	118

Figure		Page
30	Pig 1002. Age 3 days. Midjejunum. Germ-free control.	120
31	Pig 9046. Age 2 days. Duodenum. Germ-free control.	121
32	Pig 9046. Age 2 days. Midjejunum. Germ-free control.	122
33	Pig 9046. Age 2 days. Duodenum. Germfree control	123
34	Pig 1005. Age 3 days. Anterior jejunum. Germfree control.	125
35	Pig 1303. Age 4 days. Terminal jejunum. *E. coli* was introduced *per os* 4 hours before necropsy.	126
36	Pig 9041. Age 2-1/2 days. Duodenum. *E. coli* was introduced *per os* 14 hours before necropsy.	127
37	Pig 9041. Age 2-1/2 days. Terminal ileum. *E. coli* was introduced *per os* 14 hours before necropsy.	128
38	Pig 1009. Age 6-1/2 days. Terminal ileum. *E. coli* was introduced *per os* 96 hours before necropsy.	129
39	Pig 9042. Age 2-1/2 days. Midjejunum. *E. coli* was introduced *per os* 16 hours before necropsy.	130
40	Pig 9046. Age 2 days. Terminal jejunum. Germfree control.	132
41	Pig 9047. Age 2-1/2 days. Terminal ileum. Germfree control.	133
42	Pig 9040. Age 2-1/2 days. Duodenum. *E. coli* was introduced *per os* 12 hours before necropsy.	134
43	Pig 9044. Age 3 days. Terminal ileum. *E. coli* was introduced *per os* 20 hours before necropsy.	135
44	Pig 9045. Age 3 days. Terminal ileum. *E. coli* was introduced *per os* 24 hours before necropsy.	137
45	Pig 9042. Age 2-1/2 days. Terminal ileum. *E. coli* was introduced *per os* 16 hours before necropsy.	138

Figure		Page
46	Pig 9047. Age 2-1/2 days. Terminal ileum. Germfree control.	139
47	Pig 9040. Age 2-1/2 days. Terminal ileum. *E. coli* was introduced *per os* 12 hours before necropsy.	140
48	Pig 9043. Age 3 days. Terminal ileum. *E. coli* was introduced *per os* 18 hours before necropsy.	142
49	Pig 9046. Age 2 days. Terminal ileum. Germfree control.	143
50	Pig 9040. Age 2-1/2 days. Terminal ileum. *E. coli* was introduced *per os* 12 hours before necropsy.	144
51	Pig 9042. Age 2-1/2 days. Duodenum. *E. coli* was introduced *per os* 16 hours before necropsy.	146
52	Pig 9042. From the same field as Figure 51	147
53	Pig 9044. Age 3 days. Terminal ileum. *E. coli* was introduced *per os* 20 hours before necropsy.	148
54	Pig 9046. Age 2 days. Duodenum. Germfree control	149
55	Pig 9046. Age 2 days. Duodenum. Germfree control	150
56	Pig 9046. Age 2 days. Terminal jejunum. Germfree control.	151
57	Pig 9047. Age 2-1/2 days. Terminal jejunum. Germfree control.	152
58	Pig 9038. Age 2-1/2 days. Terminal ileum. *E. coli* was introduced *per os* 8 hours before necropsy	153
59	Pig 9039. Age 2-1/2 days. Terminal ileum. *E. coli* was introduced *per os* 10 hours before necropsy.	155
60	Pig 9041. Age 2-1/2 days. Terminal jejunum. *E. coli* was introduced *per os* 14 hours before necropsy.	156
61	Pig 9042. Age 2-1/2 days. Terminal jejunum. *E. coli* was introduced *per os* 16 hours before necropsy.	157
62	Pig 1005. Age 3 days. Duodenum. Germfree control	158

Figure		Page
63	Pig 1002. Age 3 days. Duodenum. Germfree control	160
64	Pig 1005. Age 3 days. Terminal ileum. Germfree control.	161
65	Pig 1000. Age 3-1/2 days. Terminal ileum. *E. coli* was introduced *per os* 24 hours before necropsy.	162
66	Pig 1004. Age 3-1/2 days. Terminal jejunum. *E. coli* was introduced *per os* 28 hours before necropsy.	164
67	Pig 1008. Age 4 days. Terminal jejunum. Midvillus. Germfree control.	168
68	Pig 1008. Age 4 days. Terminal jejunum. Midvillus. Germfree control.	170
69	Pig 1008. Age 4 days. Terminal jejunum. Midvillus. Germfree control.	172
70	Pig 1002. Age 3 days. Terminal ileum. Basal third of villus. Germfree control	174
71	Pig 1002. Age 3 days. Terminal jejunum. Germfree control.	177
72	Pig 1310. Age 4-1/2 days. Terminal jejunum. Midvillus. Germfree control.	179
73	Pig 1306. Age 4-1/2 days. Terminal jejunum. Midvillus. *E. coli* was introduced *per os* 8 hours before necropsy	181
74	Pig 2251. Age 2-1/2 days. Terminal ileum. Midvillus. *E. coli* was introduced *per os* 14 hours before necropsy	183
75	Pig 1005. Age 3 days. Terminal ileum. Basal third of villus. Germfree control.	186
76	Pig 1310. Age 4-1/2 days. Terminal jejunum. Midvillus. Germfree control.	188
77	Pig 2253. Age 2 days. Terminal jejunum. Basal third of villus. Germfree control.	191
78.	Pig 1310. Age 4-1/2 days. Terminal jejunum. Midvillus. Germfree control.	193

Figure		Page
79	Pig 1001. Age 3-1/2 days. Terminal jejunum. Midvillus. *E. coli* was introduced *per os* 24 hours before necropsy	195
80	Pig 1001. Age 3-1/2 days. Terminal jejunum. Midvillus. *E. coli* was introduced *per os* 24 hours before necropsy	197
81	Pig 2251. Age 2-1/2 days. Terminal ileum. Basal third of villus. *E. coli* was introduced *per os* 14 hours before necropsy	199
82	Pig 2251. Age 2-1/2 days. Terminal ileum. Basal third of villus. *E. coli* was introduced *per os* 14 hours before necropsy	203
83	Pig 2251. Age 2-1/2 days. Terminal ileum. Midvillus. *E. coli* was introduced *per os* 14 hours before necropsy	205
84	Pig 2251. Age 2-1/2 days. Terminal ileum. Midvillus. *E. coli* was introduced *per os* 14 hours before necropsy	207
85	Pig 2251. Age 2-1/2 days. Terminal ileum. Midvillus. *E. coli* was introduced *per os* 14 hours before necropsy	209
86	Pig 2252. Age 2-1/2 days. Terminal jejunum. Midvillus. *E. coli* was introduced *per os* 14 hours before necropsy	211
87	Pig 2251. Age 2-1/2 days. Terminal jejunum. Midvillus. *E. coli* was introduced *per os* 14 hours before necropsy	214
88	Pig 2251. Age 2-1/2 days. Terminal jejunum. Midvillus. *E. coli* was introduced *per os* 14 hours before necropsy	216
89	Pig 1001. Age 3-1/2 days. Terminal jejunum. Midvillus. *E. coli* was introduced *per os* 24 hours before necropsy	218
90	Pig 1001. From the same field as Figure 89	220
91	Pig 1001. Age 3-1/2 days. Terminal jejunum. Midvillus. *E. coli* was introduced *per os* 24 hours before necropsy	222
92	Pig 2258. Age 3 days. Terminal jejunum. Midvillus. *E. coli* was introduced *per os* 18 hours before necropsy	224

Figure		Page
93	Pig 1001. Age 3-1/2 days. Terminal jejunum. Midvillus. *E. coli* was introduced *per os* 24 hours before necropsy	228
94	Pig 2258. Age 3 days. Terminal jejunum. Midvillus. *E. coli* was introduced *per os* 18 hours before necropsy	230
95	Pig 2251. Age 2-1/2 days. Terminal jejunum. Midvillus. *E. coli* was introduced *per os* 14 hours before necropsy	232
96	Pig 1001. Age 3-1/2 days. Terminal ileum. Basal third of the villus. *E. coli* was introduced *per os* 24 hours before necropsy	234
97	Pig 1001. Age 3-1/2 days. Terminal ileum. Basal third of the villus. *E. coli* was introduced *per os* 24 hours before necropsy	236
98	Pig 1001. Age 3-1/2 days. Terminal ileum. Basal third of the villus. *E. coli* was introduced *per os* 24 hours before necropsy	238
99	Pig 1001. Age 3-1/2 days. Terminal ileum. Basal third of the villus. *E. coli* was introduced *per os* 24 hours before necropsy	240
100	Pig 1001. Age 3-1/2 days. Terminal ileum. Midvillus. *E. coli* was introduced *per os* 24 hours before necropsy	243
101	Pig 1001. Age 3-1/2 days. Terminal ileum. Midvillus. *E. coli* was introduced *per os* 24 hours before necropsy	245
102	Pig 1006. Age 4 days. Terminal ileum. Midvillus. *E. coli* was introduced *per os* 32 hours before necropsy	247
103	Pig 1006. Age 4 days. Terminal jejunum. Midvillus. *E. coli* was introduced *per os* 32 hours before necropsy	250
104	Pig 1006. Age 4 days. Terminal ileum. Midvillus. *E. coli* was introduced *per os* 32 hours before necropsy	252
105	Pig 1009. Age 6-1/2 days. Terminal jejunum. Midvillus. *E. coli* was introduced *per os* 96 hours before necropsy	254

PREFACE

"I can't believe *that*!" said Alice. "Can't you?" the Queen said in a pitying tone. "Try again: draw a long breath, and shut your eyes." Alice laughed. "There's no use trying," she said: "one *can't* believe impossible things." "I daresay you haven't had much practice," said the Queen. "When I was your age, I always did it for half-an-hour a day. Why, sometimes I've believed as many as six impossible things before breakfast."

Through the Looking-Glass
Lewis Carroll (Charles Lutwidge Dodgson)
1832-1898

INTRODUCTION

There is a growing body of opinion in favor of the use of the domestic pig (*Sus scrofa*) as an experimental animal model for enteric diseases of man. Infantile colibacillary diarrhea is one of the major diseases of childhood in the underdeveloped countries of the world, and it is occasionally associated with high mortality in intrahospital epidemics in the United States of America and other of the so-called developed countries, yet the pathogenesis of this disease remains undetermined.

Colibacillosis is a term applied to a group of diseases in man and the higher animals caused by infection with bacteria classified as *Escherichia coli*. This definition has been widened by some biologists to include infections of the mammary gland and urinary tract of these species and coliform infections of birds. In this study, the term colibacillosis is used to refer to postnatal and weanling diarrheal disease of man and higher animals specifically associated with growth of *E. coli* in the gastrointestinal tract of the host.

Pigs derived by hysterectomy or hysterotomy and maintained free of all detectable microorganisms, or specifically contaminated with known organisms, are called "gnotobiotic" pigs. The increased availability of germfree and gnotobiotic pigs as experimental animals for investigation of infectious diseases has facilitated research into

basic pathology and pathogenesis of diseases, uncomplicated by secondary infections and microflora in general and, most important, in the absence of nonpathogenic microbial flora of the same genus and species as the pathogen under investigation. Until recently, pathologists studying colibacillosis have been confused somewhat by the presence of the microflora of the "normal" intestine and particularly by the presence of serotypes of *E. coli* of unknown pathogenicity.

Very little significant work has been done to clarify the pathogenesis of colibacillosis of human infants despite the enormity of this public health problem. In veterinary research, an increasing interest has been shown in this field over the past five years. Since it is likely that the basic problem of pathogenesis can most successfully be investigated in experimental animal models, elucidation of the pathogenesis of the diarrhea of colibacillosis in an experimental animal such as the gnotobiotic pig should have application in the solution of the wider problem of colibacillary diarrhea of man and the higher animals.

Recent studies of colibacillosis were concentrated on bacteriologic and immunologic aspects of the disease. Some preliminary studies of the pathology and pathogenesis of colibacillosis in gnotobiotes have been reported within the last two years and the substance of this thesis is the direct development of one of these studies.

In this laboratory, using *E. coli* 0138:K81:NM, the means whereby the organism established itself as an infection in the host following

both oral and subcutaneous contamination were established (Christie, 1967). Concurrent hematologic studies and observations of the clinical syndrome were made and recorded, relating these to the development of the infection. In addition, the gross and microscopic lesions of colibacillosis in gnotobiotes were recorded. From these observations it was hypothesized that in coliform diarrheal disease -- the most commonly seen manifestation of colibacillosis in neonatal pigs -- the pathogenesis of the condition was most likely one of malabsorption due to alteration of the absorptive component of villal epithelial cell activity.

Fordtran (1967) defined diarrhea in terms of water and electrolyte malabsorption as: interruption of the transport processes of the normal mucosal cell; deranged permeability of the intestine; the presence of nonabsorbable, osmotically active substances in the lumen of the intestine; and abnormal intestinal motility. There is, in addition, some evidence that the diseased villus loses excess fluid into the intestinal lumen, thereby contributing directly to the increased volume of chyme. The clinical state of diarrhea will depend on the region of the intestine involved in the disease process and on which of the above factors has initiated and is contributing to the disease process.

In this thesis I propose to describe the villal changes contributing to alteration of the complex dynamic process of fluid and electrolyte movement and, using as experimental models the gnotobiotic pig and a specific serotype of *E. coli*, to speculate on the

pathogenesis of diarrhea in enteric colibacillosis of human and animal neonates.

LITERATURE REVIEW

"For out of olde feldes, as men seith,
Cometh al this newe corn fro yeer to yere
And out of old bokes, in good feith,
Cometh al this newe science that men lere."

Geoffrey Chaucer
1340-1400

Escherichia coli is an organism of many races, of great serological variety 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.

A natural diarrheal disorder of newborn calves, commonly called scours, was studied by Jensen in 1892. It was this man who pioneered the concept that there were enteropathogenic strains of a common intestinal organism (Jensen, 1892). Jensen distinguished between pathogenic and nonpathogenic strains of *E. coli* by oral exposure of neonatal calves with suspect strains (Barnum *et al.*, 1967).

Von Bahr in 1912 suggested that there was an etiological relationship between *E. coli* scours in animals and human diarrheal disease and this theory was endorsed by Adam (1927).

Theobald Smith and Orcutt (1925) studied the condition described previously by Jensen, and attributed it to special strains of colon bacilli which were subsequently identified bacteriologically and immunologically (Smith, 1927, 1928). Theobald Smith and others, from 1927 on, attempted to distinguish between pathogenic strains by biochemical means but were unsuccessful (Barnum *et al.*, 1967).

Certain serotypes of *E. coli* have been designated as "enteropathogenic", this designation referring to those strains isolated from animals and man with diarrhea associated with the presence of these strains in the alimentary tract (Kohler and Bohl, 1966). Adam (1927) characterized these strains by their behavior in fermentation tests, but the first serious epidemiological study of human infantile gastroenteritis due to *E. coli* is attributed to Goldschmidt (1933), who identified enteropathogenic serotypes by serological methods.

It was in 1927 that Adam isolated one or two strains of coliform bacilli from human patients with infantile gastroenteritis (Barnum *et al.*, 1967). Further experiments with animals were carried out by Lovell in the United Kingdom, whose reports strengthened the concept of an etiological relationship of *E. coli* to diarrheal disease in man (Lovell, 1937; Lovell, 1951). Lovell (1937) employed precipitin tests and was able to distinguish serologically between pathogenic and nonpathogenic bovine strains of *E. coli*.

Of more recent import, in that it gave impetus to modern interest in human colibacillosis, is the work of Bray (1945) and Bray and Beavan (1948) in the United Kingdom, who demonstrated enteropathogenic *E. coli* in 88% of cases of infantile diarrhea, but in only 3% of controls.

Material gains in the understanding of the clinical and epidemiological significance of such infections were made possible by the publication of the serological classification of *E. coli* by Kauffman (1944, 1947) and by Kauffman and Dupont (1950). Their discovery of the "O" agglutination masking effect of the "L" antigen and subsequent classification of the antigenic structure of *E. coli* according to the serological investigations of Kauffman - Knipschildt - Vahlne, was subsequently expanded by Ewing in the United States of America, and by Ørskov in Europe (Sojka, 1965). This schema was based on the fact that three kinds of antigen occur in *E. coli*, and that these antigens can be selectively activated or inactivated by specific heat treatments.

Since 1950 the 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).

Recent excellent and succinct accounts of the bacteriology and serology of *E. coli* are available in the work of Sojka (1965) and Barnum *et al.* (1967).

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.

It is not yet known what factors are associated with virulence in a particular strain of *E. coli*, 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 of *E. coli* 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 confirmed 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 Human Medicine

In human medicine the epidemiologic entity, infantile diarrhea or "weanling diarrhea" (Gordon *et al.*, 1963), is generally observed with the transition of babies from a breast-fed existence to that of a mixed diet. This clinical syndrome is still considered one of the major health problems of the world, and in the underdeveloped nations is considered to be the major cause of death in infants less than two years of age (W.H.O. Report, *International List of Causes of Death*, 1957; Gordon *et al.*, 1963; Pearse, personal communication, 1968). In a number of authoritative studies the diarrheas and the dysenteries are recognizable as outstanding among the health problems of children (Wasti, 1959; Silhar and Maru, 1960; Carter, 1961).

No more than an eighth of infants and young children of the world are free from appreciable risk of a lethal diarrhea (Cruickshank, 1961), and despite the lesser freqency of this condition in school children and adults, a survey conducted by the Pan American Sanitary Bureau found this condition to be the leading cause of death in 8 of 17 countries studied (Anon., 1956).

The weanling diarrhea syndrome includes several etiologic entities. In most cases in babies no accepted pathogenic agent can be identified, and significant numbers carry agents of questionable disease-producing capacity or variously involved in multiple disease processes (Gordon *et al.*, 1963). Although the clinical manifestations of weanling diarrhea differ widely according to the environmental sanitation and the state of nutrition of the infant, the infectious

agents broadly encountered are much the same, suggesting that clinical variations relate mainly to host resistance and infecting dose.

Not all acute diarrheas are of infectious origin, but most are (Public Health Dept., London County Council, 1953). Shigellosis or bacillary dysentery is the most common specific enteric infection among diarrheas of children (Hardy and Watt, 1945; Goodwin *et al.*, 1960; Gordon, 1960), although *Shigella* is less common in the diarrheas of inhabitants of preindustrial countries where diarrheal disease is more prevalent (Gordon *et al.*, 1963).

Within recent years it has become increasingly apparent that a considerable porportion of the acute diarrheal disease of infants during their first year is due to particular enteropathogenic strains of *E. coli* (Hodes, 1956; Shanks and Studzinski, 1952). *Escherichia coli* is the causative agent of epidemic diarrhea of newborn infants in the United States of America. It kills 25 to 50% of affected babies in this age group. *Escherichia coli* also causes diarrhea in two- and three-year-old infants in the United States of America and is the major cause of infantile diarrhea in this country, constituting from 5 to 40% of all cases of diarrhea in the age group, according to the region in the U.S.A. that is sampled (Wheeler, 1967).

In human colibacillosis enteropathogenic serotypes are usually present only during the period of the illness (Kauffmann and Perch, 1943; Wallick and Stuart, 1943; Perch, 1944; Sears *et al.*, 1950). A causative relationship between implicated strains of *E. coli* and epidemic and sporadic occurrences of colibacillosis in humans was established by experiments in which adult volunteers and infants

were fed different strains of *E. coli* (Taylor *et al.*, 1949; Neter and Shumway, 1950; Taylor and Charter, 1952; June *et al.*, 1953; Wright and Boden, 1953; Ross and Dawes, 1954; Ferguson and June, 1955; Kessner *et al.*, 1962). These observations were made in countries where general nutritional standards were adequate (Europe and U.S.A.), but there is confirmatory evidence of the role of colibacillosis in diarrheal disease in other countries such as India (Anon., 1962), Indonesia (Joe *et al.*, 1960), Guatemala (Gordon *et al.*, 1962) and Chile (Rodriguez-Leiva, 1960).

Intrahospital epidemics of diarrheal disease associated with *E. coli* enteric infection are reported (Barnum *et al.*, 1967) and representative series of sporadic cases among infants of a general population regularly include infections of this nature (Yekutiel, 1959).

The ability to serotype enteropathogenic strains of *E. coli* enabled investigators to determine that they were relatively rare in the feces of normal infants (Giles *et al.*, 1949; Ewing *et al.*, 1955) but that there existed nonclinical carriers of pathogenic *E. coli* among the contact group of cases of infantile diarrhea due to organisms of the same serotype (Payne and Cook, 1950).

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).

In human medicine certain serotypes of this organism are important etiologic agents of focal pyogenic skin infections, omphalophlebitis, polyarthritis, meningitis, 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).

Experimental Animal Models for Diarrheal Disease

The pathogenicity of strains of *E. coli* has been tested in embryonated eggs, white mice, guinea pigs, neonatal and weaned calves and "conventional" pigs and gnotobiotic pigs. Studies thus far have led to the conclusion that differences in the susceptibility of the test species, the source of the organism, the enhancement of virulence by host passage of the organism, the route of exposure, the host defense mechanism (including intrinsic as well as extrinsic factors such as nutrition) and the serologic characteristics of the *E. coli* strain, all lead to variation in the clinical and pathologic manifestations of infection.

In 1966, Bustad emphasized the physiologic relationship of pigs to man and the value of these animals as models for human disease investigation (Bustad, 1966).

In earlier papers Dubos (1962) and Gordon *et al.* (1963) speculated that the young germfree animal offered possibilities for exploring the result of the first invasion of organisms, ordinarily nonpathogenic for man, into the malnourished subject.

Bustad and McClellan (1966) have reported a much closer physiologic similarity between the domestic pig and man than that between the carnivores and man. Haelterman and Hooper (1967) consider that the baby pig is a particularly suitable model for infantile diarrhea studies, whereas Bustad has a broader opinion and considers pigs to be an ideal experimental model for many diseases of man (Bustad, 1966). Maronpot and Whitehair (1967) record their enthusiasm for the baby pig as a "useful experimental model for obtaining additional basic information on enteric disturbances" and go on to point out the particular advantages of these animals especially in the investigation of enteric disturbances related to infection and nutrition.

The unique qualities of the germfree neonatal pig as detailed by the above authorities, and in my own experience, make it an excellent tool for research into some or all of the abovementioned factors associated with infantile and weanling diarrhea.

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 *E. coli* 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. These authors (Jubb and Kennedy, 1963) list the predisposing factors in young non-primates: (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.

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 duodenitis and jejunitis with no obvious lesions in other organs. He isolated *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 reddening 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 and was followed at the end of the first or 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 lesion at necrospy. 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 findings 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 postmortem 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 study of 100 pigs submitted to a diagnostic laboratory, Gossling and Rhoades (1966) obtained isolates of *E. coli* 0138:K81:NM from 6 pigs with "enteritis". These authors stated that "two serotypes, 08:K85:H19 and 0138: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) postweaning enteritis of pigs, 10 to 12 weeks of age or shortly after weaning, manifested chiefly as "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.

Colibacillosis in "Specific Pathogen Free" and Gnotobiotic Pigs

Experimental reproduction of colibacillosis in "specific pathogen free", "germfree" or gnotobiotic pigs is generally not well documented. Saunders *et al.* (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 5 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 pigs exposed at ages ranging from 2 to 12 days. The outstanding lesion reported by these workers was a serofibrinous or fibrinopurulent polyserositis and 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 intestinal tract. The villi of the intestines of pigs monocontaminated with *E. coli* were generally similar to those in the germfree control animals. Pigs contaminated with two serotypes of *E. coli* had branched and moderately foreshortened villi on the intestinal mucosa, and slight edema of the lamina propria. 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), and the basic dynamic process of cell necrobiosis and replacement (Creamer, 1967).

In three experiments, using 62 gnotobiotic pigs, Christie (1967) determined the clinical effects, and gross and microscopic lesions of colibacillosis produced by 2 strains of *E. coli* -- 0138:K81:NM isolated in Michigan and 0138:K81 isolated in Minnesota. Precluding oral exposure of some test litters, he determined that both serotypes

readily colonized the intestinal tract from experimental infection simulating neonatal contamination of the umbilical stump. It was observed that 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 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 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* 0138:K81 (Minnesota origin) 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* 0138:K81:NM (Michigan origin) produced 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 (Christie, 1967).

Christie (1967) reported that 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 an acute hemorrhagonecrotic enteritis to a histological picture, in persistently scouring pigs, microscopically indistinguishable from that seen in clinically normal germfree animals. He observed the

most common lesion to be in the villal epithelial cell and described it as a marked degenerative vacuolation of the cytoplasm that he termed "hydropic degeneration". He observed and described evidence of basic changes in apparent function of the villi, and hypothesized that these changes were responsible in part for malabsorption associated with colibacillary diarrhea (Christie, 1967).

Extrinsic Factors Complicating Colibacillary Diarrhea

Diarrhea is less likely to be caused by nutrient excess than it is by nutrient deficiency, as exemplified in pellagra and beri-beri. Protein deficiency is the outstanding factor in the etiology of kwashiorkor, and "kwashiorkor without diarrhea" is likely not to be kwashiorkor (Gordon *et al.*, 1963). Recent attempts were made in Ontario to determine the role played by high and low energy diets in the development of enteric colibacillosis in experimental pigs (Barnum, D. A., personal communication). Although some changes in intestinal coliform populations were observed, little of clinical or pathologic significance was recorded.

In addition to colibacillosis and malnourishment, factors singly or severally associated with diarrhea in infants include *Shigella spp.*, *Salmonella spp.*, many enteroviruses (including poliomyelitis virus, Coxsackie Group A, Coxsackie Group B, and many echoviruses), many adenoviruses, general infections, bacterial toxins, herbal medicines (used in tropical and underdeveloped countries), saline purgatives and indiscriminate use of antibiotics.

The Mucosa of the Small Intestine

The transfer of the products of digestion from the intestinal lumen to the blood or lymph is the process of intestinal absorption. In a normal animal the major factors involved in absorption are gastrointestinal motility, integrity of the intestinal wall and blood flow. Circular contractions of the muscularis mucosa have the effect of moving ingesta through the intestinal tube, but they also squeeze the villi together, diminishing the surface available for chyme-mucosal contact and decreasing the opportunity for absorption.

The small intestine is the main organ for absorption of dietary nutrients; the villus, and the epithelial cells clothing the villus, constitute the functional absorptive unit (Kenworthy, 1967). The mucosa of the small intestine consists of that of the tubular crypts in continuity with that of the villi, the long finger-like processes which project into the lumen. The integrity of the anatomy and physiology of the villus is essential to normal function of the intestinal tract.

Movements of the villus are considered to be both undulating and vertically contractile. These movements are the result of passive movement with the flow of ingesta, active secondary movement as a result of movements of the intestine muscular coats in peristalsis, and active primary movement due to contraction of the Breucke fibers. Finger-shaped villi are said to "pump" actively (Verzár and McDougall, 1936), whereas leaf-shaped villi never show pumping movements (Kenworthy, 1967).

Unlike the brain and kidney, organs of the body composed of almost permanent cell populations, the gastrointestinal tract epithelium works on the basis of rapid cell replacement. The most dynamic part of the gastrointestinal tract epithelium is the small-intestinal mucosa. The intestinal epithelial cell has, as D. H. Smyth (1967) says, "a short life, but a merry one, if merriment is the equivalent in cellular terms of a remarkable range of activities possessed by few other cells in the body". The life of the mucosal cell is brief, for the whole epithelial lining is renewed every few days.

The specialized function of the crypt epithelium is that of cell division, so that as cells are lost from the terminal portion of the villus, they are replaced by a continual streaming of villal epithelial cells from the crypts. The villal epithelial cells arise from the repeated division of relatively undifferentiated cells in the crypts of Lieberkühn, and once division has taken place differentiation is complete relative to eventual function (Kenworthy, 1967). The concept that the crypts are almost solely for epithelial cell production whilst the villi provide a large surface area almost wholly for absorption was first positively propounded by Bizzozero (1888), was amply confirmed by the exhaustive studies of Leblond and various co-workers (1948, 1955, 1958) and has been recently summarized in an excellent review (Creamer, 1967).

The cells lost from the tips of the villi disintegrate rapidly so that little is ever seen as whole cells, or even recognizable residue, in the lumen of the small intestine (Leblond and Messier,

1958). Indeed, this disintegration in the small intestine of the normal human is so rapid that cells labelled with [^{3}H] thymidine did not survive long enough, once shed into the lumen, for experimental biopsy in the cell migration studies reported by Deschner *et al.* (1963).

The very fast turnover of intestinal epithelial cells in general is illustrated by the following examples of the shortest and longest recorded turnover times, the shortest time being that of the villal epithelial cells of the ileum of the mouse -- 24 hours (Creamer, 1967) -- and the longest being that of the villal epithelial cells of the duodenum of man -- 5 to 6 days (MacDonald, Trier and Everett, 1964). Lipkin (1965) has estimated that the time taken for complete replacement of the epithelial lining of the gastrointestinal tract in man is 3 to 6 days. A more graphic expression of this dynamism is the determination of the flow-rate of villal epithelial cells in "positions per hour". Creamer (1967) has recorded a flow-rate for the human ileum at 0.8 cell positions per hour and for the human jejunum at 1.0 cell position per hour. For man and for mammals the turnover time is "remarkably constant" (Creamer, 1967).

Germfree animals have an "abnormal" small intestine compared to that of conventional animals, that is, animals not reared in germfree conditions. Germfree animals have shorter crypts, much longer villi and a paucity of lymphoid and inflammatory tissue in the epithelium and the laminae propriae (Dubos, 1966). The villal epithelial cell turnover time is approximately twice that of the conventional animal, and the generation cycle of crypt cells in the germfree animal is

slower than that of the conventional animal (Abrams *et al.*, 1963; Lesher *et al.*, 1964).

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 intestinal tract. The villi of pig intestine monocontaminated with *E. coli* 0141:K85a,c(B):H4 were generally similar to those in the germfree control animal. Pigs contaminated with *E. coli* serotypes 0141:K85a,c(B):H4 and 08:H had intestinal villi with considerable variation in shape, including some branching forms. Kenworthy and Allen (1966) introduced a third group of axenic pigs into a piggery and reported that these animals developed a large, mixed, intestinal flora and a striking change in the mucosa with long crypts, increased mitoses and short villi, the picture, according to Creamer (1967), characteristic of very rapid cell turnover.

Smyth (1967) has raised the question of mode of desquamation of cells in the zone of extrusion. Alternative suggestions are that either cells at the top of the villus are pushed off by the continuum of new cells streaming from the crypts, or they are produced in response to a need to replace those sloughing at the apex. Smyth (1967) suggested that histochemical and microscopic studies of cells at the very tip of the villus, should they reveal basic pathologic change, would suggest an answer to this problem.

Intestinal absorption is complex in a number of ways -- iron and vitamin B_{12} are absorbed into the epithelial cells and kept there for a variable time, then later released from the cell, through the

basement membrane into the lamina propria. Dipeptides, disaccharides and glycerides are absorbed into the villal epithelial cell and hydrolyzed intracellularly. Conversion of fructose to glucose, glucose to lactic acid, and glutamic acid and aspartic acid to alanine are well documented examples of the detailed metabolic digestion processes occurring within the villal epithelial cells as an integral part of the serosal transfer of nutrients (Levin, 1967).

Apart from the absorption of protein, carbohydrates and lipids the villal epithelial cell, being provided with membranes permeable to electrolytes and water, exchanges fluid and electrolytes with its environment in order to facilitate absorption of digestion products and as a means of stabilizing cell volume.

Intestinal epithelial cells, in normal function, move substances against their electrochemical potential and for this purpose use the energy of metabolism mediated through adenosine triphosphate synthesis and breakdown. Since all cells are required to regulate individual cell composition and volume, they utilize the energy of metabolism for osmotic work, a process aptly called "chemi-osmotic coupling" (Mitchell, 1961).

The mechanism of membrane transport in relation to intestinal absorption is one of great complexity and to this date, investigation has been primarily directed to the elucidation of the individual steps of membrane transport in model systems such as the erythrocyte, nerve axon, and cholecyst epithelium (Diamond and Tormey, 1966; Smyth and Whittam, 1967).

Molecules and molecular aggregates such as soluble enzymes, intermediates of metabolism, and organelles carrying a surface charge function as electrolytes to which the villal epithelial cell membrane is impermeable. As a consequence there is an osmotic stress which tends to favor the imbibition of fluid by the villal epithelial cell. In a normal cell there is a stabilization of volume dependent upon the regulation of the concentration of diffusible ions within the cell in two ways; by active transport systems -- or "pumps" -- for ions, particularly of sodium and potassium, and loss of ions through leaks in the cell membranes. The operation of the pumps requires a continual supply of free energy. Interference with, or destruction of such energy-producing systems, leads to a cessation of pump activity, a loss of cell potassium and increased intracytoplasmic sodium, chloride and water (Robinson, 1960; Parsons, 1967).

In 1957, Lifson and Parsons demonstrated in the small intestine of experimental rats that sodium transport depended on aerobic glycolysis, and that this glycolysis was stimulated by glucose or mannose but not by acetate, pyruvate or lower fatty acids. They observed, however, that in the ileum and colon, fluid absorption was stimulated, under aerobic conditions, by the addition of glucose and acetate, pyruvate and the lower fatty acids (Lifson and Parsons, 1957).

A direct coupling exists between inward movement of sodium at the brush border of villal epithelial cells and accumulation and transport of sugars and amino acids (Crane, R. K., personal communication). Parsons (1967) considered that the absorption of either

sodium, or sugars and amino acids is stimulated when active absorption of the other is proceeding, and he concluded that the energy for sugar and amino acid transport is derived from the movement of sodium and potassium ions down an electrochemical gradient that is maintained by cellular metabolism involving the hydrolysis of energy-rich substances such as adenosine triphosphate. Membrane adenosine triphosphatases implicated in sodium and potassium transport have been described in the brush border and other bordering membranes of the villal epithelial cells and Parsons (1967) postulated that there is an outward oriented sodium pump in the brush border of the anterior jejunum. The obvious practical need for such a pump is that more sodium, than that provided by diet and exocrine digestive systems, is required in the chyme to drive the absorption of sugars and amino acids.

Within the villal epithelial cell, alterations in the energy-producing systems of the mitochondria could lead to inability of these organelles to excrete metabolic water. Such distention of the mitochondria has been reported (Rouiller, 1960) and is considered to be the cause of the finely granular cytoplasm of the cell undergoing cloudy swelling. Still further distention of the mitochondria will continue to that point at which the organellar membrane ruptures releasing its store of metabolic water to the cytoplasm, and eventually resulting in the phenomenon of hydropic degeneration of the cell (Robinson, 1960).

The Villus

Under the optical microscope the structure of the villus is deceptively simple. The villus is clad with a simple columnar epithelium bearing a faintly discernible brush border of microvilli. In the neonatal pig the brush border is more apparent in the duodenal and jejunal epithelial cells than it is on those of the ileum. Interspersed amongst the villal epithelial cells are goblet cells, increasing in relative number caudally. The epithelium rests on a clearly demonstrable basement membrane.

The lamina propria of the villus consists of connective tissue cells and noncellular stroma, capillaries, lymphatic vessels, a central chyle vessel termed a "lacteal" (it can be single or multiple), contractile fibers superficially resembling smooth muscle fibers (termed Breucke fibers), and variable numbers of reticuloendothelial cells and lymphocytes.

It is not uncommon to find marked vacuolation of the villal epithelial cells in germfree and monocontaminated neonatal pigs. This vacuolation is more marked in infected pigs and is more common in sections taken from more caudal sites in the small intestine. These vacuoles have been described as the result of either normal intestinal absorption or of necrobiosis in the germfree pig and the more marked vacuolization seen in pigs monocontaminated with *E. coli*, as absorption vacuolization, evidence of necrobiosis, or hydropic degeneration associated with the toxic activities of the enteric organisms (Christie, 1967).

Other than the basic cellular structures -- nucleus, cytoplasm, cell wall, brush border, various vacuoles including fat droplets, hyaline bodies, glycogen and other substances there is little else in the villal epithelial cell that is visible to the optical microscopist. The advent of the electron microscope has enabled cytologists to observe the absorptive cell in much greater detail. As would be expected from the knowledge of the highly specialized functional demands of this cell, the ultrastructure is quite complex.

The brush border consists of microvilli about 1 μ long and 0.1 μ in diameter (Granger and Baker, 1949). In the jejunal epithelial cell of the 3-week-old pig, the individual microvilli are enclosed within a hirsute layer, the "glycocalyx". The glycocalyx is not present in neonatal pig villal epithelial cells, and Staley *et al.* (1968a) suggested that it was synthesized after whole protein absorption ceased since it was present in the absorptive cell of the 3-week-old pig. A trilaminar membrane constituting what is virtually the basement membrane for the glycocalyx is continuous over the microvilli. The filamentous central core of each microvillus is termed the "cytoskeleton" and runs from the tip, through the shaft, to the base and into the apical cytoplasm, condensing in that region into well-defined "rootlets" (Staley *et al.*, 1968a). Microvilli occasionally have a common base and in the crypts of Lieberkühn and at the tips of the villi, the microvilli are shorter and somewhat club-shaped. At the level of the rootlets, in the extreme apical cytoplasm of the cell, there is a mass of interlacing fibers around the rootlets, constituting what is called the terminal web

(Palay and Karlin, 1959). The terminal web is not clearly apparent at the apex of the jejunal villal epithelial cell of the neonatal pig (Staley *et al.*, 1968a).

The subapical zone of the jejunal villal epithelial cell was observed by Staley *et al.* (1968a) to be free of organelles but with a faintly granular appearance. Apical tubules were seen, particularly in cells near the tip of the villus. These tubules were usually parallel, but did branch and anastomose, and penetrate the cell a distance of 2 or 3 μ. The tubules were slender; near the luminal plasmalemma they were 30-60 mμ wide but were sometimes dilated by their contents to form vacuoles. It was observed that these tubules had a trilaminar membrane 95 to 100 A thick with fine extensions projecting into the lumen of the tubules. The apical tubules were absent from the absorptive epithelial cells in the 3-week-old pigs. It is clear that the apical tubule system described here corresponds to the "smooth-surfaced cisternae" described by Kenworthy *et al.* (1967).

Staley *et al.* (1968a) described vacuoles in the jejunal villal epithelial cells as being prominent in the apical two thirds of the villus and, within the individual cell, generally small in the supranuclear position and larger below the nucleus. These workers suggested that the apical vacuoles originated either by pinocytosis or by dilatation of the apical tubular system; that these large vacuoles delaminated into the lumen forming "prominent myelin-like whorls"; and that they contained "little material except free membranes". The so-called "basal vacuoles" were of a somewhat

different nature in that they contained a flocculent material that was "either dense and homogeneous or clumped in bizarre arrangements."

Staley *et al.* (1968a) described the Golgi apparatus of the villal epithelial cell of the neonate as being subnuclear and associated with the basal vacuoles. In less mature villal epithelial cells, the Golgi apparatus was in a paranuclear position. In the jejunal absorptive cells of pigs at 21 days of age, the Golgi apparatus was peripheral to the nucleus in the apical cytoplasm.

In neonates, the mitochondria and granular endoplasmic reticulum were present at the base of the cell, whereas in pigs at 3 weeks of age, these organelles were generally dispersed throughout the cell (Staley *et al.*, 1968a).

The other ultrastructural features of villal epithelial cells, namely desmosomes, mural invaginations, intramembranous space, smooth endoplasmic reticulum, lysosomes, micropinocytotic vesicles, multivesicular bodies, ribosomes, and the various intranuclear structures, have been described elsewhere in consummate detail in a number of standard references (Rhodin, 1963; Ham, 1965; Fawcett, 1966).

Permeability of the Intestinal Mucosa

Macromolecular Absorption.

Since there is no transplacental movement of antibody in swine, cattle, or sheep, consumption of colostrum by the neonates of these species precedes a rise in serum gamma globulin in the first day of life to levels usually found in adult animals. These globulins are apparently absorbed intact into the lacteals through the intestinal

epithelium during the first 36 hours of life. The ability of the neonate to absorb intact proteins generally is lost between 8 and 16 hours of life (Brambell, 1958; Clark, 1959). This phenomenon of "closure" (Lecce, 1966) was considered due to decrease or loss of pinocytotic activity. According to Barnum *et al.* (1967), the proteins (other than immunoglobulins) absorbed from the gastrointestinal tract of newborn herbivora prior to closure are cleared from the renal circulation, resulting in proteinuria during the second and third day after birth.

Comprehensive studies of the intestinal absorption of protein by neonatal (but not gnotobiotic) pigs have been reported by Mattisson and Karlsson (1964, 1965, 1966) and by Pierce and Smith (1967), who demonstrated the predominance of the jejunum over the duodenum and ileum in the intestinal absorption of immune lactoglobulins. Mattisson and Karlsson (1966) described a nongranular membrane system of tubules and vacuoles in the apical cytoplasm of jejunal epithelial cells. They further observed that, after colostrum feeding, lipid and granular electron dense material, suggestive of protein, was present in pinocytotic tubules and vacuoles in the apical cytoplasm of the cells.

The concept of absorption of proteins by the intracellular tubular system is not new. It was described in the epithelium of guinea pig yolk sac by Dempsey in 1953, in the neonatal jejunal cell of rats and mice by Clark (1959), in the absorption of ferritin by the intestinal cells of rats and rabbits (Graney, 1965; Kraehenbuhl *et al.*, 1967), in the proximal convoluted tubules of the kidney

(Maunsbach, 1966) and presumptively in the intestine of 1-day-old puppies (Anderson, 1965).

In neonatal pigs fed colostrum 3 to 6 hours earlier, Staley *et al.* (1968b) observed in duodenal villal epithelial cells protein-containing vacuoles, up to 10 μ in diameter and "almost entirely filling the cytoplasm". These authors demonstrated, however, that duodenal villal cells no longer contain visible intracytoplasmic colostral vacuoles 48 hours after exposure to colostrum, and that at this time epithelial cells cannot be distinguished from mature cells. The jejunal absorptive cell of the neonatal pig is characterized by a poorly developed hirsute layer over the microvilli, an abundance of pinocytotic vacuoles and apical tubules, and a subnuclear location of the Golgi apparatus. The apical tubules are lined with spinous processes and are retained only until the pig is first fed, or if unfed, for approximately 42 hours (Staley *et al.*, 1968b). The disappearance of the tubular system after feeding suggests that this is the system involved with the absorption of protein macromolecules in the newborn pig.

The decreased ability to absorb proteins was suggested by Payne and Marsh (1962) to be a sign of maturation of the villal epithelial cell, as does the development of normal enzyme (particularly alkaline phosphatase) content in the cell during its migration from the crypt and maturation en route along the shaft of the villus (Wilson, 1962).

Entry of Microorganisms to the Villal Mucosa

The neonatal intestinal epithelial barrier to intraluminal bacteria has not been defined, and the ease with which macromolecules

enter the neonatal villal epithelial cells indicates the possibility of a similar route of entry for microorganisms.

Dixon (1960) was the first to clearly demonstrate the ability of viable bacteria to pass intact through the apparently normal intestinal epithelium.

Using guinea pigs preconditioned by starvation and the administration of opium, Takeuchi and Sprinz (1967) gave *Salmonella typhimurium* organisms *per os* and observed subsequent changes in the mucosa of the ileum. The enteritis was characterized histologically by blunting, swelling, and foreshortening of villi; elongation of crypts and emptying of goblet cells; and increased and abnormal extrusion of epithelial cells, compensated in part by increased mitoses. Although the continuity of the epithelial lining was retained, the individual epithelial cells became flattened and their brush border attenuated concomitant with loss of cytoplasmic components. Inflammatory changes of the lamina propria were also reported by these authors (Takeuchi and Sprinz, 1967). Takeuchi and Sprinz (1967) described the process whereby *Salmonella typhimurium* adhere to the brush border, disrupt the microvilli, and enter the cytoplasm of the apical portion of the cell as membrane-enclosed bodies. Within 14 hours the number of bacilli within the villal epithelial cell had decreased, but many were seen in the lamina propria. By 48 hours after challenge, there were reduced numbers of bacteria in the lamina propria; in some instances degeneration of the phagocytized bacilli was noted; in others the organisms persisted in the lamina propria and even proliferated within macrophages. Associated with these bacterial migrations there

were a number of significant morphologic changes in the villal epithelial cells. At first, the brush border was reduced in height; later the microvilli of affected cells were completely denuded. The short tubules of the granular endoplasmic reticulum were transformed into long curvilinear patterns or concentric whorls with intermittent vacuolar dilatations, and there were fewer free ribosomes present. Coincident with the epithelial changes, progressive intravascular clotting of blood in the microvasculature of the lamina propria was observed, associated with fragmentation of cellular membranes, sloughing of endothelial cells and the extension of plasma fluid into the extravascular space (Takeuchi and Sprinz, 1967).

Staley *et al.* (1968c, 1968d) observed the migration of *E. coli* 055:B5:H7 organisms through the mucosa of the small intestine of the neonatal pig. It was seen that the organisms were aligned along the surface of a villal epithelial cell in close proximity to a goblet cell and became attached to the brush border in contact with the apices of the microvilli. In 20-hour-exposed ileum, all bacteria (nonfimbriated prior to the 20-hour interval) both in the lumen and attached to the villal epithelial cell, had a well developed fimbriated coating. The microvilli nearest the bacterium exfoliated to form a microerosion -- the authors termed it a "microulcer" -- which developed into an invagination of the plasma membrane containing the organism. The organism became fully enclosed by the "pinching off" of the invagination, to form an intracytoplasmic vacuole. The trilaminar structure of the vacuole wall was lost and appeared just as a single thickened membrane. The microvilli of the nearby cells were

shortened and swollen and appeared as though "budding". The organisms within vacuoles then could move throughout the cytoplasm down to the basement membrane, but were not seen within the basement membrane, the intercellular spaces or the lamina propria. The enclosed organisms apparently did not come into association with lysosomes, nor was there evidence of formation of secondary or compound lysosomes. There was no evidence of intracellular digestion of *E. coli* observed by these authors (Staley *et al.*, 1968c, 1968d).

Absorption and Malabsorption

Absorption implies movement of fluid substance across the apical plasmalemma through the cytoplasm (possibly involving passage through the compartmental boundaries of organelles) and out of the cell at the serosal pole, which can be defined as those boundaries of the cell not in contact with the intestinal lumen or adjacent cells. The substances absorbed by the intestine are water, hydrophilic substances in solution, and lipids in various forms.

Movement of molecules and ions through the villal epithelial cell (so-called "translocation") implies a functional asymmetry of the cell, with transport systems distributed asymmetrically between the two faces bordering the two fluid compartments between which transport is occurring. It has been shown by Newey (1967) and by Fisher (1967) that absorption of sugars and amino acids required active pumping systems in the limiting membranes of the villal epithelial cells, and that for translocation of these substances it was necessary that they be maintained in the mucosal cell cytoplasm at

a concentration greater than the extracellular environment so that they would leak out at the opposite face of the cell. Parsons (1967) recorded that with sodium, translocation required that the cytoplasmic sodium ion concentration be lower than that of the environment, so that sodium would leak into the cell at one face, then outward from the cell through a pump at the opposite face.

In normal circumstances, the duodenum and jejunum differ from the ileum in that the former will add or subtract sodium chloride from the chyme until isotonicity with blood is established, then net absorption of the isotonic solution occurs. In the ileum, net absorption of sodium chloride will occur, even from hypotonic solutions (Vischer *et al.*, 1944; McHardy and Parsons, 1957; Code, 1965; Parsons, 1967).

Parsons (1967) has summarized the proof of active transcellular transport of sodium and the evidence for membrane function in translocation of sodium, potassium, sugars and amino acids.

Absorption of Water and Salts

Intestinal epithelial cell membranes are basically lipoidal in nature but allow non-lipids to penetrate by two pathways: through aqueous channels, or pores, in the membranes; or for some electrolytes (and non-electrolytes such as glucose) transport via a membrane carriers. There are no carriers for water, so its absorption is exclusively through these aqueous channels (Fordtran, 1967). The forces controlling passive movement of ions and water through the membrane pores are: electrochemical gradients, osmotic pressure gradients and "solvent drag" (Fordtran, 1967).

It is of significance that, in man, pore size decreases with increasing distance from the ligament of Treitz. In the duodenum and anterior jejunum, active transport of non-electrolytes such as glucose and amino acids constitutes the major driving force for sodium absorption and, in turn, water flow with its particular tendency to further absorb sodium ions by solvent drag. Sodium transport involves the active processes of bicarbonate absorption or hydrogen ion secretion. An active transport of sodium per se has been proposed for the duodenum and jejunum, but the ease of diffusion of the sodium ions back through the relatively large pores into the intestinal lumen makes this a very inefficient process (Fordtran, 1967). In the ileum and colon, however, the opposite is true. The effective aqueous pore size is approximately 3A, and actively transported sodium molecules are effectively restrained. Passive movement and solvent drag are relatively unimportant in sodium ion absorption in the ileum. However, the rate and direction of sodium movement affects net anion movement. In the anterior jejunum, bicarbonate is absorbed in preference to chloride and against concentration gradients whereas, in the ileum and colon, chloride is absorbed against steep concentration gradients and is in part coupled to bicarbonate secretion. Fordtran (1967) maintains that this reciprocal anion movement is brought about by an exchange carrier in the membrane.

In the proximal jejunum, potassium is passively absorbed whereas, in the ileum and colon, potassium is secreted (Fordtran and Locklear, 1966).

The data of Watten *et al.* (1959) and of Cooke (1957) indicated that as diarrhea increased in severity there was a progressive rise in fecal sodium and a decrease in fecal potassium. They showed that the degree of sodium loss varied according to the cause of the diarrhea, whereas potassium loss relative to water loss seems more or less fixed regardless of the cause of the diarrhea.

In a number of diarrheal diseases of man, including laxative diarrhea and "infantile gastroenteritis", the sodium and potassium concentrations in feces, together, exceed the total chloride content (Evanson and Stanbury, 1965).

The type of food ingested is a major determinant of the direction and rate of water and electrolyte movement in the intestine. Those characteristics of chyme most important to absorption being osmolality, the rate of addition of osmotically active solutes (the products of intraluminal digestion) and the relative concentrations of absorbable and nonabsorbable solutes in the chyme (Fordtran, 1967).

In infantile diarrhea, fecal osmolality has been recorded to be half the osmalality of plasma (Teree *et al.*, 1965). The source of fecal osmolality is considered to be malabsorption of hydrolyzed dietary products and sodium and potassium ions (Fordtran, 1967). Fordtran explained this phenomenon as possibly being due to a decrease in size of the aqueous channels in the jejunal absorptive cells and a marked loss of jejunal surface area coupled with oral ingestion of hypotonic fluid.

An increased rate of movement of ingesta through the intestinal tract is an almost invariable consequence of enteritis. Under this

circumstance sodium is less able to be reabsorbed and, as a result, sodium loss exceeds potassium loss during the course of infantile diarrhea (Darrow, 1946). It has long been known that diarrhea, even of slight duration, will result in depletion of sodium that is by far the major extracellular base in the body (Butler *et al.*, 1933; Black, 1953). Relatively more of an infant's body weight is "highly labile" extracellular space. Sodium loss from this component leads to water loss, reduced extracellular fluid volume, disturbed renal function and eventual shift of water from an intracellular to an extracellular disposition (Darrow, 1945). In consequence, cellular dehydration involves loss of cell potassium to tissue fluids and serum (Elkinton *et al.*, 1948). Shock and cellular anoxia have been observed at 30 to 50% falls in levels of extracellular electrolyte (Darrow, 1946).

At a particular but undetermined cell potassium concentration, sodium and hydrogen ions emigrate from serum to cell according to an approximate but not quantitative inverse correlation in the osmotic activity of these cations (Elkinton *et al.*, 1948).

There occurs, further, a metabolic acidosis as serum sodium levels fall. Potassium moves from the cells to the serum as a consequence of this (Smith and Etteldorf, 1961). Serum potassium so derived is lost in renal excretion (Darrow, 1945). Pitts (1963) has summarized the sequence as follows: a loss of extracellular sodium leads to acidosis, a decrease in extracellular volume, then a decrease in cellular volume with loss of cellular potassium. Intracellular migration of sodium occurs and bicarbonate follows sodium into the

cell, producing an intracellular alkalosis and an extracellular acidosis. Falling serum bicarbonate levels stimulate renal mechanisms to increase serum chloride levels in an attempt to maintain electrical balance, but in addition this accentuates extracellular acidosis. The end result, of grave consequence to the patient, is depletion of cellular potassium.

It is obvious that abnormalities of the villal epithelial cells, and change in the intestinal absorptive surface area, will alter the rate of absorption of water, electrolytes and other products of digestion. The enteric diseases of man and animals now recognized to be malabsorptive in nature are celiac disease, idiopathic steatorrhea, nontropical sprue, tropical sprue (Wiseman, 1964), transmissible gastroenteritis of swine (Trapp *et al.*, 1966; Maronpot and Whitehair, 1967) and sprue-like syndrome in dogs (Vernon, 1962; Kaneko *et al.*, 1965). In these diseases the actual loss of cells and foreshortening and "clubbing" of the villi, in addition to the ultrastructural changes in the microvilli and the intracellular organelles, provide readily acceptable reasons for the malfunction of the absorptive mechanism. In colibacillosis, villal changes under optical microscopy are slight, so slight in fact that Moon *et al.* (1966) stated that "diarrhea occurs in *E. coli* infection without morphologic evidence of enteritis...." This opinion coincides with that of Stowens (1966), who reported that the intestines of babies dead from uncomplicated colibacillary diarrhea were without signs of inflammation. It is only in recent years that some authors (Kenworthy and Allen, 1966; Christie, 1967; Barnum *et al.*, 1967) have described changes in the villi or the lamina propria associated with *E. coli* infection.

Although there has been much evidence, particularly with ligated intestinal loop preparations, of the diarrhea of colibacillosis being associated with a massive outflow of fluid from the circulation to the intestinal lumen, it has been recently hypothesized that another possible major factor in the pathogenesis of colibacillary diarrhea has been malabsorption due to hydropic degeneration of the villal epithelial cells (Christie, 1967). Also Fordtran *et al.* (1967) reported that, although cell loss does significantly reduce the absorptive surface area of the jejunal mucosa in sprue, the permeability of the remaining mucosal cells is markedly decreased to levels lower than that seen in the normal ileum. The resulting malabsorption of water, sodium chloride, and other small, water soluble solutes contributes to the diarrhea associated with the abnormal osmotic pressure and ionic content of the chyme (Fordtran *et al.*, 1967).

Absorption of Carbohydrates, Proteins and Fats

In vitro intestinal techniques have made the intestinal epithelial cell available for detailed study of a number of systems such as amino acid and hexose transport, which systems also are found in kidney and other tissues.

It is apparent from the work of Auricchio *et al.* (1963) and Dahlqvist *et al.* (1963) that a major component of maldigestion and malabsorption is deficiency in the carbohydrases of the intestinal absorptive cell. Basically, the transport of carbohydrates from the lumen of the intestine through the brush border into the villal epithelial cell depends in part on the presence and function of the carbohydrases: alpha-dextrinase (isomaltase), maltase, lactase, and

sucrase (Gray, 1967). Gray (1967) presented evidence that complete digestion of disaccharides is accomplished by mucosal enzymes at the luminal surface.

Specific carbohydrases are located at the luminal portion of the columnar absorptive cell (Miller and Crane, 1961). Oligosaccharides -- alpha-dextrins, maltotriose, maltose, and others -- resulting from intraluminal digestion must be further hydrolyzed during physical contact with the intestinal mucosal surface, otherwise carbohydrate malabsorption will occur. Any mechanism preventing contact of the substrate with the brush border, or alternatively, disruption of the luminal border enzymes either spatially or chemically, will interfere with carbohydrate absorption.

Glucose comprises the bulk of monosaccharide that must be absorbed, and the villal epithelial cell appears to have a specific carrier mechanism with which glucose or galactose can combine, and by which monosaccharide can enter the otherwise impermeable lipoprotein membrane of the cell (Gray, 1967).

The specificity of intestinal absorption is such that the slight structural difference between glucose and mannose is sufficient to determine that the intestinal epithelium will absorb the former and reject the latter. Such specificity requires special mechanisms, and one hypothesis is that there are present at the cell membrane carrier sites to which particularly structured chemical groups can attach. The attachment of large hydrophilic substances to lipid carriers may partly explain the movement of these complex particles, such as vitamin B_{12}, into the cell. It is of interest to note that

the large vitamin B_{12} molecule cannot enter the epithelial cell until it is attached to its carrier molecule which is itself a larger molecule (Matthews, 1967).

There are well documented specific acquired, secondary and congenital carbohydrate malabsorption states associated with enzyme inadequacy, disaccharidase deficit, lactase deficiency and sucrase-isomaltase deficiency, and these have been tabulated by Gray (1967) in their associations with 14 of the described human malabsorption syndromes, including "infant diarrhea".

In infantile diarrhea, and its associated disaccharidase deficiency, sugar malabsorption leads to bacterial conversion of unabsorbed carbohydrates to organic acids which accumulate and cause metabolic acidosis (Weijers *et al.*, 1961; Torres-Pinedo *et al.*, 1966).

After "closure" mammals, including man, can absorb no more than traces of intact protein or peptide (Dent and Schilling, 1949). Proteolytic enzymes hydrolyze dietary protein to peptides and amino acids. Peptides are then bound to or taken up by the microvilli where, with peptidases, they are further hydrolyzed to amino acids (Holt and Miller, 1961). Amino acids are absorbed by the mucosal cells -- most of them by an active system -- and transported to the capillary blood (Wiseman, 1953).

Pancreatic lipase and bile salts interact with dietary triglycerides in the intestinal lumen, thus hydrolyzing them to fatty acids and monoglycerides. Bile salts are essential in the formation of micelles which serve to effectively solubilize fatty acids and monoglycerides and lead to a clear solution rather than an emulsion

(Hoffman and Borgstrom, 1962; Isselbacher, 1967). Fatty acids and monoglycerides in the micellar phase are then brought to the surface of the villal epithelial cell, attach thereto, and the micelle discharges its lipid contents such that they enter the cell in a molecular manner rather than in a micellar form (Isselbacher, 1967). This is a nonenergy-dependent process (Strauss, 1966). The monoglycerides and fatty acids are reesterified to triglycerides in reactions mediated by enzymes in clearly defined reactions (Senior, 1964) within the agranular reticulum of the apical cytoplasm of the villal epithelial cell (Porter, 1969).

All of the glyceride synthesizing enzymes are located in the microsomes, and more specifically, in the smooth endoplasmic reticulum, and it is significant that as soon as fat is seen beneath the terminal web it is almost always surrounded by membranes which appear to be components of the endoplasmic reticulum (Isselbacher, 1967). Lipid passes through the basal intercellular wall of the villal epithelial cells in the form of a chylomicron, a central triglyceride core bound by an outer coating of protein, cholesterol and phospholipid (Zilversmit, 1965).

Lipid droplets accumulate in the region of the Golgi zone prior to their exit from the cell (Dobbins, 1966) and in this area each droplet is enclosed by a membrane most representative of the rough endoplasmic reticulum. Isselbacher (1967) speculated that the "entire packing process" of the chylomicron takes place while the triglyceride is surrounded by endoplasmic reticulum -- "the smooth providing the triglyceride synthetase mechanism, the rough the protein synthetic machinery".

Histochemistry of the Intestinal Mucosa

As recently as 1966, Kohler and Bohl have stated,

> "the illumination of the mechanism by which diarrhea and subsequent dehydration are caused without concurrent enteritis will undoubtedly require further extensive study. In fact, histochemical studies may well be required to detect changes other than morphological changes in the affected cells" (Kohler and Bohl, 1966a).

The relationship between functional activity of the villal epithelial cell and its changing cytochemistry has not been studied to any significant extent. According to Pearse and Riecken (1967),

> "...the evidence presented is restricted to a number of cytochemical phenomena associated with or related to absorption in the normal or diseased mucosa, and the significance of these is often obscure".

Many cytochemical phenomena observed in studies of the small intestine still lack biochemical interpretation (Pearse and Riecken, 1967).

Much of the histochemistry of the intestinal tract has been concerned with the establishment of normal distribution patterns of enzymes -- the hydrolytic, oxidative and proteolytic enzymes -- and cell inclusions such as lipids, mucopolysaccharides, amyloid and others. The distribution of enzymic activities and reactive groups bound to membranes has enabled workers such as Pearse and Riecken (1967) to map, histochemically, the functional organellar pattern of villal epithelial cells. The accuracy of such methods compared with electron microscopy is at present being studied. The relationship of these results to organellar function is suggested by some histochemists, but not yet established (Pearse and Riecken, 1967).

There is no scientific literature, known to this author, concerning the enzyme histochemistry of the neonatal gnotobiotic pig.

Hydrolytic Enzymes

The Phosphatases.

The hydrolysis of phosphoric acid esters such as glycerol-1-phosphate to glycerol and inorganic phosphate is catalyzed by the phosphatases. They are in fact esterases, but this term is retained for those enzymes that hydrolyze esters of the carboxylic acids.

Alkaline Phosphatase.--Relatively few of the known enzymes at the present time can be conclusively demonstrated in animal tissues by histochemical means. Two of the alkaline phosphatase methods that are available are for phosphomonoesterase I, and for nonspecific alkaline phosphatase. Alkaline phosphatases hydrolyze most orthophosphomonoesters at an optimum pH of 9.0 to 9.6. Phosphate esters, namely glycerophosphate, glucose-1-phosphate, creatinine phosphate (and other phosphoamide linked esters), diphosphate esters such as hexose diphosphate, and the nucleotides (adenylic acid and adenosine triphosphate) are split by alkaline phosphatase activity (Gomori, 1949; Feigin and Wolfe, 1955; Friedenwald and Gryder, 1958).

It is possible that alkaline phosphatase is a number of closely related enzymes, all of which split phosphate at an alkaline pH (Emmel, 1950; Burgos *et al.*, 1954; Burgos *et al.*, 1955). The probable function of both acid and alkaline phosphatase is the dephosphorylation necessary for absorption, transport and control of metabolism, possibly in the synthesis of some esters, and the maintenance of levels of intracellular inorganic phosphate sufficient for osteogenesis (Morton, 1961).

Alkaline phosphatase has been described as being present in the villal epithelial cells; at the apical border in tissues from man (Shnitka, 1960; Padykula *et al.*, 1961), specifically localized in the brush border in cells from man (Pearse and Riecken, 1967), in the brush border and apical cytoplasm of 3- to 5-week-old conventional pigs (Maronpot and Whitehair, 1967) and at the apical border in 1- to 2-week-old conventional pigs (Thake, 1968).

Acid Phosphatase.--Phosphomonoesterase II and nonspecific acid phosphatase hydrolyze a variety of phosphoric esters, all being hydrolyzed at an optimum pH of 5.0. There are several enzymes that constitute this group (Barka, 1960; Barka, 1961a; 1961b; 1961c).

Acid phosphatase has been described as being present in the villal epithelial cells in a number of locations: in the lysosomes confined to a narrow zone beneath the terminal web in man (Fric and Lojda, 1964; Riecken *et al.*, 1966), as a loose scattering of granules in the apical cytoplasm in the rat and the mouse (Pearse and Riecken, 1967), continuous from the "villous apex to the crypt base except for goblet cells" of 3- to 5-week-old conventional pigs (Maronpot and Whitehair, 1967), and predominantly in the apical cytoplasm in 1- to 2-week-old conventional pigs (Thake, 1968).

Adenosine Triphosphatase.--At an alkaline pH, adenosine triphosphate is hydrolyzed to adenosine diphosphate and phosphoric acid in the presence of adenosine triphosphatase. This reaction is notable for the release of free energy with the breaking of the phosphate bond. More than one enzyme can break this bond, including

nonspecific alkaline phosphatase, but each active enzyme essentially requires calcium or magnesium ions for activation (Hunt, 1966).

Adenosine triphosphatase has been described as being present in these locations in the villal epithelial cells: in the brush border and mitochondria in man (Pearse and Riecken, 1967), from the base to the apex of the villus, and specifically in the brush border and throughout the cytoplasm, in 3- to 5-week-old conventional pigs (Maronpot and Whitehair, 1967), and uniformly throughout the cells in 1- to 2-week-old conventional pigs (Thake, 1968).

Proteolytic Enzymes.

The group of proteolytic enzymes is divided into 2 major subgroups, the proteases (proteinases and endopeptidases) and the peptidases (exopeptidases) which hydrolyze the -CO-NH- linkages of proteins and peptides. Peptidases generally do not attack proteins but act on peptides or peptide derivatives which contain free carboxyl or amino groups.

Leucine aminopeptidase.--This enzyme is a proteolytic enzyme found in high concentrations in the liver, pancreas, kidneys and small intestine (Ticktin and Trujillo, 1966). Sites of leucine aminopeptidase activity have been reported to occur in the columnar cells of the rat, human and monkey small intestine (Nachlas *et al.*, 1957; Nachlas *et al.*, 1960). It is specifically localized at the level of the microvilli in man and does not appear until after birth (Pearse and Riecken, 1967). Maronpot and Whitehair (1967) reported leucine aminopeptidase to be localized in the villal epithelium, specifically

in the brush border, with strong activity in the supranuclear cytoplasm of the villal epithelial cell. They did not detect enzyme activity in the crypts of Lieberkühn in 3- to 5-week-old conventional pigs.

Oxidative Enzymes

The Dehydrogenases.

The oxidative process involves either the addition of oxygen to a substance or the loss of hydrogen (and/or the loss of electrons) from that substance. The enzymes concerned with biological oxidation through hydrogen removal are termed dehydrogenases, the hydrogen generally being passed through the cytochrome system and combined with oxygen to form water.

Succinate dehydrogenase.--This enzyme catalyzes the transformation of succinic acid to fumaric acid in the tricarboxylic acid cycle (Krebs cycle) with oxidation of the succinate fraction and the transfer of the hydrogen ions to the electron transport substance flavine adenine dinucleotide. Nachlas *et al.* (1957) have reported high levels of succinic dehydrogenase activity in the epithelium of the duodenum, jejunum and ileum, moderate activity in Brunner's glands and the colonic epithelium, and slight activity in Peyer's patches. These reactions were observed in tissues from both rats and dogs. All of the positive reactions were confined to intracytoplasmic structures resembling mitochondria.

Pearse and Riecken (1967) found succinate dehydrogenase at three levels within the cytoplasm of the intestinal absorptive cells of man. These three zones corresponded to the distribution pattern of the bulk of the mitochondria, separated by the Golgi region and nucleus. Succinate dehydrogenase has been described as being present in these locations in the villal epithelial cells: in the subnuclear epithelial cytoplasm, both on the villi and in the crypts, of 3- to 5-week-old conventional pigs (Maronpot and Whitehair, 1967), and throughout the cytoplasm, but more concentrated toward the base, in 1- to 2-week-old conventional pigs (Thake, 1968).

Lactate dehydrogenase.--Lactate dehydrogenase is a glycolytic enzyme that catalyzes the reversible reaction of pyruvate to lactate in the presence of diphosphopyridine nucleotide. Although lactate dehydrogenase activity has been demonstrated in urine and other body fluids, the pathways of degradation and excretion are still obscure (Ticktin and Trujillo, 1966).

Lactate dehydrogenase is very widely distributed in a high proportion of the mitochondria of most tissues, including mucosal cells of the gastrointestinal tract of rats (Nachlas *et al.*, 1958; Hess *et al.*, 1958). There are no other reports of lactate dehydrogenase activity's being used as a measure of mitochondrial activity in the villal epithelial cells in health or disease.

Cytochemical Stains for Cell Vacuoles and Inclusions and the Brush Border

The literature relating to the demonstration of lipoidal inclusions and hydrophilic substances within cells and for specialized structures,

such as the brush border, is voluminous and beyond the scope of this review. Complete histologic and biochemical details are available from many sources. The most encyclopedic recent review is available in the text of Thompson and Hunt (1966).

The Pathology of Enteric Colibacillosis

In Human Infants and Calves

The difficulties inherent in obtaining suitable tissues from human patients with colibacillary diarrhea whose condition is uncomplicated by malnutrition, secondary infection, multiple microbial contamination or medical treatment is readily understandable. The literature of the pathology of enteric colibacillosis in man is inadequate as exemplified in the papers of McKay and Wahle (1955), Handforth and Sorger (1961) and Stowens (1966).

Stowens (1966), in describing the pathology of colibacillary diarrhea (epidemic diarrhea of the newborn) due to *E. coli* O111, stated "... not only is there complete absence of signs of inflammation either in the bowel or elsewhere, but there appears to be a depression of the lymph nodes." He defined lymph node depression as depopulation of lymphocytes with disappearance of follicles and germinal centers. In 2 of 3 unrelated epidemics, extensive virologic studies of these dead infants were pursued with negative results. Stowens considered lymph node depression to be significant in epidemic diarrhea of the newborn in that he has not observed this phenomenon "in any other disease in infants" (Stowens, 1966).

In a recent experiment, Osborne (1967) studied the lesions produced in neonatal calves following a composite inoculum of *E. coli* serotypes 078:K-:NM, 015:54:NM, 09:35:10, 026:60:11 and 0119:69:4. He suggested that the pathogenesis was primarily a disseminated intravascular coagulation with thromboembolism being widespread in the microvasculature of the lungs, liver, kidneys and the gastrointestinal tract. In his experimentally infected calves a severe gastroenteritis developed, and 18 of 33 calves died. Osborne has speculated that bacterial endotoxin moves across the intestinal membrane, primarily by passive diffusion, that the direct action of the *E. coli* endotoxin activates the clotting mechanism by the release of a platelet factor (Factor III), or that the endotoxin activates Hagemann factor (Factor XII) which acts as a trigger mechanism, in the presence of other clotting factors, for intravascular coagulation. Osborne also reported that *E. coli* endotoxin has been shown to effect directly selective vasoconstriction, and anticipating that this phenomenon would most likely favor thrombus formation where small vessels branch, considered that vasoconstriction at these sites was contributory to the thromboembolism that he observed in the pulmonary, renal and splanchnic microvasculature of experimental calves. His conclusion was that blockage of the microvasculature caused local tissue and vascular endothelial hypoxia, and in turn necrosis; or relaxation of vascular tissue cells, vasodilatation, endothelial cell separation, edema and hemorrhage. Such "overwhelming pathophysiological changes apparently stabilize in an irreversible state and the internal cellular environment becomes incompatible with life" (Osborne, 1967).

There are important similarities between Osborne's findings and those of McKay and Wahle (1955), who described the pathology of colibacillosis as observed in 9 infants who had died during an epidemic of infantile diarrhea associated with infection by *E. coli* O111:B4.

In Conventional Pigs

Barnum *et al.* (1967) stated, "The histological appearance of the intestinal tract of piglets with colibacillosis is usually normal", but they qualified the statement somewhat by claiming that there is occasionally a catarrhal enteritis and that after prolonged diarrhea there can be atrophy of intestinal villi. They described the villi as being short and blunt, the mucosa approaching that normally found in the colon, the normal eosinophilic columnar epithelium of these atrophic villi being replaced by a basophilic, cuboidal epithelium. Their description concluded with, "The atrophic change is similar to that described for 'sprue' in man and transmissible gastroenteritis (TGE) in piglets" (Barnum *et al.*, 1967). This similarity was also drawn by Maronpot and Whitehair (1967). Barnum *et al.* (1967) described similar atrophic changes of the villi of the small intestine in their report of lesions in weaned pigs with persistent coliform diarrhea. In addition they referred to hyperemia and sloughing of the mucosa of the gastric fundus associated with infarction by granular thrombi lodged in the mucosal veins and capillaries. Their description of weanling colibacillosis of swine included that of an acute catarrhal enteritis with an accumulation of epithelial and inflammatory cells mixed with masses of bacteria, fibrin and mucus

on the mucosal surface and accumulation of inflammatory cells in the lamina propria.

Kenworthy *et al.* (1967) have reported their observations of ultrastructural changes in jejunal, apical-zone villal epithelial cells of conventional pigs with postweaning diarrhea associated with *E. coli* 0147:K89(B),K88a,c(L):H19 and other unnamed serotypes. They described absence of microvilli or microvilli "enormously variable in length" and saw no evidence of rootlets or terminal web, or else "a disorganized zone of shadow immediately below the microvilli". In animals with mild diarrhea the terminal web remained organized, but the distance between the upper surface of the web and the cell membrane was increased. There was distortion of the free surface of the cell membrane and obliteration of microvilli, mitochondrial bodies were elongated and few in number and they described degeneration of the cell membranes whereas the desmosomal attachments remained *in situ*. There was loss of organelles from the cytoplasm reminiscent of the lesions of *Shigella spp.* infection in the guinea pig as described by Takeuchi *et al.* (1965).

In Gnotobiotic Pigs

A mild neutrophilic infiltration of the laminae propriae of the villi of gnotobiotic pigs in response to specific infection with *E. coli* 0138:K81:NM was reported by Christie (1967). At times aggregates of neutrophils in the lamina propria formed microabscesses that distended the shaft of the villus. This observation has since been confirmed in gnotobiotic pigs infected with *E. coli* 08:K.:H21 in that a

mild neutrophil infiltration of the villi of the small intestine was a consistent finding (Kohler, 1967). Both authors observed that the infiltration was very mild and was only apparent when compared with tissue sections from germfree control animals (Christie, 1967; Kohler, 1967).

It is apparent, as would be expected, that different serotypes of *E. coli* would produce different lesions and of different degrees of severity. Christie (1967) described a range of lesions extending from a mild generalized hydropic degeneration to an acute hemorrhago-necrotic enteritis. Kohler (1967) reported that enteritis was not detected in his experimental animals and described only mild neutrophil infiltration of the cecum and colon and the presence of small numbers of the organisms, usually in the capillaries.

The Pathogenesis of Enteric Colibacillosis

There have been no definitive studies of the pathogenesis of colibacillary enteritis in the human subject. It is only very recently that an attempt has been made to differentiate, in terms of pathology, the diarrheal diseases of man, and those studies of any significance are few.

Experimental colibacillosis in domestic and laboratory animals provides a significant basis for speculation, and confirmation of such speculation, on the pathogenesis of colibacillary enteritis and diarrheal disease associated with *E. coli* infections.

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 baby pigs 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 site 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 infectious 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 coli-septicemia 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 (Neter and Shumway, 1950; Ferguson, 1956).

Stevens (1963) has classified the syndrome of porcine colibacillary diarrhea into 3 groups, namely, "piglet enteritis", "enteritis of unweaned pigs -- three-week enteritis", and "post-weaning enteritis". The so-called "three-week enteritis" of Stevens' classification 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.

The most comprehensive of the recent reviews of the role of *E. coli* in disease (Sojka, 1965) does not include an adequate statement of the pathogenesis of colibacillosis, despite the author's statement that

> "...a theory of how enteritis (associated with *E. coli*) is produced in pigs has been recently advanced by Stevens (1963 a,b). It seems to be the most logical explanation of the sequence of events leading to development of various forms of enteritis and will be described in some detail."

The concluding statement common to most of this author's chapters on the various diarrheal syndromes of colibacillosis are generally listed as "Diarrhoea and/or death due to toxic effect of *E. coli*" (Sojka, 1965).

In a more succinct review, Barnum *et al.* (1967) have incorporated a number of hypotheses in their chapter, "Pathogenesis", and have attempted to establish alternative possible sequential processes in the development of diarrheal disease associated with *E. coli* infection.

Escherichia coli organisms normally enter neonates through skin wounds, localized infections such as omphalophlebitis or, more usually, by ingestion (Jubb and Kennedy, 1963). In neonatal pigs certain serotypes of *E. coli* can move directly along the intestine to their multiplication sites in the terminal small intestine (Christie, 1967) and in certain instances can enter the circulation and persist for a few hours in the bacteremic state in gnotobiotic pigs (Christie, 1967) and conventional pigs (Barnum *et al.*, 1967).

It has also been shown in calves (Fey *et al.*, 1962) and in gnotobiotic esophagectomized pigs (Christie, unpublished data) that *E. coli* can leave the alimentary tract (presumably at the tonsils, through epithelial defects in the oral cavity, through the intact oro-pharyngeal epithelium, or via the respiratory tract), enter the circulation and become established in the gastrointestinal tract.

Speculation, as reported by Jubb and Kennedy (1963) and Barnum *et al.* (1967), that intestinal infection may be preceded by, and result from bacteremia, was confirmed in one recent limited experiment with *E. coli* 0138:K81:NM in gnotobiotic pigs (Christie, 1967).

A recent published account of pathogenesis (Barnum *et al.*, 1967) emphasizes the bacteriologic aspects and factors concerned with the proliferation of the organism in the lumen of the intestine. These authors stated that, "The mechanisms by which certain strains of *E. coli* produce diarrhea when present in large numbers in the intestine are unknown". Barnum *et al.* (1967) stated that gastric acid destroys the coliforms in the stomach, and the acid that floods the upper small intestine partially inhibits the growth of *E. coli* in this area. The high susceptibility of neonates to colibacillosis may be, in part, due to low gastric acidity during their first one or two days of life. Intestinal secretions and motility move bacteria caudally, constantly cleaning the tract of organisms and their metabolic products. Interference with secretion or motility would theoretically allow for some build-up of potentially pathogenic populations of organisms. The presence of fimbriae on some serotypes of *E. coli* enables the organisms to adhere to the thin layer of seromucin on the brush border of the villal epithelial cells.

Strains of *E. coli* in man are known to be both resident and transient. The transient nature of the sojourn of *E. coli* strains in animals is well documented (Smith, 1960; Smith and Jones, 1963; Smith, 1965). The intestinal flora changes with age, change in diet, and interactions among intestinal bacteria and their products (such as colicines) (Barnum *et al.*, 1967).

Barnum *et al.* (1967) reviewed the concept that the absorption of endotoxins produced in the intestine is responsible for the physiological disturbances resulting in diarrhea. They speculated further that *E. coli* can produce toxins other than classical endotoxin and that these toxins might, as is the toxin recently isolated from *Vibrio cholerae*, be involved in the pathogenesis of diarrhea.

Independently, Christie (1967), from his observations of the lesions of colibacillosis in gnotobiotic neonatal pigs, postulated that some of the pathologic changes that he saw were less characteristic of endotoxic phenomena than they would be if exotoxin were present and suggested that colonies of *E. coli* 0138:K81:NM in the lumen of the pig intestine produced both endo- and exotoxin.

The sequence of events suggested by Barnum *et al.* (1967) in the clinical syndrome of diarrhea due to *E. coli* is as follows: diarrhea; dehydration; hemoconcentration, leading to increased blood viscosity and resistance to blood flow; electrolyte losses (namely water, sodium, bicarbonate, chloride and at times potassium) and consequent electrolyte imbalance. If untreated, severe uncompensated acidosis ensues, with detectable fall in blood pH, interference with cardiac impulse conduction, and death apparently due to cardiac failure. Terminal

uremia has been observed in some instances, probably due to increased utilization of body protein and decreased renal circulation as a result of cardiac insufficiency.

MATERIALS AND METHODS

"The difficulty with which enteric colibacillosis is produced under controlled experimental conditions remains the single greatest argument against a primary causative role for *E. coli* in the disease".

D. A. Barnum, P. J. Glantz and H. W. Moon, 1967

General Plan

In a study that involves following the progress of a specific infection, the development of a lesion or syndrome in body tissues or systems, and the collection of tissues from the test animals so that the tissue changes can be correlated with the development of the disease, 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 tissue 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 generally reliable picture of the development of the disease in 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 *et al.*, 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

The experimental animals consisted of 5 litters of gnotobiotic pigs, using some as control (uninfected) animals and some as test (infected) animals in an accepted experimental design. Between-litter

---

* Department of Pathology, Michigan State University.

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

variation was minimized so far as possible. The pigs used in this series of experiments were from related sows and were the progeny of one of two boars; 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, bacterial serotype, number of organisms, and age of pigs at exposure are given in Table 1.

Age of Experimental Animals

Clark (1959) had described in mice and rats an apical tubular system in the neonatal jejunal cell which transported undigested proteins into the cell. The ability of neonatal pigs to absorb unaltered, colostral protein during the first 36 hours of life (Brambell, 1958) was possibly a function of a similar apical tubular system. It was considered in this study that the neonatal germfree pigs should be at least 48 hours old before being exposed to the test organism. By this time the phenomenon of "closure" (Lecce, 1966) should have occurred and one complicating factor in the pathogenesis of colibacillosis, that of the direct absorption by the apical tubular system of endotoxin, exotoxin, or in the parlance of Barnum (1968) "enterotoxin", should be eliminated.

Two other factors were also considered. It takes approximately 24 hours for the experimental pigs in isolators to learn to properly drink out of the feed pans, and by 48 hours after birth, the pigs are drinking effectively and have an established feeding regime. It is

---

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

Table 1. Details of experimental animals

Exposed Pigs				Germfree Control Pigs		
Litter Number	Number and Sex of Test Pigs	Number of Organisms x 10^6	Age of Pigs at Exposure (days)	Number and Sex of Pigs	Identification Number	Age at Necropsy (days)
1	3 M 2 F	2.4	28	1 M 1 F	0661 0662	29 30
2	4 M 3 F	3.0	2-1/2	1 M 1 F 1 F 1 F	1005 1002 1008 1010	3 3 4 6
3	2 M 4 F	2.4	4	1 M 1 F	1309 1310	4-1/2 4-1/2
4	4 M 4 F	1.8	2	1 M 1 F 1 F 1 F	2253 2256 2259 2262	2 3 3 4
5	5 M 3 F	2.7	2	1 M 1 M	9046 9047	2 2-1/2

also more likely that the presence of material other than meconium in the gastrointestinal tract, prior to the introduction of the infective organisms, more closely approximates the situation that might obtain in natural infections of man or animals.

In all litters in this experiment, there was a time lapse of at least 48 hours from delivery until the infective dose of organisms was administered. In Litter 1 it was observed that the clinical response to the organisms was unsatisfactory in terms of the production of a diarrheal syndrome, and in subsequent experiments all litters were infected within 5 days of delivery.

Determination of Initial Sterility of Test Animals

Immediately prior to the introduction of the infective material into the pigs in 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, 4 and 5 the tubes were incubated only at 37 C and at room temperature.) These tubes were examined daily for 14 days. If there was no growth after 14 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.

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

Control Animals

Some pigs from each litter were 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, since within a total of 5 litters this would give more than the necessary number of tissue controls for the experiment (see Table 1).

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 litters constituting the whole experiment. Since between-litter variation was negligible in terms of diet and environment, and genetic variation was minimal, 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 Agent

The organism used in all experimental animals (in this series) was *E. coli* 0138:K81:NM and was isolated in August, 1959, from experimental pigs at Michigan State University. These organisms had been cultured, lyophilized in glass ampules, and stored at -20 C until required.

Seventeen hours before the pigs were due to be infected, an ampule of the culture was broken open, and its contents were inoculated into liquid brain-heart infusion medium* and streaked onto a human blood

---

* Bacto Brain Heart Infusion (B 37); Difco Laboratories, Detroit.

agar plate and a MacConkey Agar plate.* Only MacConkey Agar plates were inoculated prior to infecting litters 4 and 5. These culture plates were incubated at 37 C. After 16 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 compared with a series of McFarland nephelometer tubes, from which comparison was derived an estimate of the number of bacteria present. The tube culture was then aseptically diluted with sterile saline (0.85% NaCl) until the desired number of organisms was contained in an appropriate volume of inoculum (see Table 1). This saline suspension of the organisms was the material used in the isolators to inoculate the test animals.

The screw-capped tubes containing the inoculum were thoroughly sprayed with 2% peracetic acid (with approximately 0.1% wetting agent added).** The inoculum tubes (2 per isolator) were then introduced into the port of the sterile isolator, and the port was resprayed and closed. After 30 minutes the tubes were introduced into the isolator through the internal cap, and the inoculum was then ready for use. A standard dose volume of 1 ml. per pig was used throughout the experiment.

The inoculum was measured into a calibrated syringe, and a 1-ml. dose was squirted onto the back of the tongue of each pig in the isolator. After each pig had been inoculated, 2 fluid ounces of the

---

* Bacto MacConkey Agar (B 75); Difco Laboratories, Detroit.

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

standard milk formula diet were offered.

Infective Dose of Organisms

Almost invariably, research workers have used infective doses in excess of 1×10^7 *E. coli* organisms. Saunders *et al.* (1963b) used graded doses of organisms from 1×10^3 to 1×10^8 and established infections with numbers of organisms as low as 1×10^3. Otherwise, much larger numbers of organisms have been used; 5×10^8 (Lecce and Reep, 1962), 1×10^8 up to 1×10^{12} (Saunders *et al.*, 1963a), 1×10^7 (Kohler and Bohl, 1966), 4.5×10^{10} daily for up to 10 days (Osborne, 1967), 0.42×10^6 up to 135×10^6 viable organisms (Christie, 1967). In a recent study, Staley *et al.* (1968d) gave 1- to 2-hour-old pigs 100×10^9 *E. coli* 055:B5,H7 organisms. These organisms had been packed by centrifugation and resuspended in saline prior to being administered to the pigs through a stomach tube.

A number of theoretical objections to such large doses are immediately apparent, in that large numbers of organisms, submitted to extraordinary procedures such as centrifugation or prolonged incubation time, would lead to the introduction, together with the viable organisms, of dead organisms and preformed endotoxin and/or enterotoxin.

In this experiment the infective dose was made from high dilutions of a young (16-hour) culture of organisms, and the diluted culture was given directly, thereby minimizing the possibility of introducing organic material other than the viable organism itself. The highest infective dose used in this experiment was 3×10^6 organisms. It was hoped that this dose would more nearly approximate that ingested under natural conditions of exposure.

Necropsy and Laboratory Procedures

Immediately prior to necropsy, oral and rectal contents of pigs were sampled with swabs, which were then placed in sterile tubes and removed from the isolator with the particular animal to be killed.

These swabs were streaked onto MacConkey Agar plates and human blood agar plates and incubated at 37 C for 16 to 24 hours.

Immediately after the pigs were removed from the isolators, they were killed by cerebral concussion and exsanguination (by severing the axillary vessels). A terminal blood sample of approximately 10 ml. was taken at this time. The identification numbers and the time intervals between inoculation of test animals and necropsy are given in Table 2.

The body cavities were opened within a minute by excising the ventral thoracic and abdominal walls. The entire small intestine was freed from its attachments at the pylorus, the root of the mesentery and the ileocecal valve. The intestine was then quickly stretched out on a clean surface, and four 1-cm.-long sections were taken from each of five sites: the duodenum, jejunum (approximately 50 cm. from the pylorus), jejunum (approximately 1 m. from the pylorus), jejunum (approximately 2 m. from the pylorus), and the terminal ileum at the level at which it bears two diametrically opposed serosal reflections, one to the mesentery and the other to the cecum. Those sections of intestine intended for electron microscopic study were taken first and immediately opened along the mesenteric border. With fine scissors, groups of between 5 and 20 villi were snipped from the mucosal surface and immersed in cold (4 C) osmium tetroxide fixative (Palade, 1952).

Table 2. Times of exposure and necropsy of pigs

Interval Between Exposure and Necropsy (hours)	Pig No.	Age at Necropsy (days)	Interval Between Exposure and Necropsy (hours)	Pig No.	Age at Necropsy (days)
4	1303	4	20	2260	3
	1304	4		2261	3
				9044	3
8	1305	4-1/2	24	1000	3-1/2
	1306	4-1/2		1001	3-1/2
	9038	2-1/2		9045	3
10	9039	2-1/2	28	1003	3-1/2
				1004	3-1/2
12	1307	4-1/2	32	1006	4
	1308	4-1/2		1007	4
	9040	2-1/2			
14	2251	2-1/2	48	0657	30
	2252	2-1/2		0658	30
	9041	2-1/2			
16	2254	2-1/2	64	0659	31
	2255	2-1/2		0660	31
	9042	2-1/2			
18	2257	3	93	0663	32
	2258	3			
	9043	3	96	1009	6-1/2

These samples were the first to be taken from the five levels of the intestine so that all tissues for electron microscopy were in cold fixative within no more than 3 minutes of the concussion of the experimental animal.

Preservation and fixation procedures for all experimental tissues are tabulated according to litter number in Table 3.

Histopathologic Technique

Tissues from the five selected intestinal sites, taken for routine histologic examination, were sliced approximately 0.5 cm. thick, identified, fixed in 10% neutral formalin buffered with sodium acetate, and processed for examination by procedures described in the *Manual of Histologic and Special Staining Technics*, Second Edition, of the Armed Forces Institute of Pathology, Washington, D.C. (1960). Sections for the following stains: Movat's pentachrome (Thompson and Hunt, 1966), Alcian Blue (Pearse, 1961), Mayer's mucicarmine, Brown and Brenn stain for bacteria, hematoxylin and eosin, and the periodic acid-Schiff reaction (Armed Forces Institute of Pathology, 1960) were cut from Paraplast*-embedded tissues at a thickness of 6 μ.

Formalin-fixed tissues were frozen, 12 μ sections were cut on an Ames Labtech Cryostat, and the sections were stained with Oil Red O Fat stain (Armed Forces Institute of Pathology, 1960).

From the pigs in Litters 4 and 5, 1-cm. lengths from each of the previously selected sites of the small intestine were fixed in

---

* Paraplast Embedding Paraffin, Scientific Products, 1210 Leon Place, Evanston, Ill., U.S.A.

Table 3. Preservation and fixation of tissues

Methods	Litter Number 1	2	3	4	5
Carnoy's fluid fixative				+	+
Fresh, unfixed tissue frozen over dry ice and stored in sealed containers at approx. -70 C				+	+
Tissue frozen in a mixture of acetone and dry ice, then stored in sealed containers at approx. -70 C	+	+	+		
10% neutral formalin buffered with sodium acetate, and kept at approx. 4 C	+	+	+	+	
10% neutral formalin buffered with sodium acetate kept at room temp.	+	+	+	+	+
Osmium tetroxide fixative (Palade, 1952)	+	+	+	+	+

Carnoy's fluid and were subsequently stained by Best's method for the demonstration of glycogen (Mallory, 1942).

Histochemical Methods

All tissues for histochemical study, save for the Paraplast-embedded tissues, were cut at 12 μ, except where stated otherwise, on either an International Harris Cryostat (cold acetone fixed tissues), an Ames Labtech Cryostat (unfixed frozen tissues) or a Pearse-Slee Cryostat (unfixed frozen tissues and cold formalin fixed tissues) and mounted on clean glass slides or coverslips and stored at -20 C prior to histochemical staining in litter batches.

Adequate controls for each enzyme reaction were prepared by: (1) incubating duplicate tissue sections with and without substrate, (2) processing tissues from similar organ sites from germfree litter mates of the same age and kept under the same environmental and dietary conditions as the monocontaminated gnotobiotes, (3) processing all tissues of a particular litter and treatment at the same time, in one batch of incubating medium (except where specifically stated otherwise), (4) processing tissues known to be positive for the test material at the same time as the experimental tissues were being processed.

Alkaline phosphatase.

Pearse (1961) described the disadvantages of using unfixed frozen tissue in the histochemical visualization of alkaline phosphatase, and stated that during incubation the enzyme, together with other protein and nonprotein materials, is lost into the incubating

medium, leading to false localization of the enzyme by adsorption at other sites and deposition on the section of products of enzymic activity in the medium.

Mowry (1949) recommended fixation in cold acetone and storage at -70 C prior to processing for demonstration of alkaline phosphatase.

Pearse (1961) has suggested preservation of tissues in cold formalin and preparation of frozen sections as being "particularly suitable" for the demonstration of alkaline phosphatase. The particular advantages of this technique were claimed to be sharpness of localization of the enzyme and excellence of the morphologic picture (Pearse, 1961).

In view of these conflicting reports, tissues were preserved in acetone at -70 C, and duplicate specimens were stored in 10% neutral formalin buffered with sodium acetate at 4 C. In addition, fresh tissues from Litter 5 were frozen over dry ice and stored in individual screw-cap aluminum vials prior to processing.

Tissues from each of the four preservation methods were cut and mounted on clean glass slides. Alkaline phosphatase activity at pH 9.2 was determined, using the calcium-cobalt method for alkaline phosphatase according to Gomori (1952). Tissues were incubated at 37 C for approximately two hours. One batch of fresh frozen unfixed tissues (Litter 5) was incubated at room temperature for 30 minutes in the hope that there would be less nonspecific artifactual staining of sections.

Acid phosphatase.

Two methods for the demonstration of acid phosphatase were used. Unfixed frozen tissue sections were cut at 12 μ and mounted on clean glass coverslips. The enzyme was localized using the "Standard Coupling Azo Dye Technique" (Pearse, 1961). The acid phosphatase activity was determined at pH 5.0. Sodium alpha naphthyl phosphate in veronal acetate buffer served as the incubating medium; the diazonium salt employed was the stable diazotate of O-amino azotoluene. The sections were incubated for 60 minutes at room temperature.

In the second method, formalin-fixed, Paraplast-embedded tissues were cut at 6 μ and mounted on clean glass slides. Acid phosphatase was demonstrated according to the post-coupling technique devised by Rutenberg and Seligman (1955). Acid phosphatase activity was determined at pH 5.2. Sodium 6 benzoyl-2- naphthyl phosphate in veronal acetate buffer served as the incubating medium, and the released insoluble naphthol was coupled to diazo blue B in phosphate buffer at pH 7.6. Tissue sections were incubated for 4 hours at room temperature.

Adenosine triphosphatase.

Tissues to be tested for the presence and distribution of adenosine triphosphatase were immediately frozen at -70 C and maintained at that temperature until processed to completion. The calcium method for adenosine triphosphatase of Padykula and Herman (1955a,b) was employed.

Tissue sections were cut at 12 μ and mounted on clean glass coverslips. A sodium barbiturate buffer at pH 9.4 was used, and the

tissue sections were incubated in the substrate solution for three hours at room temperature.

Leucine aminopeptidase.

The method used for demonstration of leucine aminopeptidase activity was that devised by Nachlas, Crawford and Seligman (1957). Tissues were incubated for three hours at room temperature in L-leucyl-4-methoxy-beta-naphthylamide solution containing sodium chloride, potassium cyanide and fast blue B salt. Acetate buffer at pH 6.5 was used.

Succinate dehydrogenase.

To localize succinate dehydrogenase, 12 μ sections, cut from unfixed frozen tissues, were incubated for 30 minutes at 37 C in Tris-buffered sodium succinate with 3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) according to the method of Pearse (1961).

Lactate dehydrogenase.

For the intramitochondrial demonstration of lactate dehydrogenase it is necessary that cold formalin-fixed tissues be used. When fresh, unfixed frozen sections are incubated with nitro-blue tetrazolium, a crystalline artifact outside the mitochondria is produced (Novikoff and Masek, 1958). Pearse (1961) condemned the use of formalin fixation because some of the enzyme activity is destroyed by the fixative. Because of these conflicting opinions, both unfixed frozen sections and formalin-fixed tissues were cut and processed for lactate dehydrogenase demonstration.

Lactate dehydrogenase activity was determined according to the method of Hess *et al.* (1958), using sodium DL-lactate as the substrate with diphosphopyridine nucleotide, sodium cyanide adjusted to pH 7.2, and MTT added.

Electron Microscopy Methods

After initial fixation of villi for 30 minutes to 2 hours in cold osmium tetroxide fixative (Palade, 1952) the tissue samples were immersed in fresh osmium tetroxide fixative at 4 C for 6 hours, then washed in 4 changes of Palade's sodium barbital buffer solution at pH 7.4 (Palade, 1952). The tissues were then dehydrated in a graded series of cold ethanol-water mixtures and embedded in Beem* capsules in Epon 812* according to the method of Luft (1961). The ratio of A:B mixture of the Luft (1961) resins was 5:5. The tissue capsules were cured at 37 C for 16 hours and at 58 C for 24 hours.

Sections were cut with glass and diamond DuPont knives on a Sorvall Porter-Blum MT-2 Ultramicrotome. The Epon blocks were trimmed so that cells at the base of the villi were first to be sectioned, then as these tissues were examined, the blocks were retrimmed so that sections of cells at the midvillus were taken.

Sections were cut at a thickness between 700 and 900 A. Naked 400-mesh and Parlodion*-carboned grids of 100- and 200-mesh were used. Sections were stained with Reynolds' lead citrate (Reynolds, 1963), uranyl acetate (Pease, 1964), or a combination of both (Pease, 1964). Grids were observed on a Philips EM-100 B electron microscope and

---

* Ladd Research Industries, Inc., Burlington, Vermont, U.S.A.

photographed on 35mm. Kodak Fine Grain film.

Reclamation of the Test Serotype

After the blood agar and MacConkey Agar plate cultures of the swabs removed from the isolators had been examined and read, a single sterile loop was used to sample a typical colony from each of the cultures grown from samples from a particular isolator.

This loop of organisms, selected from different plates but all from the one isolator, was inoculated onto a nutrient agar slant, incubated for 24 hours at 37 C, sealed with a foam plug, and stored at room temperature until all samples were ready to be serotyped. In this way it was possible not only to check the validity of the monocontamination but also to ensure 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.

At the conclusion of all experiments, cultures harvested from each of the nutrient agar slants were serotyped according to the methods for O and K serotyping recorded by Sojka (1965).

RESULTS

"The fact that diarrhea occurs in *E. coli* infection without morphologic evidence of enteritis should be considered in any attempt to explain the pathogenesis of the diarrhea".

H. W. Moon, D. K. Sorenson, J. H. Sautter and J. M. Higbee, 1966

The Experimental Plan

In this experiment animals from 5 litters of germfree pigs were orally contaminated with an approximately equal number of *E. coli* 0138:K81:NM organisms and maintained under gnotobiotic conditions for a variable number of hours until necropsy. Clinical observations were made at this time. In this way it was hoped to simulate natural infection of neonatal pigs and to study the pathogenesis of the associated disease in colostrum-deprived animals.

Clinical and Gross Pathologic Findings

The gross pathologic findings of infection of neonatal gnotobiotic pigs with *E. coli* 0138:K81:NM (Mich.) have been reported in detail elsewhere (Christie, 1967).

In this experiment the observations of gross lesions were confined to the intestinal tract in an attempt to relate the observed clinical manifestation of diarrhea with the gross appearance of the intestinal tract at necropsy.

The first gross lesion of disease was seen in Pig 1305 when it was killed 8 hours after inoculation. The mesentery of the entire length of the small intestine at the point of attachment to the intestine was markedly edematous. The intestinal tube was relatively flaccid and moderately filled with homogeneous, very fluid contents.

The clinical appearance of the pigs monocontaminated with *E. coli* 0138:K81:NM in this experiment is tabulated (see Table 4).

The first clinical sign was seen in Pig 1306 when it was killed 8 hours after inoculation. This pig did not have apparent diarrhea, but the rectal contents evacuated by the animal after concussion were a mixture of thin watery fluid, mucus and suspended curds. At necropsy the intestine was flaccid and distended with fluid containing suspended milk curd and mucus. Hyperemia of the intestine was not grossly apparent.

One pig (1307) died during the course of the experiment 2 hours before it was due to be killed. At necropsy there was a flaccid, fluid-filled small intestine, hyperemia of the entire gastrointestinal tract, particularly of the gastric mucosa, and a marked ascites.

Twelve hours after inoculation the fluid ingesta persisted throughout the intestinal tract and was sometimes the only gross abnormality that was seen at necropsy. From 12 hours after inoculation, most inoculated pigs scoured profusely. At first, the perineum and hindquarters were pasted with the soft yellow feces characteristic of postmeconial feces of germfree neonatal pigs. As the diarrhea continued, the feces washed the perineum leaving it relatively clean, until in animals that had scoured for many hours (Pig

Table 4. Clinical appearance of pigs monocontaminated with *E. coli* 0138:K81:NM

Pig Number	1303	1304	1305	1306	9038	9039	1307	1308	9040	2251	2252	9041	2254	2255	9042	2257	2258
Age at necropsy (days)	4	4	4.5	4.5	2.5	2.5	4.5	4.5	2.5	3	3	3	3	3	3	3	3
Hours after inoculation	4	4	8	8	8	10	12	12	12	14	14	14	16	16	16	18	18
No clinical signs	+	+	+		+	+						+					
Mixed watery fluid and mucous curds in feces				+					+				+				
Profuse watery diarrhea								+		+	+			+		+	+
Raised hair coat									+						+	+	+
Projectile watery diarrhea; arching of back													+		+		
Dehydration																	
Death							+										

Table 4--continued

Pig Number	9043	2260	2261	9044	1000	1001	9045	1003	1004	1006	1007	0657	0658	0659	0660	0663	1009
Age at necropsy (days)	3	3	3	3	3.5	3.5	3	3.5	3.5	4	4	30	30	31	31	32	6.5
Hours after inoculation	18	20	20	20	24	24	24	28	28	32	32	48	48	64	64	93	96
No clinical signs												+			+	+	
Mixed watery fluid and mucous curds in feces													+	+			
Profuse watery diarrhea					+	+		+	+	+	+						+
Raised hair coat	+	+	+	+			+		+	+	+						
Projectile watery diarrhea; arching of back	+	+	+	+			+										
Dehydration	+			+			+										+
Death																	

+ = clinical state immediately prior to necropsy

1009) "scalding" of the perineum (erythema venenatum) was apparent.

Diarrhea was observed in every pig exposed to 3 x 10^6 *E. coli* 0138:K81:NM at least 16 hours beforehand.

The fecal material from those pigs 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 among pigs, and the suspended curds were less commonly seen, even though the pigs continued to drink well throughout the experiment. Occasionally mucus was observed in the intestinal contents. However, there was no gross evidence that excess mucus production contributed to the diarrhea in any way.

Anorexia was not observed in any of these experimental pigs, but in few was the disease permitted to run its course. However, those animals left for a relatively long time before euthanasia did not lose appetite at any stage.

Loss of "bloom" in the hair coat, erection of the hair coat, and degrees of arching of the back and straining in an apparent attempt to defecate were observed variously from 16 through 24 hours after inoculation of the gnotobiotes with the organism. The first signs of dehydration were seen in pigs 20 hours after inoculation.

In a previous experiment (Christie, 1967) significant temperature changes were not observed in neonatal pigs infected with this serotype, except in terminal stages of the infection when the body temperature fell. Rectal temperatures were not recorded in either control or infected pigs in this experiment.

At necropsy of those animals killed 14 hours or longer after inoculation, the small intestine was seen to be flaccid and distended with fluid, with or without suspended matter such as milk curd, mucus and gas. Hyperemia of the tract was generally not grossly apparent.

Throughout the experiment the control animals remained free of demonstrable bacteria, continued to pass soft or typically pasty feces, and maintained apparent good health until the time of euthanasia.

Recovery and Serotyping of the Organism

Fourteen cultures were grown on nutrient agar slants. Each individual culture represented a composite of organisms harvested from the positive cultures of the experimental pigs of a particular litter that were housed in the same plastic isolator. The cultures harvested from the slants were serotyped according to the methods recorded by Sojka (1965) and all cultures were found to belong to the *E. coli* O group 138 and had the K 81 antigen.

Histopathologic Findings

Histopathologic observations in this study were confined to the intestinal tract. The lesions observed ranged from those of a mild acute enteritis (Figures 1, 2 and 3) to a histologic picture, in persistently scouring pigs, microscopically indistinguishable from that seen in clinically normal germfree control animals.

The Small Intestine

Sections were routinely made from duodenum, jejunum at approximately the 50-cm., 100-cm., and 200-cm. levels, and the ileum close

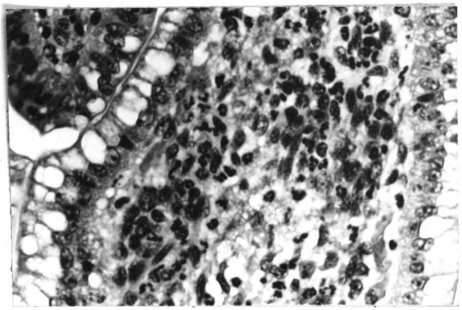


Figure 1. Pig 2258. Age 3 days. Terminal ileum. *E. coli* was introduced *per os* 18 hours before necropsy. Mild acute enteritis. Neutrophils are invading the lamina propria. Hematoxylin and eosin. x 750.

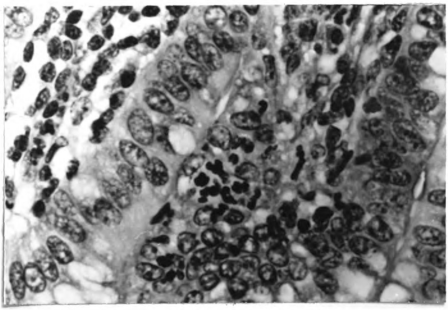


Figure 2. Pig 2257. Age 3 days. Duodenum. *E. coli* was introduced *per os* 18 hours before necropsy. Neutrophils are present in the tissue spaces and have formed a microabscess in the lamina propria. Hematoxylin and eosin. x 750.

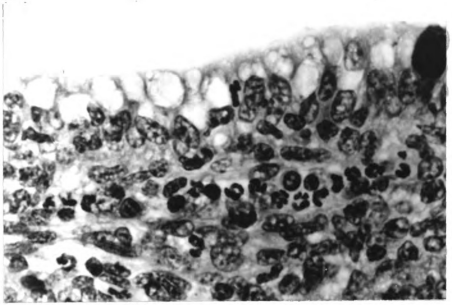


Figure 3. Pig 2257. Age 3 days. Duodenum. *E. coli* was introduced *per os* 18 hours before necropsy. There is marked vacuolization of epithelial cells with cell breakdown and mucosal disarray. Neutrophils abound in a vessel within the lamina propria, and are free in the interstitium. Movat's pentachrome. x 750.

to the ileocecal valve. It is apparent that marked variations from the normal can occur as a result of infection with this serotype 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 an intestinal tract colonized with *E. coli* 0138:K81:NM.

The mucosa and lamina propria.

In some infected pigs and some germfree control animals the surface epithelium was ordered and regular with little or no vacuolization (Figures 4 and 5) and with occasional active goblet cells filled with mucin and becoming more frequent in more caudal sites (Figures 6, 7 and 8). According to the level of the intestine from which the section was taken, lymphocytes were present singly or in aggregates in the submucosa and in increasing numbers caudad (Figures 6 and 9).

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 centrifugally placed in relation to the cell cytoplasm and the axis of the villus (Figure 10); in other sections the epithelial cell nuclei were uniformly axially placed, close to the basement membrane of the epithelium (Figure 11). The cytoplasm of these cells was negative to Mayer's mucicarmine stain.

A more advanced degenerative change frequently observed was that in both control and infected animals columnar epithelial cells of the villi were markedly distended or vacuolate either singly or *en masse* (Figures 12 and 13).

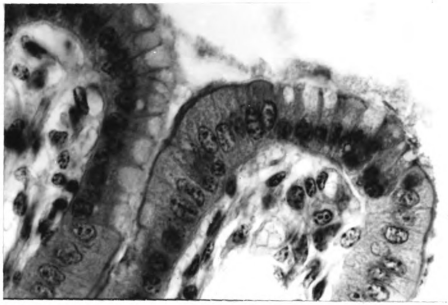


Figure 4. Pig 9047. Age 2-1/2 days. Duodenum. Germfree control. The duodenal epithelium is ordered and regular with little vacuolization. Movat's pentachrome. x 750.

Figure 5. Pig 2253. Age 2 days. Terminal ileum. Germfree control. The epithelium is ordered and regular, with moderate vacuolization in some areas. Intercellular fluid is indicative of active fluid absorption. Moderate subepithelial edema of the basal lamina is present. The lacteal canal is distended. Hematoxylin and eosin. x 470.

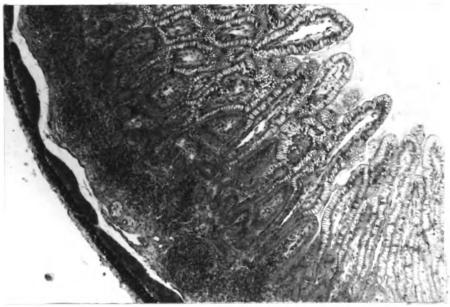


Figure 6. Pig 9046. Age 2 days. Terminal jejunum. Germfree control. The epithelium is ordered with consistent moderate vacuolization characteristic of this region of the intestine. Goblet cells are more frequent here than in more anterior sites. Aggregates of lymphocytes are present in the submucosa. Movat's pentachrome. x 75.

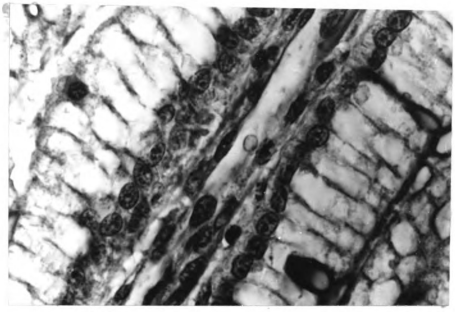


Figure 7. Pig 9047. Age 2-1/2 days. Terminal ileum. Germfree control. The surface epithelium is markedly vacuolate, with interspersed goblet cells filled with mucin granules. This degree of vacuolization is not unusual in this region of the intestine of neonatal germfree pigs. Movat's pentachrome. x 750.

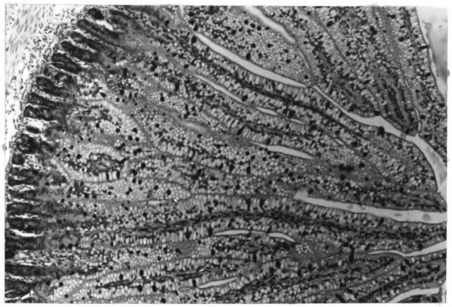


Figure 8. Pig 1006. Age 4 days. Terminal ileum. *E. coli* was introduced *per os* 32 hours before necropsy. Villi are long and slender. Breucke fibers are not contracted. Although the epithelium is regular it is consistently moderately vacuolate. Active goblet cells filled with mucin are plentiful. The villi tend to be compressed together. Hematoxylin and eosin. x 75.

Figure 9. Pig 9047. Age 2-1/2 days. Terminal ileum. Germfree control. The epithelial cells are markedly vacuolate; goblet cells are frequent. Aggregates of lymphocytes are present in the submucosa. Movat's pentachrome. x 75.

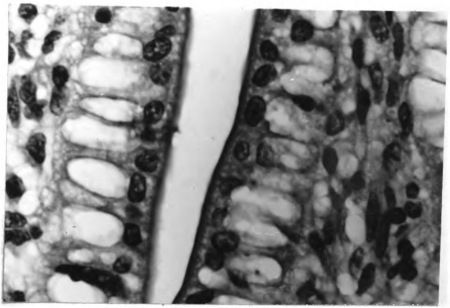


Figure 10. Pig 1010. Age 6 days. Terminal ileum. Germfree control. Villal epithelial cells are moderately vacuolate with centrifugally placed nuclei. There is no evidence of rupture of individual cells. Hematoxylin and eosin. x 750.

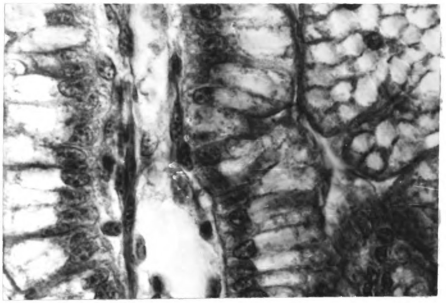


Figure 11. Pig 9046. Age 2 days. Terminal ileum. Germfree control. These markedly vacuolate cells represent the maximum distention seen in the epithelium of the ileum of germfree neonatal pigs. Epithelial nuclei are situated axially to the villus at the cell base. Movat's pentachrome. x 750.

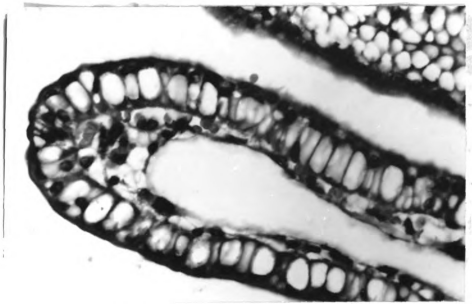


Figure 12. Pig 1005. Age 3 days. Midjejunum. Germfree control. Marked vacuolization and distention of the epithelial cells. Mild subepithelial edema, with moderately distended lacteal vessel. Hematoxylin and eosin. x 470.

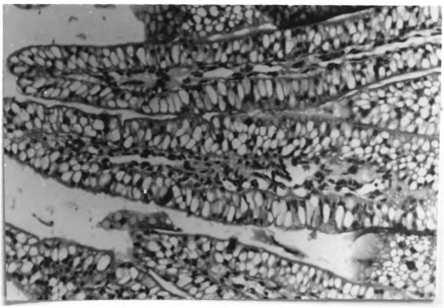


Figure 13. Pig 1306. Age 4-1/2 days. Terminal jejunum. *E. coli* was introduced *per os* 8 hours before necropsy. There is marked vacuolization and distention of the villal epithelial cells. Hematoxylin and eosin. x 190.

Varying degrees of subepithelial edema of the villi were seen to be associated with 1 or more of the epithelial changes described above (Figures 5, 12 and 14). Commonly observed was edema of the tips of the villi, forming subepithelial bullae (Figures 5, 12 and 14). 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.

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 vacuolization bounded by fibrous stroma (Figures 15 and 16). At times, edema of the axial tissues of the lamina propria of the villus was seen.

Other changes observed in the intestinal villi of infected but not control pigs were hyperemia with or without hemorrhage and/or necrosis of individual cells (Figures 17 and 18). In rare instances there was surface necrosis with sloughing of individual cells, and where sufficient cells had been lost from the tip of a villus there was an apparent replacement of villal epithelial cells with apparently normal juvenile cells.

A further condition 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

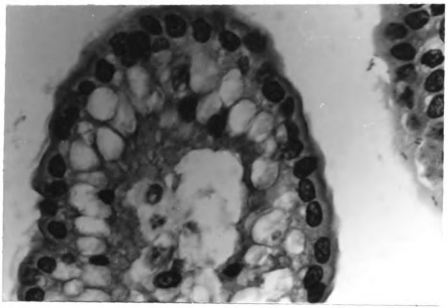


Figure 14. Pig 1004. Age 3-1/2 days. Anterior jejunum. *E. coli* was introduced *per os* 28 hours before necropsy. Mild subepithelial edema with distention of the vessels of the lamina propria. Hematoxylin and eosin. x 470.

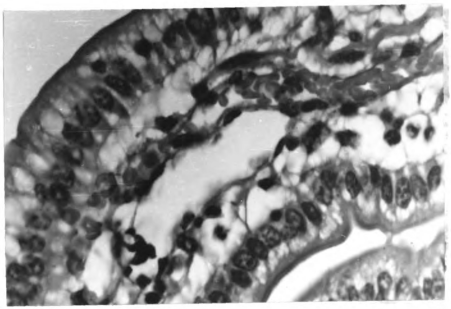


Figure 15. Pig 0657. Age 30 days. Anterior jejunum. *E. coli* was introduced *per os* 48 hours before necropsy. Subepithelial vacuolization bounded by fibrous stroma, constituting subepithelial edema and edema of the intramembranous space of the basement membrane. Hematoxylin and eosin. x 750.

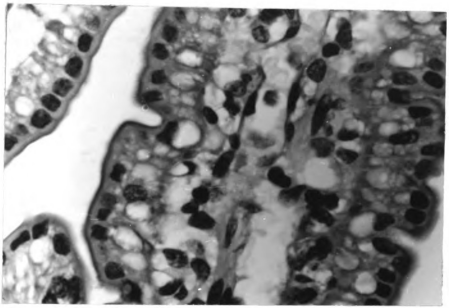


Figure 16. Pig 1306. Age 4-1/2 days. Anterior jejunum. *E. coli* was introduced *per os* 8 hours before necropsy. Subepithelial vacuolization bounded by fibrous stroma, indicating edema of the basal lamina and the lamina propria. Hematoxylin and eosin. x 750.

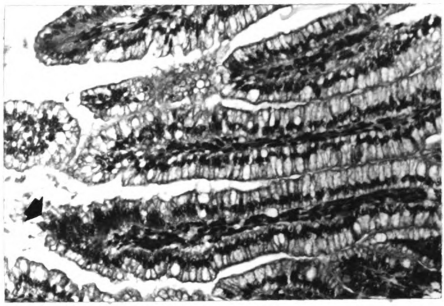


Figure 17. Pig 2255. Age 2-1/2 days. Terminal ileum. *E. coli* was introduced *per os* 16 hours before necropsy. Some epithelial cells are distended to the point of rupture, others have ruptured and become confluent with neighboring cells. Effete cells at the tip of the villus are sloughing (arrow). There is moderate hyperemia of the lamina propria. Hematoxylin and eosin. x 190.

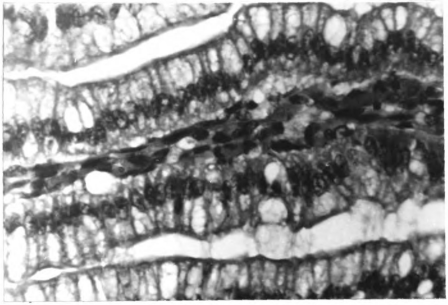


Figure 18. Pig 2255. From the same field as Figure 17. Epithelial cell distention to the point of confluence with neighboring cells, or rupture with loss of cytoplasm into the lumen. There is moderate hyperemia. Hematoxylin and eosin. x 470.

were in very close apposition and appeared adherent to each other, the microvilli forming a dense eosinophilic boundary common to both villi (Figures 8, 19, 20 and 21). The gross appearance in this case was of a thicker-walled intestine that was firm to the touch compared to the flaccid intestine that was commonly seen in colibacillosis.

At times in the infected intestine the laminae propriae of some of the villi were infiltrated with inflammatory cells, primarily neutrophils (Figures 1 and 2), 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 (Figure 21), eosinophils, plasma cells and occasional macrophages.

In some instances, the villi were seen to be very long and slender. There was almost uniform, advanced vacuolization of the epithelium of the villi in these sections, there was little or no subepithelial vacuolization (Figure 22), and Breucke fibers were generally not contracted. Frequently the laminae propriae of these villi were infiltrated with foci of neutrophils.

The villal epithelial cell of the small intestine.

The outstanding feature of villal epithelial cells of the small intestine of both neonatal germfree pigs and neonatal pigs monocontamined with *E. coli* 0138:K81:NM was the degree of vacuolization of the cytoplasm of cells clothing the midshaft and luminal end of the intestinal villus. Although this phenomenon is almost always present, and is in fact used routinely by histologists as one of the signs

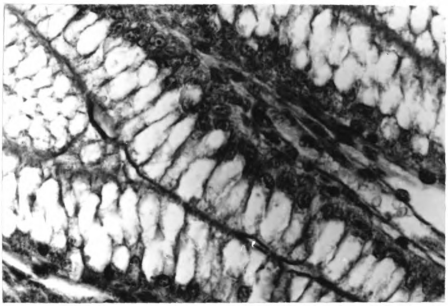


Figure 19. Pig 9043. Age 3 days. Terminal jejunum. *E. coli* was introduced *per os* 18 hours before necropsy. Marked vacuolization and rupture of intercellular walls with confluence of mucosal cells, and distention of the villi to the point of apposition of brush borders. Breucke fibers are not active. Movat's pentachrome. x 470.

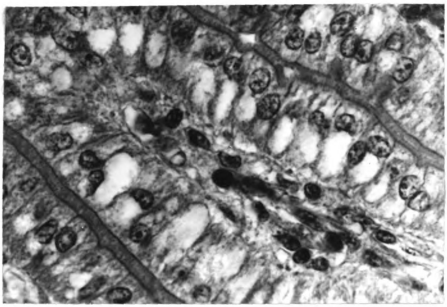


Figure 20. Pig 9043. Age 3 days. Midjejunum. *E. coli* was introduced *per os* 18 hours before necropsy. Epithelial vacuolization with villal distention and apposition of brush borders. Movat's pentachrome. x 750.

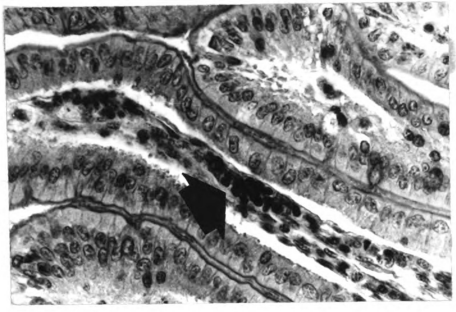


Figure 21. Pig 9038. Age 2-1/2 days. Duodenum. *E. coli* was introduced *per os* 8 hours before necropsy. Distention at the tip of the villus is due to collection of absorbed material or inflammatory edema. The clear space midshaft is probably artifact. There is apposition of the brush borders with those of neighboring villi. The lamina propria is infiltrated with lymphocytes (arrow). Movat's pentachrome. x 470.

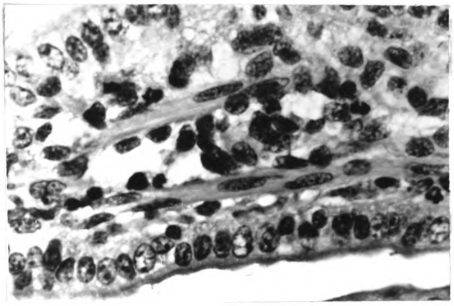


Figure 22. Pig 0657. Age 30 days. Terminal jejunum. *E. coli* was introduced *per os* 48 hours before necropsy. Edema of the interstitium of the lamina propria. Some neutrophils are present. Breucke fibers are contracted in this villal shaft. Movat's pentachrome. x 750.

for identification of the site of origin of the tissue, rarely is even the slightest reference made to this feature in the literature of the pathology of colibacillosis.

In germfree neonatal pigs it can be assumed that the villal epithelial cells from the more caudal sites in the small intestine will be more often vacuolate.

In these germfree neonatal pigs, rarely were duodenal epithelial cells vacuolate to any marked degree; more often they were regularly columnar with evenly stained cytoplasm, centrally placed nuclei, detectable brush border, and well defined basement membrane (Figure 23). In those instances in which active absorption was taking place immediately prior to the collection and preservation of tissues, the accumulation in the intercellular spaces of fluids en route from the epithelial cells to the lacteal and capillaries was readily apparent (Figures 5 and 24). This phenomenon was also observed in infected animals in which cellular disruption had not yet occurred (Figure 25).

In the germfree neonatal pigs the degree of vacuolization of the villal epithelial cells became more marked with distance along the intestinal tract (Figures 7 and 26). Although in some instances the vacuolization of individual cells was considerable in villal mucosa from germfree control pigs it was not associated with marked disruption of the mucosa (Figures 7, 10 and 11), whereas in villal mucosa from infected pigs, vacuolization of the same order of magnitude seemed to be associated with a degree of individual cell breakdown and mucosal disarray (Figures 3, 19 and 27).

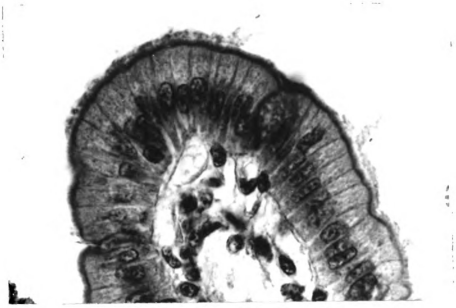


Figure 23. Pig 9046. Age 2 days. Duodenum. Germfree control. Regularly columnar villal epithelial cells with evenly stained cytoplasm, centrally placed nuclei, detectable brush border, and well defined basal lamina. Active absorption is indicated by the small clear cytoplasmic spaces, and by the fluid present in the intercellular space. Movat's pentachrome. x 750.

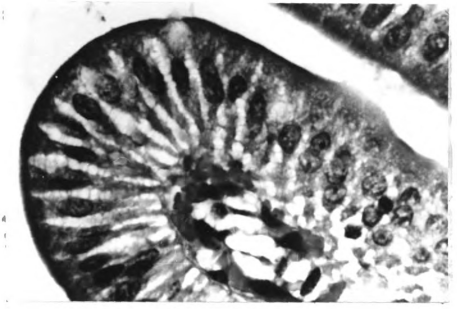


Figure 24. Pig 2262. Age 4 days. Duodenum. Germfree control. Active fluid absorption is indicated by intercellular collection of fluid. Lack of edema or accumulation of fluid in the lamina propria is a sign of normal function. Hematoxylin and eosin. x 750.

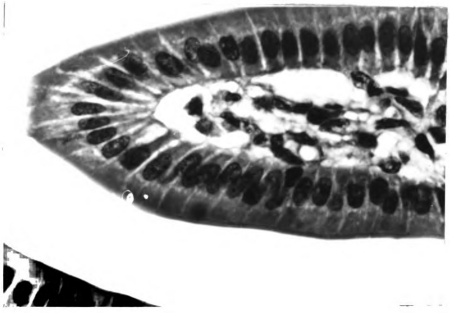


Figure 25. Pig 1305. Age 4-1/2 days. Duodenum. *E. coli* was introduced *per os* 8 hours before necropsy. As in Figure 24, there is evidence of epithelial cell function and active absorption. There is some accumulation of fluid in the lamina propria, possibly an early sign of developing infection. Hematoxylin and eosin. x 750.

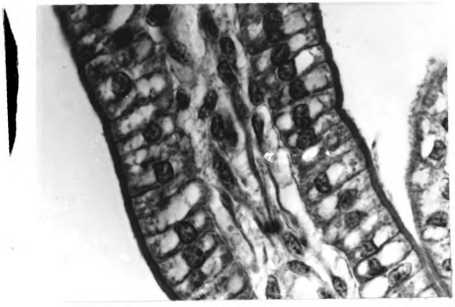


Figure 26. Pig 9047. Age 2-1/2 days. Midjejunum. Germfree control. Moderate vacuolization of villal epithelial cells typical of this region of the intestine of germfree neonatal pigs. Movat's pentachrome. x 750.

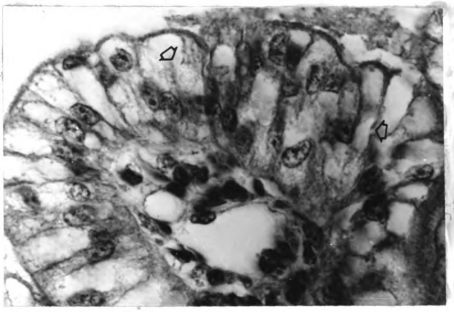


Figure 27. Pig 9043. Age 3 days. Terminal ileum. *E. coli* was introduced *per os* 18 hours before necropsy. Vacuolization of epithelial cells, although of the same magnitude as that seen in germfree neonates, is here seen to be associated with cell breakdown (arrow) and mucosal disarray. Movat's pentachrome. x 750.

Generally in the pigs monocontaminated with *E. coli* 0138:K81:NM vacuolization was more pronounced and extensive. The effect of the organism on the mucosa of the more cranially situated portions of the small intestine was less apparent. In many instances, particularly in those animals inoculated only a few hours beforehand, there was no apparent difference between the duodenal mucosa of the monocontaminated gnotobiotes and the axenic control group.

Mucosal cells from more caudal sites in the intestine were more markedly vacuolate (Figures 8, 13, 14, 19 and 27) than were those from comparable sites in axenic control pigs (Figures 7, 9, 10, 11 and 12), and the vacuolization was much more extensive.

At times, in the mucosa of infected pigs, the cells were seen to be so vacuolate as to be disrupted by the breakdown of the cell membrane either at the luminal surface or at the intercellular wall (Figures 19 and 27). Despite this degree of disruption, at no stage was the lamina propria seen to be denuded of epithelial cells. Those epithelial cells which were excessively distended up to and beyond the point of rupture were apparently still retained as part of the covering epithelium of the villus. There was no evidence of excessive shedding of prematurely effete villal epithelial cells, nor was there evidence of denudation of the villus, exposure of uncovered lamina propria nor of foreshortening or clubbing of the villus.

At no stage during the examination of tissues with the optical microscope were bacteria seen on the mucosal surface or within the villal epithelial cells, neither in hematoxylin-and-eosin stained sections, nor in those submitted to the Brown and Brenn stain for

bacteria nor the other special stains used on tissues in this experiment.

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 (Figures 17 and 18), distended lymphatic vessels of the submucosa, subserosal edema (Figure 28) 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 germ-free control pigs, but there were increased numbers of lymphocytes in these tissues in the pigs heavily contaminated with the test organisms.

Enzyme Histochemistry

Histochemistry of the Villus and Villal Epithelial Cells

The Phosphatases.

Alkaline Phosphatase.--Although there was considerable loss of alkaline phosphatase activity in the formalin-fixed tissues, the histochemical reaction was intense enough to be observed, and there was satisfactory sharpness of localization of the enzyme with excellent morphologic detail of tissues (Figure 29).

More of the enzyme was retained in the tissues fixed in cold acetone and stored at -70 C, but morphologic detail was less

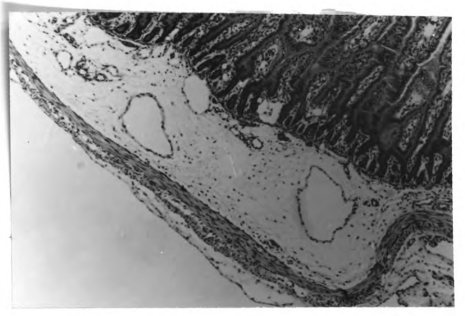


Figure 28. Pig 1306. Age 4-1/2 days. Anterior jejunum. *E. coli* was introduced *per os* 8 hours before necropsy. Subserosal and submucosal edema and distention of submucosal lymphatic vessels. Hematoxylin and eosin. x 75.

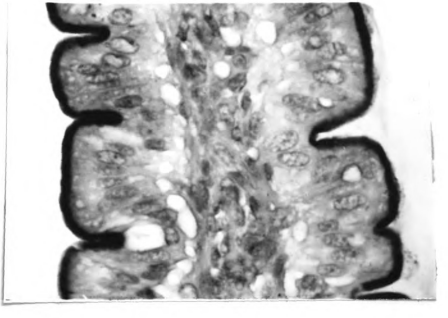


Figure 29. Pig 0661. Age 29 days. Terminal ileum. Germfree control. There is satisfactory localization of alkaline phosphatase in the brush border and excellent morphologic detail of the tissue. Cold neutral formalin fixation. Calcium-cobalt method for alkaline phosphatase. x 750.

satisfactorily, and the stain was less sharply localized in the tissues and within the cells (Figure 30).

Least satisfactory in terms of specific localization of enzyme and of morphologic detail were those tissues fresh frozen over dry ice and stored at -70 C (Figures 31 and 32).

In the germfree neonatal pig, alkaline phosphatase was restricted to the villal epithelial cells and was not seen in the epithelial cells of the crypts (Figure 31).

Within the individual villal epithelial cell the enzyme was strongly localized in the brush border. There was at times a narrow clearer zone at the apex of the cell that would correspond to the region of the terminal web, in which there seemed to be a region of reduced alkaline phosphatase activity (Figures 30, 32, and 33). This phenomenon did not depend on the method of fixation, the period of storage prior to processing, or the level of intestine from which the section was cut (Figures 30 and 32).

The remainder of the alkaline phosphatase activity was demonstrated only in the frozen unfixed and cold acetone fixed tissues and was concentrated in the apical third of the villal epithelial cell and in a narrow zone axial to the cell nucleus close to the basement membrane (Figure 30).

This pattern of tissue and cell distribution of alkaline phosphatase was the same for each of the five levels of the small intestine that were sampled. In control tissues, in which the villal epithelial cells were markedly vacuolated, the normal distribution of the enzyme was somewhat altered in that the positive staining

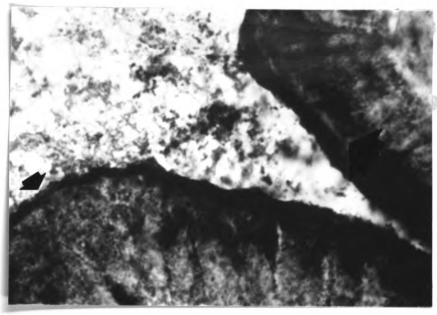


Figure 30. Pig 1002. Age 3 days. Midjejunum. Germfree control. Much enzyme activity is retained, but morphologic detail and enzyme localization is poorly defined. Alkaline phosphatase is strongly localized in the brush border and there is some enzyme activity in the apical cytoplasm. There is reduced enzyme activity at the terminal web (arrow). Tissue frozen in acetone at -70 C. Calcium-cobalt method for alkaline phosphatase. x 750.

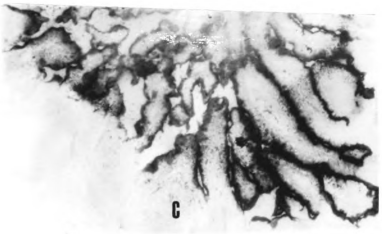


Figure 31. Pig 9046. Age 2 days. Duodenum. Germfree control. The enzyme is poorly localized and morphologic detail is minimal. Alkaline phosphatase is restricted to the villal epithelial cells and is not apparent in the epithelium of the crypts (c). Unfixed frozen (-70 C) tissue. Calcium-cobalt method for alkaline phosphatase. x 75.

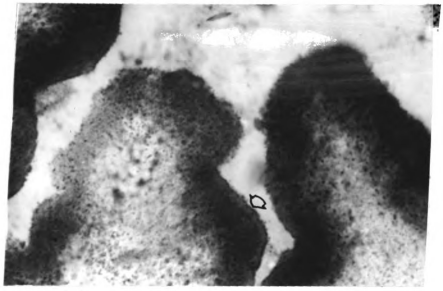


Figure 32. Pig 9046. Age 2 days. Midjejunum. Germfree control. The enzyme is poorly localized and morphologic detail is minimal. Alkaline phosphatase is localized in the brush border (arrow) and a prominent band of reaction product -- probably indicative of nonspecific phosphatase activity -- is present in the remainder of the cytoplasm. There is a band of reduced enzyme activity in the region of the terminal web. Unfixed frozen (-70 C) tissue. Calcium-cobalt method for alkaline phosphatase. x 470.

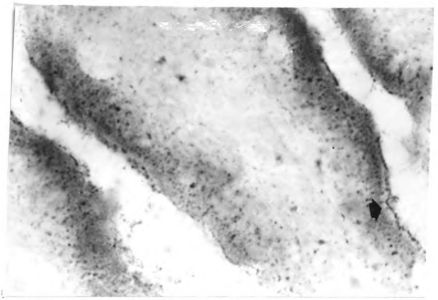


Figure 33. Pig 9046. Age 2 days. Duodenum. Germfree control. Alkaline phosphatase is localized in the brush border. There is a band of reduced enzyme activity in the region of the terminal web (arrow). Unfixed frozen (-70 C) tissue. Calcium-cobalt method for alkaline phosphatase. x 750.

reaction of the brush border was seen to be less even in intensity and there was a slight increase in intensity of the staining reaction at the margin of the vacuole (Figure 34).

In those animals killed 4, 8 or 10 hours after monocontamination with *E. coli* 0138:K81:NM there was no distinct change in the distribution or intensity of staining of the alkaline phosphatase (Figure 35). By 12 hours after inoculation there seemed to be some loss of intensity of staining, and by 14 hours the loss of intensity of the staining was more apparent in the epithelium more distally placed along the villi (Figure 36), as it was also less intense in sections taken further along the small intestine (Figure 37). Loss of this intense staining reaction and a lack of specificity of the distribution of alkaline phosphatase was seen in those animals killed at subsequent time intervals although it was apparent that by 96 hours after inoculation, there was a renewed intensity of the staining reaction in the villal epithelial cells (Figure 38).

The thin clear zone in the region of the terminal web continued to be apparent in the monocontaminated animals despite the changes in distribution of the enzyme in the remainder of the cell (Figure 39).

The changes in staining intensity and intracellular distribution of the enzyme associated with monocontamination with the organism were qualitatively similar at the five levels of the small intestine from which samples were taken.

Acid Phosphatase.--In the germfree control pigs at all of the five selected levels of the small intestine, acid phosphatase was

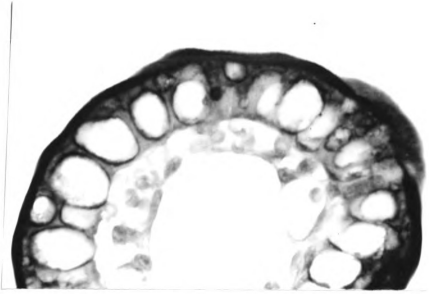


Figure 34. Pig 1005. Age 3 days. Anterior jejunum. Germfree control. Alkaline phosphatase is localized in the brush border of markedly vacuolate cells. There is some concentration of enzyme activity at the margin of the intracytoplasmic vacuoles. Cold neutral formalin fixation. Calcium-cobalt method for alkaline phosphatase. x 750.

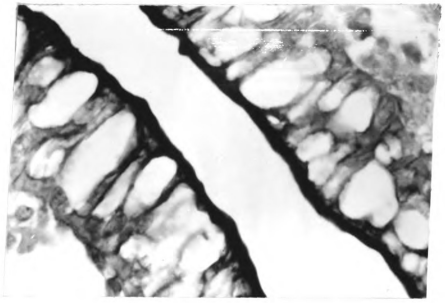


Figure 35. Pig 1303. Age 4 days. Terminal jejunum. *E. coli* was introduced *per os* 4 hours before necropsy. Although markedly vacuolate, the enzyme distribution in the villal epithelial cells is unchanged from that seen in the control animal tissues and is generally confined to the brush border. Cold neutral formalin fixation. Calcium-cobalt method for alkaline phosphatase. x 750.

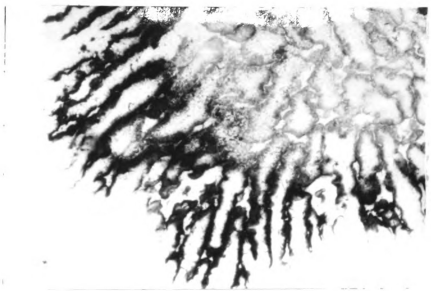


Figure 36. Pig 9041. Age 2-1/2 days. Duodenum. *E. coli* was introduced *per os* 14 hours before necropsy. There is partial loss of alkaline phosphatase from the epithelium of the middle and distal third of the villus. Note that the intensity of the reaction in this tissue is generally more marked than that observed in the terminal ileum (Figure 37). Unfixed frozen (-70 C) tissue. Calcium-cobalt method for alkaline phosphatase. x 75.

Figure 37. Pig 9041. Age 2-1/2 days. Terminal ileum. *E. coli* was introduced *per os* 14 hours before necropsy. Almost complete loss of alkaline phosphatase activity. Slight enzyme activity is apparent in the recently generated cells at the bases of the villi. Unfixed frozen (-70 C) tissue. Calcium-cobalt method for alkaline phosphatase. x 75.

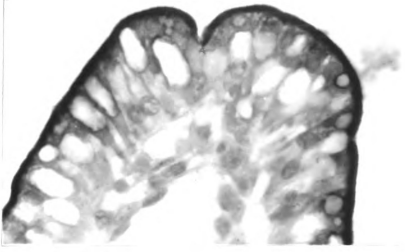


Figure 38. Pig 1009. Age 6-1/2 days. Terminal ileum. *E. coli* was introduced *per os* 96 hours before necropsy. Alkaline phosphatase activity is localized in the brush border. There is renewed intensity of the staining reaction for enzyme activity in the brush border and the enzyme is well localized in that site. Cold neutral formalin fixation. Calcium-cobalt method for alkaline phosphatase. x 750.

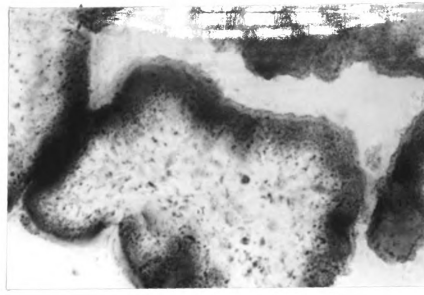


Figure 39. Pig 9042. Age 2-1/2 days. Midjejunum. *E. coli* was introduced *per os* 16 hours before necropsy. Despite the diminution in alkaline phosphatase activity in the region of the brush border in infected pigs, the thin clear zone of enzyme inactivity in the region of the terminal web is still apparent. Unfixed frozen (-70 C) tissue. Calcium-cobalt method for alkaline phosphatase. x 470.

seen to be evenly distributed throughout the villal epithelial cells and along the villi. The enzyme stained slightly less intensely in those villal epithelial cells at the tip of the villus. There was little or no acid phosphatase activity in the cells of the crypts (Figure 40). In sections of duodenum and anterior jejunum, the specific distribution of acid phosphatase within the cell was apparently more restricted than it was in those cells from tissues that were more caudally located in the small intestine. In the duodenal and anterior jejunal cells, enzyme activity was concentrated in the apical cytoplasm of the epithelial cells whereas, more caudally in the intestinal tract, the enzyme was more evenly distributed in the cell cytoplasm, although some concentration in the apical cytoplasm of the villal epithelial cell was still apparent (Figure 41). In some instances a perinuclear zone was observed in which there was no enzyme activity (Figure 41).

There was no apparent change in cellular or tissue distribution of acid phosphatase in those villi exposed to *E. coli* 0138:K81:NM at either 8 or 10 hours prior to the time of necropsy, although the general intensity of the staining reaction was reduced.

Twelve hours after contamination, at which time diarrhea was observed clinically, acid phosphatase was seen to be typically distributed within the villal epithelial cells, but the staining reaction was generally less marked (Figure 42).

Loss of staining intensity of sites of acid phosphatase activity was associated with the number of hours elapsed after contamination, and this loss was more marked in tissues more distally positioned along the intestinal tract (Figure 43).

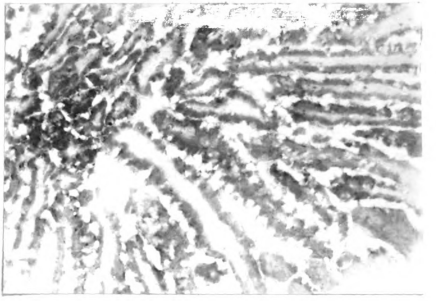


Figure 40. Pig 9046. Age 2 days. Terminal jejunum. Germfree control. Acid phosphatase is seen to be evenly distributed along the villi. There is little or no enzyme activity in the cells of the crypts (at bottom right). Acid phosphatase is distributed evenly throughout the cytoplasm of the individual villal epithelial cells. Unfixed frozen (-70 C) tissue. Standard coupling technique for acid phosphatase (Pearse, 1961). x 75.

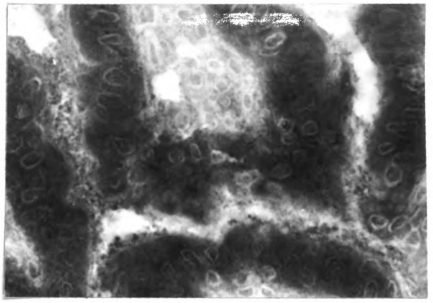


Figure 41. Pig 9047. Age 2-1/2 days. Terminal ileum. Germfree control. In more caudal sites in the small intestine, acid phosphatase activity is evenly distributed in the cytoplasm of individual cells. A perinuclear zone in which there is no enzyme activity is apparent. Unfixed frozen (-70 C) tissue. Standard coupling technique for acid phosphatase (Pearse, 1961). x 750.

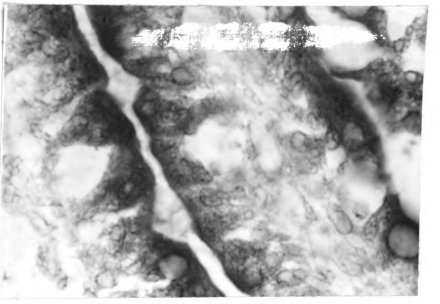


Figure 42. Pig 9040. Age 2-1/2 days. Duodenum. *E. coli* was introduced *per os* 12 hours before necropsy. Acid phosphatase is typically distributed within the villal epithelial cells, but the intensity of the stain reaction is markedly reduced as a result of infection. Unfixed frozen (-70 C) tissue. Standard coupling technique for acid phosphatase (Pearse, 1961). x 750.

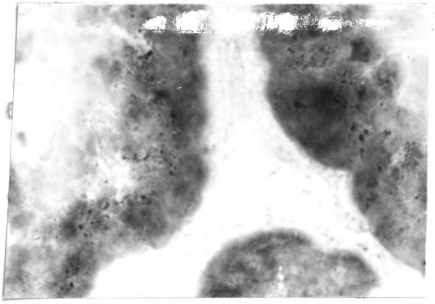


Figure 43. Pig 9044. Age 3 days. Terminal ileum. *E. coli* was introduced *per os* 20 hours before necropsy. Marked loss of enzyme activity associated both with number of hours elapsed after monocontamination and the intestinal site of the tissue sampled. Compare this reaction with that depicted in Figures 41 and 42. Unfixed frozen (-70 C) tissue. Standard coupling technique for acid phosphatase (Pearse, 1961). x 750.

The particulate or finely granular appearance of the stain reaction that is associated with histochemical reactions of membrane-bound enzymes was progressively supplanted by a smoother, more homogeneous staining reaction in the villal epithelial cells of pigs monocontaminated with *E. coli* 0138:K81:NM for 20 hours and longer (Figure 43).

Twenty-four hours after contamination, the villal epithelial cells of the ileum were uniformly stained, and there was clumping of stained material at the luminal surface in the region of the brush border (Figure 44).

As with the histochemical reactions for other enzymes, marked vacuolization of the cell resulted in displacement of the enzyme-bearing organelles to the periphery of the cell (Figure 45).

There was no acid phosphatase-positive material seen within the vacuoles.

Adenosine Triphosphatase.--In the neonatal germfree control pigs, at each of the five levels of the small intestine, adenosine triphosphatase was observed to be particularly localized in the region of the brush border but with moderate activity in the apical half of the villal epithelial cells (Figure 46).

In the monocontaminated animals, the first change was seen in pigs killed 10 or 12 hours after exposure to the organism, and this change was a partial loss of specificity of distribution and intensity of staining of adenosine triphosphatase. This change was much more marked in the terminal portion of the small intestine (Figure 47)

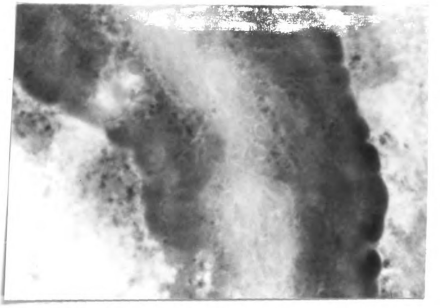


Figure 44. Pig 9045. Age 3 days. Terminal ileum. *E. coli* was introduced *per os* 24 hours before necropsy. The loss of the finely particulate staining reaction characteristic of histochemical reactions of membrane-bound enzymes is apparent. There is clumping of acid phosphatase-active material at the luminal margin of the villal epithelial cells. Unfixed frozen (-70 C) tissue. Standard coupling technique for acid phosphatase (Pearse, 1961). x 750.

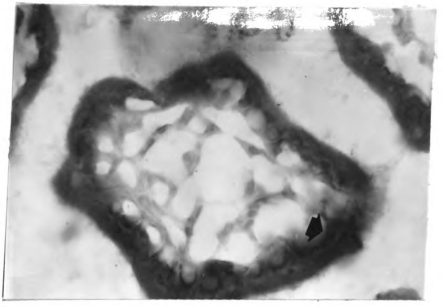


Figure 45. Pig 9042. Age 2-1/2 days. Transverse section of villus from terminal ileum. *E. coli* was introduced *per os* 16 hours before necropsy. Marked vacuolization of the villal epithelial cells resulting in the displacement of enzyme bearing organelles to the periphery of the cell or to the margin of the vacuole (arrow). Unfixed frozen (-70 C) tissue. Standard coupling technique for acid phosphatase (Pearse, 1961). x 750.

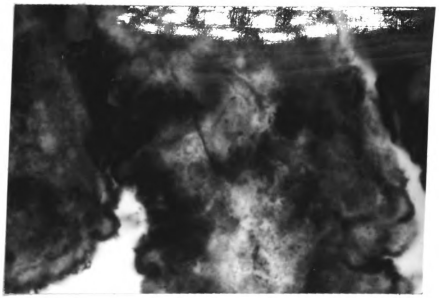


Figure 46. Pig 9047. Age 2-1/2 days. Terminal ileum. Germfree control. Adenosine triphosphatase is particularly localized in the brush border, and there is moderate enzyme activity in the apical cytoplasm of the villal epithelial cells. Unfixed frozen (-70 C) tissue. Calcium method for adenosine triphosphatase. x 750.

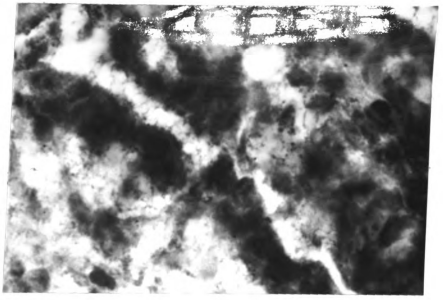


Figure 47. Pig 9040. Age 2-1/2 days. Terminal ileum. *E. coli* was introduced *per os* 12 hours before necropsy. Loss of specific concentration of adenosine triphosphatase activity from the brush border. Unfixed frozen (-70 C) tissue. Calcium method for adenosine triphosphatase. x 750.

whereas, in sections from the duodenum and anterior jejunum, stain distribution and intensity more closely resembled that of the tissues from germfree control animals.

In villal epithelial cells from animals exposed to *E. coli* 0138:K81:NM for 18, 20 and 24 hours, respectively, and in which there was marked vacuolization, the stained enzyme reaction product was seen to be pushed into the cell extremities by the intracytoplasmic vacuole. There was no positively stained material within the vacuoles. In these tissues a slightly positive stain reaction was seen in the region of the brush border, but the reaction was less intense than that seen in the germfree controls (Figure 48).

Proteolytic Enzymes.

Leucine Aminopeptidase.--Leucine aminopeptidase activity was observed to be localized at the brush border of the intestinal epithelium of neonatal germfree control pigs (Figure 49). Leucine aminopeptidase staining intensity in the microvilli of cells from the monocontaminates was sharply reduced, and 12 to 14 hours after infection only traces of the enzyme activity were detected at the apical surface of the villal epithelial cells (Figure 50).

In sections from jejunum and ileum in which there was marked vacuolization, there was no detectable enzyme at the apex of the vacuolated cells. Some leucine aminopeptidase remained in the brush border of the occasional interposed nonvacuolated cells. Sixteen hours after contamination, slight activity in the brush border of cells from the duodenum and early jejunum could be detected (Figures

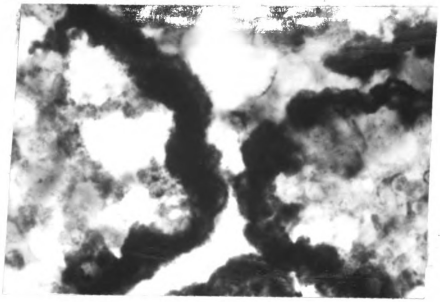


Figure 48. Pig 9043. Age 3 days. Terminal ileum. *E. coli* was introduced *per os* 18 hours before necropsy. Stained enzyme reaction products are displaced by intracytoplasmic vacuoles. There is no enzyme activity within the vacuoles. Some adenosine triphosphatase activity in the brush border and the apical cytoplasm is still apparent. Unfixed frozen (-70 C) tissue. Calcium method for adenosine triphosphatase. x 750.

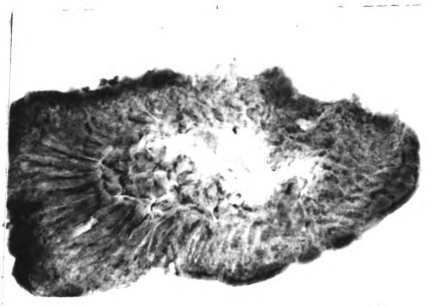


Figure 49. Pig 9046. Age 2 days. Terminal ileum. Germfree control. Leucine aminopeptidase activity is localized at the brush border. Unfixed frozen (-70 C) tissue. Method for leucine aminopeptidase (Nachlas *et al.*, 1957). x 750.

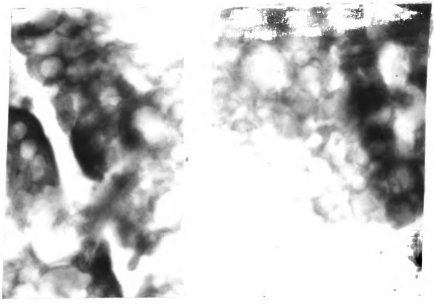


Figure 50. Pig 9040. Age 2-1/2 days. Terminal ileum. *E. coli* was introduced *per os* 12 hours before necropsy. Twelve hours after monocontamination, enzyme activity in the brush border is markedly reduced, and only traces of active enzyme are seen at the apex of the villal epithelial cells. Unfixed frozen (-70 C) tissue. Method for leucine aminopeptidase (Nachlas *et al.*, 1957). x 750.

51 and 52), whereas no enzyme activity could be demonstrated in the villal epithelial cells of more distal intestinal tissues, nor in the intestinal villi from pigs sampled 18 or more hours after monocontamination with *E. coli* 0138:K81:NM (Figure 53).

The Dehydrogenases.

Succinate Dehydrogenase.--The sites for succinate dehydrogenase activity were clearly apparent in the small intestine of the germfree neonatal pig. Throughout the small intestine succinate dehydrogenase was seen in the epithelial cells of both villi and crypts, although the staining reaction seemed to be more intense at the base and in the midshaft of the villus (Figure 54). At a cellular level, villal epithelial cells in the duodenum (Figure 55) and the anterior jejunum seemed to be more intensely stained than the villal epithelial cells in the midjejunum and the terminal ileum (Figure 56).

The specific localization of the enzyme within the villal epithelial cell was consistent in tissues taken from the germfree control pigs at all levels of the small intestine. There was a concentration of enzyme in the apical cytoplasm of the cell and a more intense, but more narrow, band of enzyme activity at the basement membrane (Figures 55 and 57).

Eight hours after infection with *E. coli* 0138:K81:NM, there was a distinct and intense reaction, with an even more clearly defined distribution of succinate dehydrogenase following the pattern of that seen in the control animals (Figure 58). Within 12 hours of

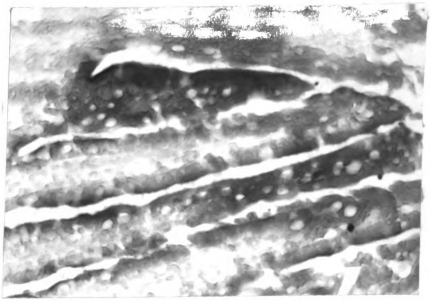


Figure 51. Pig 9042. Age 2-1/2 days. Duodenum. *E. coli* was introduced *per os* 16 hours before necropsy. Slight enzyme activity is seen in the brush border of duodenal villal epithelial cells. The loss of leucine aminopeptidase activity is uniform along the length of the duodenal villi. Unfixed frozen (-70 C) tissue. Method for leucine aminopeptidase (Nachlas *et al.*, 1957). x 190.

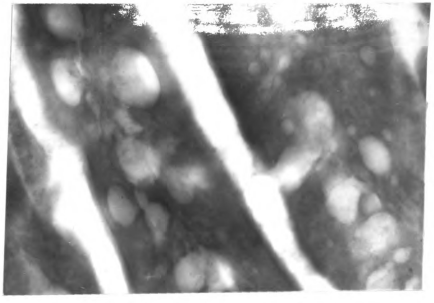


Figure 52. Pig 9042. From the same field as Figure 51. Slight leucine aminopeptidase activity is faintly discernible at the brush border of duodenal villal epithelial cells. x 750.

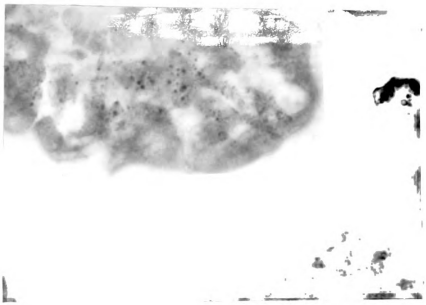


Figure 53. Pig 9044. Age 3 days. Terminal ileum. *E. coli* was introduced *per os* 20 hours before necropsy. Complete loss of leucine aminopeptidase activity from the villal epithelial cells. Unfixed frozen (-70 C) tissue. Method for leucine aminopeptidase (Nachlas *et al.*, 1957). x 750.

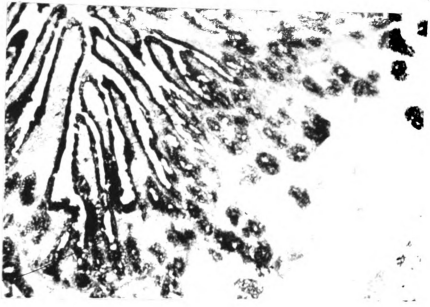


Figure 54. Pig 9046. Age 2 days. Duodenum. Germfree control. Succinate dehydrogenase activity is clearly demonstrated in the epithelium of the villi and the crypts. Unfixed frozen (-70 C) tissue. MTT (Pearse, 1961). x 75.

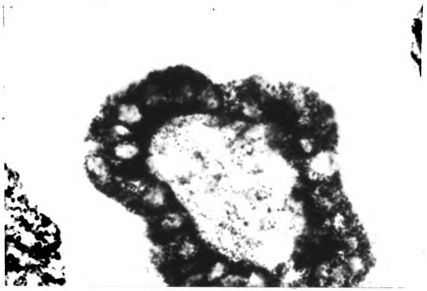


Figure 55. Pig 9046. Age 2 days. Duodenum. Germfree control. An intense staining reaction for succinate dehydrogenase is apparent throughout the cytoplasm of duodenal villal epithelial cells. MTT (Pearse, 1961). x 750.

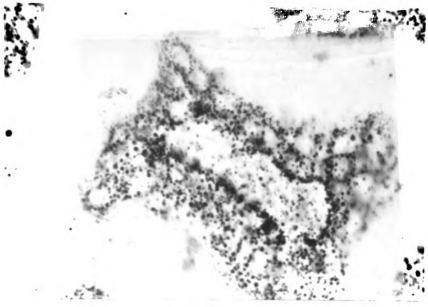


Figure 56. Pig 9046. Age 2 days. Terminal jejunum. Germfree control. Succinate dehydrogenase activity in the villal epithelial cells of the terminal small intestine is less marked than that of the anterior small intestine. Compare this figure with Figure 55. Unfixed frozen (-70 C) tissue. MTT (Pearse, 1961). x 750.

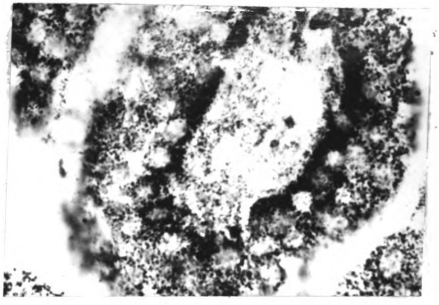


Figure 57. Pig 9047. Age 2-1/2 days. Terminal jejunum. Germfree control. Succinate dehydrogenase is localized in the apical cytoplasm and in a narrow band at the base of the villal epithelial cells. Unfixed frozen (-70 C) tissue. MTT (Pearse, 1961). x 750.

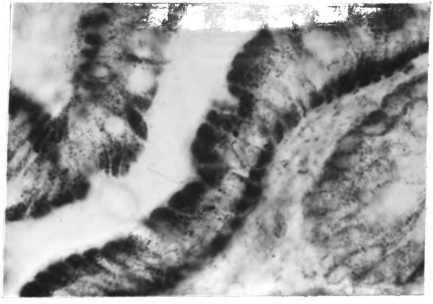


Figure 58. Pig 9038. Age 2-1/2 days. Terminal ileum. *E. coli* was introduced *per os* 8 hours before necropsy. Eight hours after monocontamination there is a distinct, intense reaction, with clearly defined distribution of succinate dehydrogenase following the pattern of that seen in germfree control animal tissues. Unfixed frozen (-70 C) tissue. MTT (Pearse, 1961). x 750.

monocontamination there was marked loss of enzyme activity (Figure 59) and appreciable amounts of staining of material in the laminae propriae was observed.

In each case the alteration, both in intensity of the staining reaction and in distribution of the stained material within the cell, was more marked in the more caudal sites in the small intestine.

Fourteen hours after inoculation, the succinate dehydrogenase reaction product was less intensely stained and was more diffusely distributed throughout the cell, although the specific distribution of the enzyme at the extremities of the cell was still apparent (Figure 60). In those villal epithelial cells in which there was marked vacuolization, the displacement of the stained material by an intracytoplasmic mass was readily observed (Figures 60 and 61).

In animals inoculated 24 hours previously, the reduction in staining intensity was apparent from the duodenum to the jejunum and terminal ileum, although the specific intracellular distribution of the enzyme in these tissues could still be seen.

In some instances, by 28 or 32 hours after inoculation there was still an apparent diminution in the amount of enzyme present in the apical cytoplasm of the villal epithelial cells.

Lactate Dehydrogenase.--In the duodenal epithelium of neonatal germfree pigs, lactate dehydrogenase was concentrated at the apical third of the villal epithelial cell and in a thin line at the basement membrane (Figure 62). Using a shorter incubation time during tissue processing there was a less intense histochemical reaction,

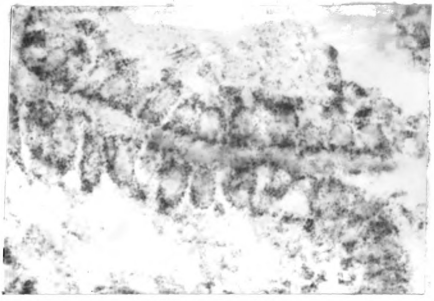


Figure 59. Pig 9039. Age 2-1/2 days. Terminal ileum. *E. coli* was introduced *per os* 10 hours before necropsy. There is appreciable loss of succinate dehydrogenase activity from the villal epithelial cells. Unfixed frozen (-70 C) tissue. MTT (Pearse, 1961). x 750.

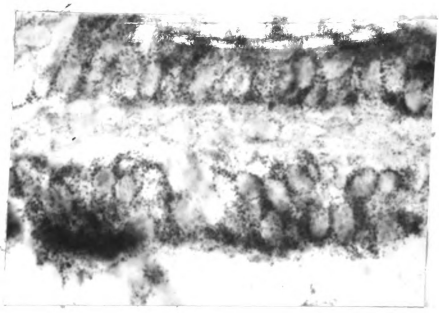


Figure 60. Pig 9041. Age 2-1/2 days. Terminal jejunum. *E. coli* was introduced *per os* 14 hours before necropsy. Succinate dehydrogenase activity is markedly reduced, although specific intracellular distribution of the enzyme is still apparent. Unfixed frozen (-70 C) tissue. MTT (Pearse, 1961). x 750.

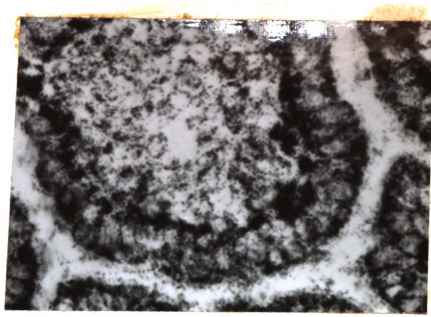


Figure 61. Pig 9042. Age 2-1/2 days. Terminal jejunum. *E. coli* was introduced *per os* 16 hours before necrospy. In vacuolate villal epithelial cells from infected pigs, the succinate dehydrogenase is reduced in activity and the enzyme is displaced to the periphery of the cell. Unfixed frozen (-70 C) tissue. MTT (Pearse, 1961). x 750.

Figure 62. Pig 1005. Age 3 days. Duodenum. Germfree control. Lactate dehydrogenase is concentrated at the apical third of the villal epithelial cell and in a thin line at the basement membrane. Cold neutral formalin fixation. MTT method for lactate dehydrogenase (Hess *et al.*, 1958). x 750.

but a more elegant picture of the distribution of the enzyme was obtained (Figure 63).

In control pigs in which the villal epithelial cells were vacuolate, the lactate dehydrogenase was distributed around the vacuoles, probably due to the mechanical disruption of normal intracytoplasmic relationships, but there was still some of the enzyme concentrated at the basement membrane (Figure 64). Some staining of lactate dehydrogenase reaction products was observed in the laminae propriae of the villi of the neonatal germfree pigs (Figure 64). There was no staining of lactate dehydrogenase reaction products in the laminae propriae of villi from control pigs in which the villal epithelial cells were apparently normal (Figures 62 and 63).

It was observed in the germfree neonatal pigs that the most intense enzyme activity was in the duodenum and the anterior third of the jejunum. In the more caudal intestinal tissue sections the intensity of the staining reaction was diminished (Figure 64).

In the monocontaminated gnotobiotes there was a noticeable difference in the specific distribution of lactate dehydrogenase within the villal epithelial cell 24 and 28 hours, respectively, after exposure to the organisms. Concentration of the enzyme in the apical third of the cell could be seen in some instances, but the enzyme was more evenly distributed throughout the cell and extended centrally around the nucleus and to the basement membrane. This change was observed throughout the small intestine (Figure 65). In some instances a slight concentration of lactate dehydrogenase was seen in the immediate subapical cytoplasm of the cell.

Figure 63. Pig 1002. Age 3 days. Duodenum. Germfree control. Half normal incubation time was used in this tissue preparation. As a result, stain intensity is reduced, but a refined depiction of enzyme activity concentration in the apical cytoplasm and at the base of villal epithelial cells is obtained. Cold neutral formalin fixation. MTT method for lactate dehydrogenase (Hess *et al.*, 1958). x 750.

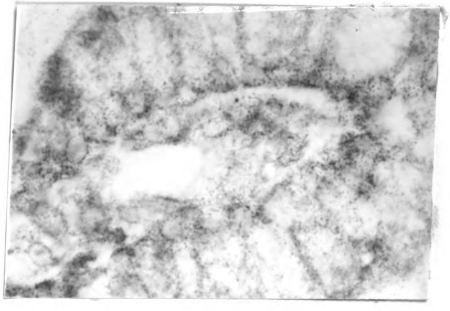


Figure 64. Pig 1005. Age 3 days. Terminal ileum. Germfree control. Villal epithelial cell vacuolization resulted in peripheral displacement of lactate dehydrogenase, but the concentration of the enzyme activity at the base of the cells is apparent. Cold neutral formalin fixation. MTT method for lactate dehydrogenase (Hess *et al.*, 1958). x 750.

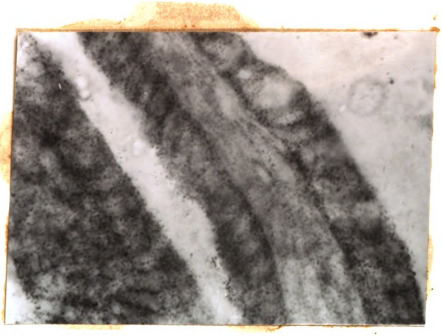


Figure 65. Pig 1000. Age 3-1/2 days. Terminal ileum. *E. coli* was introduced *per os* 24 hours before necropsy. There is more uniform distribution of active enzyme throughout the cell and extending centrally around the nucleus and to the basement membrane. Some enzyme activity in the lamina propria is apparent. Cold neutral formalin fixation. MTT method for lactate dehydrogenase (Hess *et al.*, 1958). x 750.

At 28 hours after inoculation, it was observed that there was loss of both specificity of distribution and intensity of staining of lactate dehydrogenase reaction products (Figure 66). In some instances staining of this material in the interstices of the lamina propria was observed, as it was observed occasionally in tissues from the germfree control pigs (Figure 64).

Special Stain Techniques for Intracytoplasmic Contents and the Brush Border

Oil Red O Stain

At no stage was the accumulation of fat responsible for the vacuolization of the villal epithelial cells. Oil red O failed to stain any material in the vacuoles of either the control or the monocontaminated pigs. In those pigs fed within an hour prior to being killed, positively stained material was seen in the apical cytoplasm of the villal epithelial cells of the duodenum and of the ileum, and in some instances oil-red-O-positive material was observed in the lacteals. Oil-red-O-positive material was not seen in sections from the terminal jejunum or ileum of clinically affected pigs (although some instances of fat absorption were seen in electron-micrographs of the occasional absorbing cells from comparable tissues). In no instance did the distribution of this material resemble that of the material in the vacuoles of the villal epithelial cells, and it was concluded that the vacuolization was not due to the collection of fat in the villal epithelial cells or the intramembranous space.

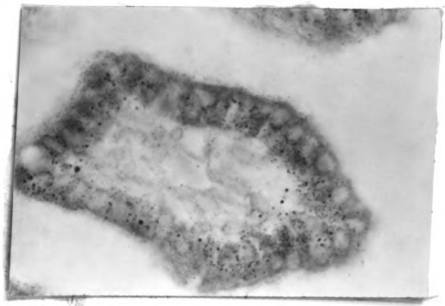


Figure 66. Pig 1004. Age 3-1/2 days. Terminal jejunum. *E. coli* was introduced *per os* 28 hours before necropsy. There is loss of specificity of distribution and intensity of staining of lactate dehydrogenase reaction products. Cold neutral formalin fixation. MTT method for lactate dehydrogenase (Hess *et al.*, 1958). x 750.

Periodic Acid-Schiff Reaction

The contents of vacuolate villal epithelial cells reacted negatively to the periodic acid-Schiff reaction. In some instances, in both control and monocontaminated pigs, the brush border reacted as strongly positive to this test as did all goblet cells and the Brunner's glands.

Best's Carmine Stain

The reaction of vacuolate cells to Best's carmine stain was negative. The only material positive for this stain was seen to be in the Brunner's glands, the goblet cells, and the brush border. In those villal epithelial cells undergoing marked vacuolization, there was no evidence that the material filling the vacuoles was Best's carmine-positive. In all instances neighboring goblet cells were positive for Best's carmine stain.

Mayer's Mucicarmine Stain

The reaction of vacuolate cells to Mayer's mucicarmine stain was negative. The extreme examples from the least to the most vacuolate cells within the tissues from the germfree control pigs did not contain stainable mucin or seromucin. Similarly, in the monocontaminated animals, none of the vacuolate cells contained stainable mucin or seromucin. In each instance the neighboring goblet cells were strongly positive for mucin.

Alcian Blue Stain

In both germfree and monocontaminated pigs, the only alcian-blue-positive material was seen to be in the Brunner's glands and

Figure 67. Pig 1008. Age 4 days. Terminal jejunum. Midvillus. Germfree control. Identifiable at this magnification are the apical tubule system, mitochondria, Golgi complex (Gc), and lysosomes (ly). The brush border is moderately deep and electron dense. Mitochondria are plentiful in both the apical and basal cytoplasm. The Golgi cisternae are moderately vacuolate. The close association of villal epithelial cells with the vasculature of the lamina propria is apparent. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 9,100.

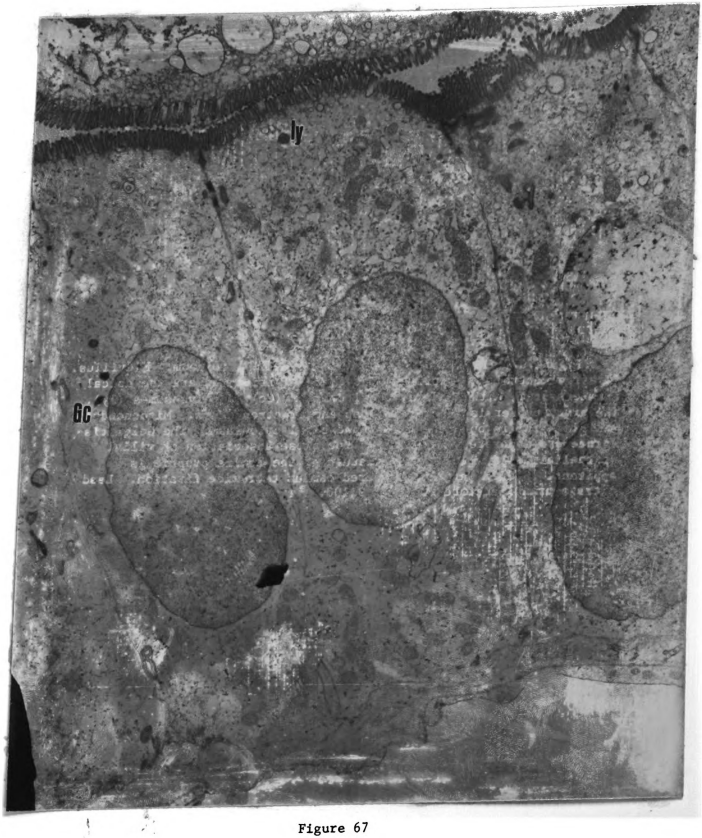


Figure 67

Figure 67. Pig 1008. Age 4 days. Terminal jejunum. Midvillus. Germfree control. Identifiable at this magnification are the apical tubule system, mitochondria, Golgi complex (Gc), and lysosomes (ly). The brush border is moderately deep and electron dense. Mitochondria are plentiful in both the apical and basal cytoplasm. The Golgi cisternae are moderately vacuolate. The close association of villal epithelial cells with the vasculature of the lamina propria is apparent. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 9,100.

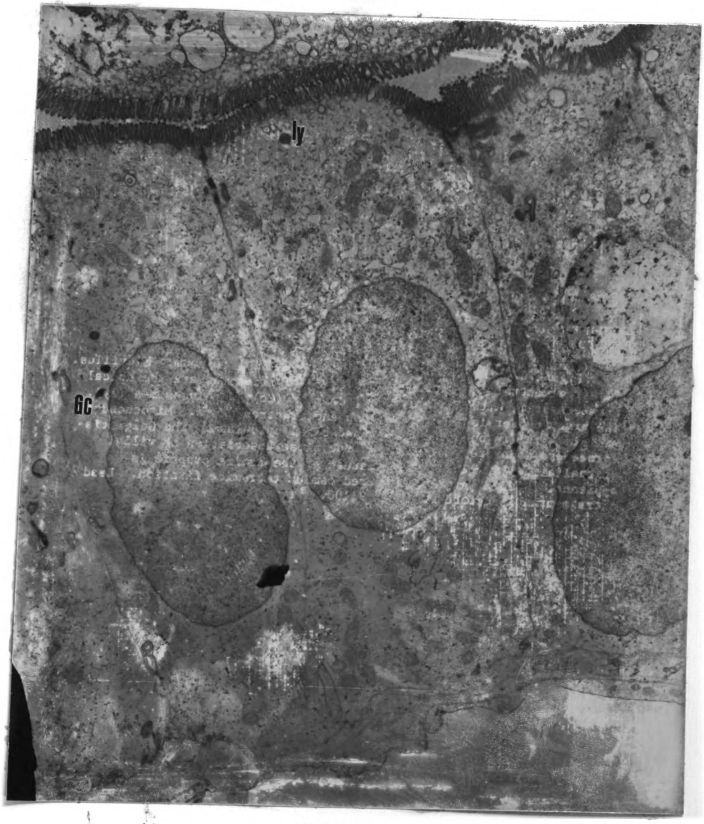


Figure 67

Figure 68. Pig 1008. Age 4 days. Terminal jejunum. Midvillus. Germfree control. Identifiable at this magnification are microvilli, mitochondria, apical tubule system (a), endoplasmic reticulum (Er), Golgi cisternae, and mural plasmalemmal interdigitations (p). The microvilli are moderately short and do not appear to be rigid. The cytoskeletal forms are indistinct and there is no evidence of a terminal web. Multiple supranuclear membrane enclosed vacuoles vary markedly in size, from obvious dilatations of the apical tubules (1), to more voluminous vacuoles probably formed within the endoplasmic reticulum (2). The contents of the vacuoles are particulate, probably protein, detached ribosomes, and absorption products. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 16,100.

Figure 68

Figure 69. Pig 1008. Age 4 days. Terminal jejunum. Midvillus. Germfree control. Identifiable at this magnification are mitochondria and their cristae mitochondriales, polyribosomes (r) and glycogen rosettes (g), Golgi cisternae, and endoplasmic reticulum. Features of the lamina propria include the basal lamina (arrow) of the epithelial cells, fluid interstitium, capillary endothelium (ca), portion of a thrombocyte (p), and cytoplasmic processes of connective tissue cells -- probably fibroblasts (F). Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 23,800.

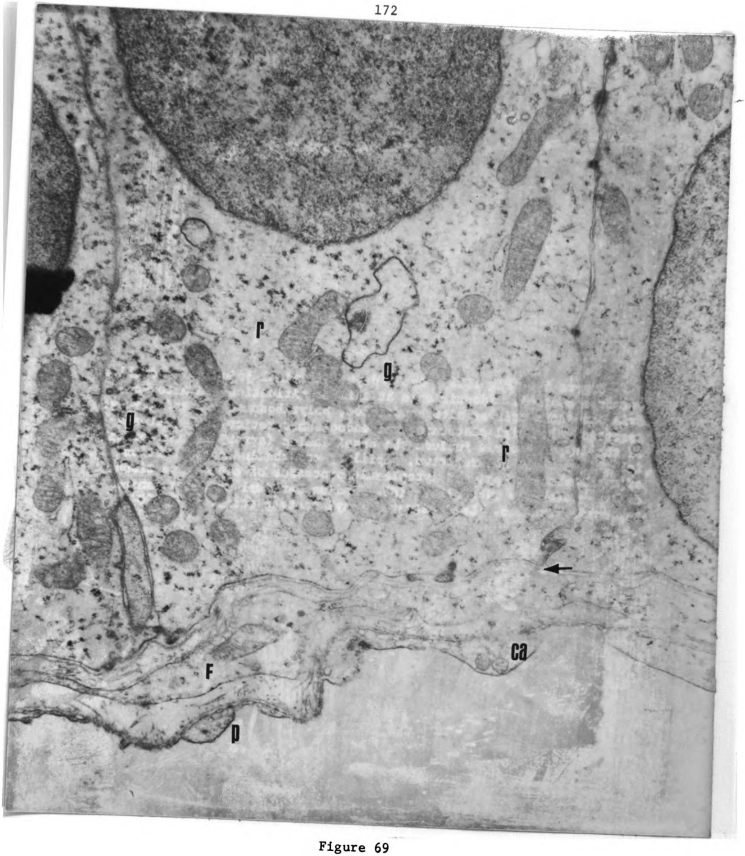


Figure 69

Figure 70. Pig 1002. Age 3 days. Terminal ileum. Basal third of villus. Germfree control. Granular reticulum (rough endoplasmic reticulum) (Gr), and lysosomes (ly) are distributed throughout the apical cytoplasm. Individual microvilli consist of a cylinder formed from the trilaminar membrane of the apical plasmalemma enclosing a structural complex, the cytoskeleton, terminating in a cytoplasmic condensation, the terminal web (tw). The microvilli, though long and slender, appear to be rigid. There is no evidence of an apical tubule system in this cell. Cristae mitochondriales are visible as parallel, nonvacuolated membranes (arrow). Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 30,600.

Figure 70

cytoskeleton consisted of a central rod surrounded by six radially placed skeletal rods of an apparently similar nature. There was some evidence of a meagre hirsute layer, the glycocalyx, on the outer surface of the microvilli (Figure 71).

Generally it was observed that in germfree neonatal pigs, the microvilli of those cells in which there was an active apical tubule system were somewhat shorter and more intensely stained (Figures 67 and 68) than those of more recently generated cells in which there was no evidence of an apical tubule system (Figure 70). In these cells the microvilli were longer with a more densely staining cytoskeleton terminating in an indistinct, finely granular layer corresponding to the terminal web (Figure 70). It was also apparent that the microvilli of these cells were more rigidly structured than those of cells containing an apical tubule system (Figures 68 and 70).

In the older, more mature cells (generated in the neonatal pig before, at, or soon after birth) in which there was an apical tubule system, the microvilli were seen to be shorter and less rigid, the cytoskeleton less electron-dense and with no apparent foundation in a terminal web (Figure 68).

In control and monocontaminated pigs 2 to 5 days old, the apical tubule system was clearly recognizable, extending 2 or 3 μ into the apical cytoplasm. The formation of pinocytotic vacuoles and the direct continuity of the tubules at the bases of the microvilli with the apical tubule system was clearly apparent (Figures 67, 72, 73 and 74).

Figure 71. Pig 1002. Age 3 days. Terminal jejunum. Germfree control. Transverse section of the microvilli. Each microvillus is a cylindrical tube formed of the trilaminar membrane from the apical plasmalemma. This membrane encloses the cytoskeleton, six rod-like structures placed radially around a central skeletal rod. There is evidence of a meagre hirsute layer, the glycocalyx (arrow). Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 134,000.

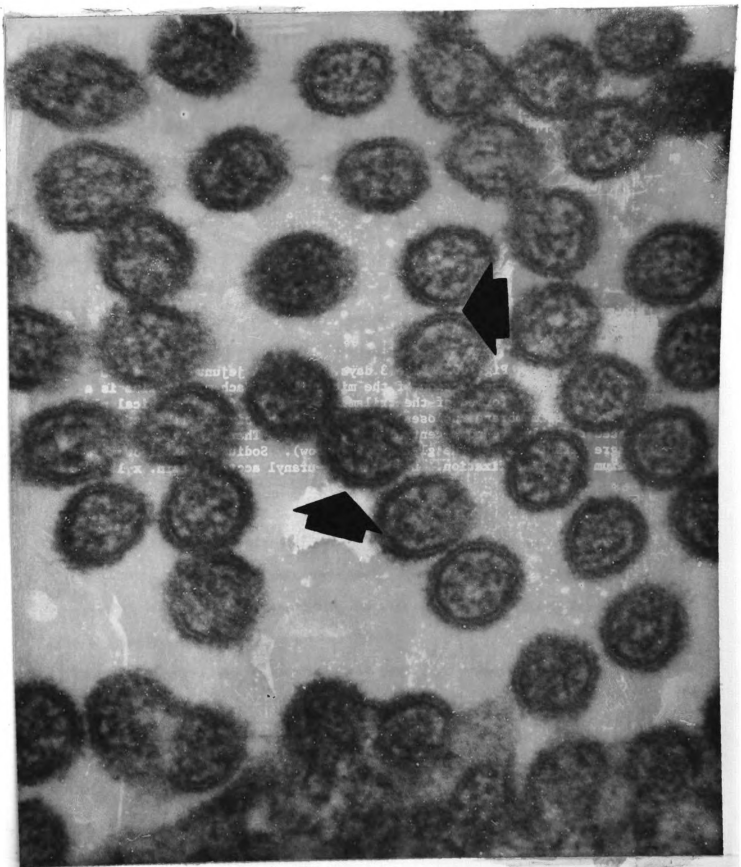


Figure 71

Figure 72. Pig 1310. Age 4-1/2 days. Terminal jejunum. Midvillus. Germfree control. The formation of pinocytotic vesicles and their relationship to the apical tubule system is apparent. The cristae mitochondriales are of uniform width and are not vacuolated. Polyribosomes are dispersed throughout the apical cytoplasm. An apical vacuole, probably associated with active absorption, is membrane bound. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 32,200.

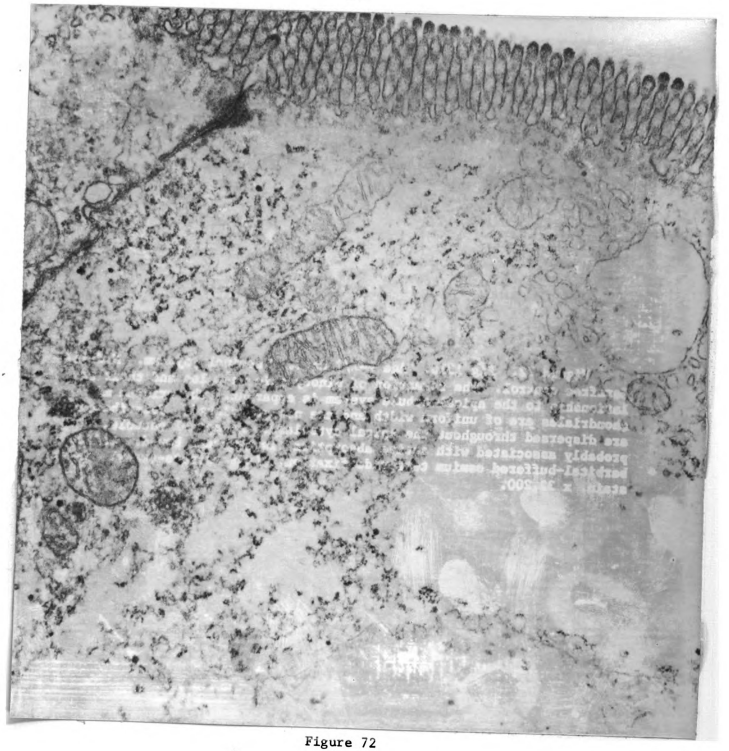


Figure 72

Figure 73. Pig 1306. Age 4-1/2 days. Terminal jejunum. Mid-villus. *E. coli* was introduced *per os* 8 hours before necropsy. Formation of pinocytotic vesicles at the bases of the microvilli and their relationship with the apical tubule system is apparent. The contents of the vacuoles are particulate, probably consisting of protein, some detached ribosomes and absorption products. These membrane lined vacuoles are indistinguishable from the absorption vacuoles seen in cells from germfree neonatal pigs. There is an indistinct, slight vacuolization of the cristae in the mitochondria of the apical cytoplasm. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 11,200.

Figure 73

Figure 74. Pig 2251. Age 2-1/2 days. Terminal ileum. Midvillus. *E. coli* was introduced *per os* 14 hours before necropsy. The formation of pinocytotic vesicles and their continuity with the apical tubule system is clearly visible (arrow). Early vacuolization of the cristae mitochondriales is apparent, as is breakdown of the laminae enclosing the absorption vacuoles. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 11,200.

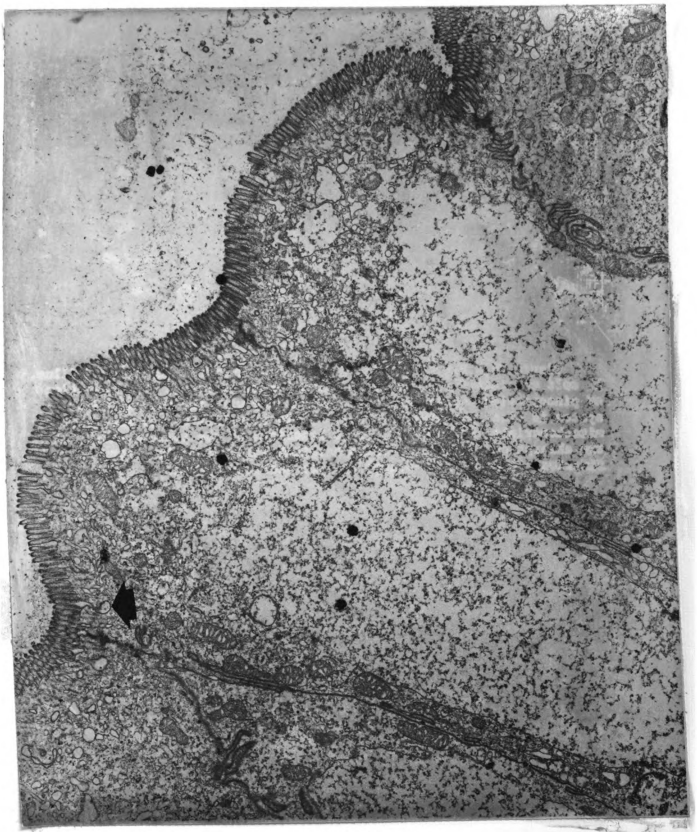


Figure 74

Early formation of membrane enclosed vacuoles, generally apical to the nucleus, was seen (Figure 67).

All stages of intracytoplasmic vacuolization comparable to those described for germfree neonates under "Histopathologic Findings" were observed with the electron microscope. These vacuoles ranged from those that were apparently dilatations of tubules of the apical tubule system (Figure 68) to multiple supranuclear vacuoles (Figures 68 and 75) and vacuoles so large as to be incapable of being contained in the field of the lowest acceptable tap-meter setting of the electron microscope (Figure 76).

These vacuoles were seen to be membrane enclosed and, where adequately resolved by the electron microscope, this membrane was seen to be a monolaminar structure, probably derived from the endoplasmic reticulum (Figures 68, 72, 75 and 76).

Particulate matter of a flocculent nature, some of it probably coagulated protein and some unattached ribosomes, was floating free in the vacuoles. Free membranes or membrane fragments were not seen in the vacuoles of cells from germfree neonatal control pigs.

In the cells examined from both jejunum and ileum of the germfree control pigs aged 2 to 5 days, organelles comprising mitochondria, lysosomes, granular endoplasmic reticulum and polyribosomes were seen to be present throughout the apical and basal cytoplasm; and mitochondria, particularly, were apparent in the subapical zone immediately beneath the terminal web (Figures 67, 70, 72 and 76). The cristae mitochondriales of cells from germfree neonates were conspicuous by their uniformity of width and lack of vacuolization (Figures 69, 70 and 72).

Figure 75. Pig 1005. Age 3 days. Terminal ileum. Basal third of villus. Germfree control. Multiple supranuclear membrane enclosed vacuoles vary markedly in size, from dilatations of the apical tubule system (3) to large vacuoles probably formed within the endoplasmic reticulum (4). The vacuole contents are particulate and probably consist of protein, detached ribosomes, and products of absorption. Free membranes or membrane fragments are not apparent. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 12,800.

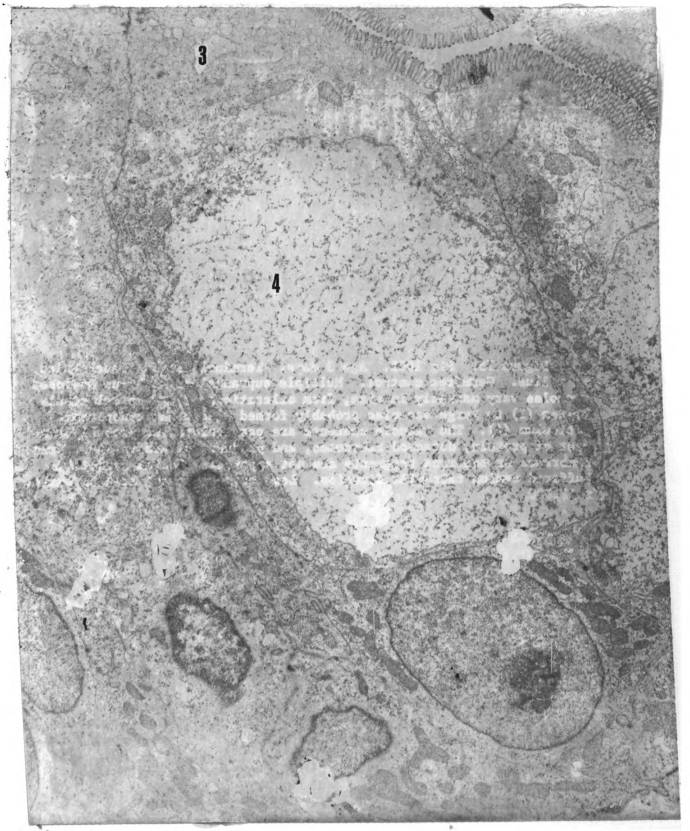


Figure 75

Figure 76. Pig 1310. Age 4-1/2 days. Terminal jejunum. Mid-villus. Germfree control. Multiple supranuclear vacuoles are membrane-enclosed and are probably derived from dilatations of the apical tubule system (5) and the endoplasmic reticulum (6). Note that free membranes or membrane fragments are not seen in the vacuoles in germfree neonatal pigs. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 9,100.

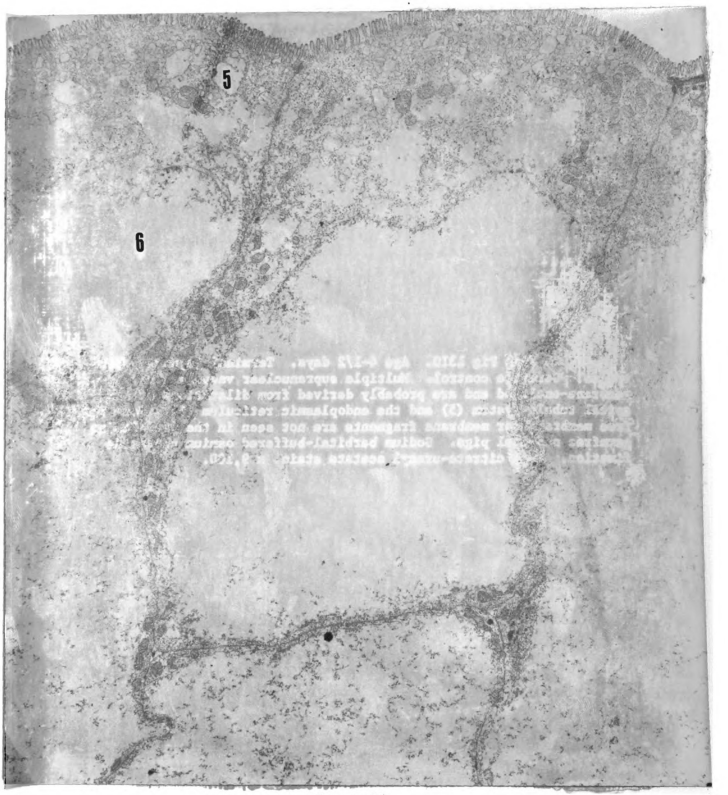


Figure 76

In germfree pigs, 2 to 5 days old, the Golgi complex was generally paranuclear and sometimes the Golgi cisternae were moderately vacuolated (Figures 67, 69 and 77). Occasional abnormal situation of the Golgi complex was associated with marked vacuolization in which the nucleus was pushed into an unusual position within the cell.

Endoplasmic reticulum, both granular and agranular, was recognizable in the apical and basal cytoplasm of the villal epithelial cells (Figures 70, 78 and 79). In some instances, endoplasmic reticulum was compressed or displaced to the periphery of the cell by intracytoplasmic vacuoles (Figures 75 and 78).

Readily identifiable lysosomes and microbodies were not common (Figures 67, 70 and 77), but numerous polyribosomes, glycogen rosettes (identifiable particularly in those sections stained with lead citrate) and absorbed material such as fat droplets, were common both in the cytoplasm and within intracytoplasmic membrane systems (Figures 69, 70, 72, 77, 79, 80 and 81).

The intricate interlocking of the mural invaginations was remarkable (Figures 68 and 82) in tissues from both germfree and monocontaminated animals, but as cells became vacuolate and swollen the complexity of the interdigitations was reduced until in some instances interdigitations were not apparent (Figures 75 and 76).

Generally the villal epithelial cells were evenly placed upon the basal lamina, in close association with the endothelium of the vasculature of the lamina propria (Figures 67 and 69).

Figure 77. Pig 2253. Age 2 days. Terminal jejunum. Basal third of villus. Germfree control. Notable in the cytoplasm are occasional lysosomes, absorbed free intracytoplasmic fat droplets (f), numerous polyribosomes and glycogen rosettes. The interstitium of the lamina propria consists of a few fine collagen strands, chylomicrons and fat droplets in a fluid matrix, a fibroblast, and the vascular structures. It is enclosed within the basal lamina of the epithelium. The endothelial cell of the lymphatic vessel is recognized by its intracytoplasmic fat droplets, relative lack of micropinocytotic vesicles and mitochondria, and delaminating internal membranes. The basal laminae of both endothelium and epithelium are marked (arrow). Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 9,100.

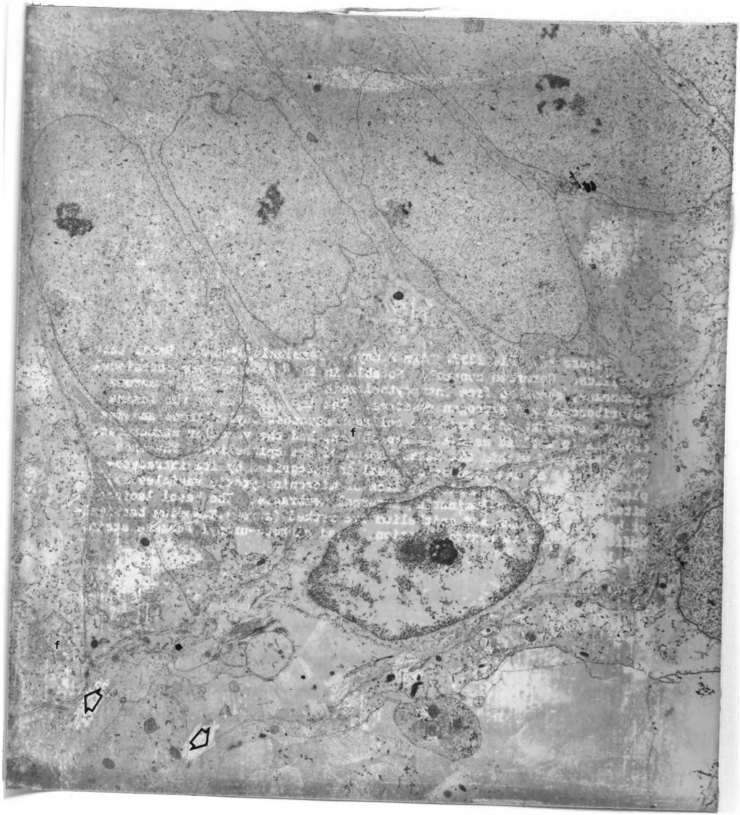


Figure 77

Figure 78. Pig 1310. Age 4-1/2 days. Terminal jejunum. Mid-villus. Germfree control. Granular reticulum (rough endoplasmic reticulum) (Gr) is plentiful in both supranuclear and subnuclear cytoplasm but is sometimes displaced to the periphery of the cell or compressed by encroaching membrane enclosed vacuoles (vm). The lamina propria is seen to consist of an epithelial basal lamina, tissue spaces filled with fluid, a few fine strands of collagen, portion of a fibroblast (F), a capillary endothelial cell and its basal lamina, and erythrocytes (within the lumen of the vessel). Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 13,300.

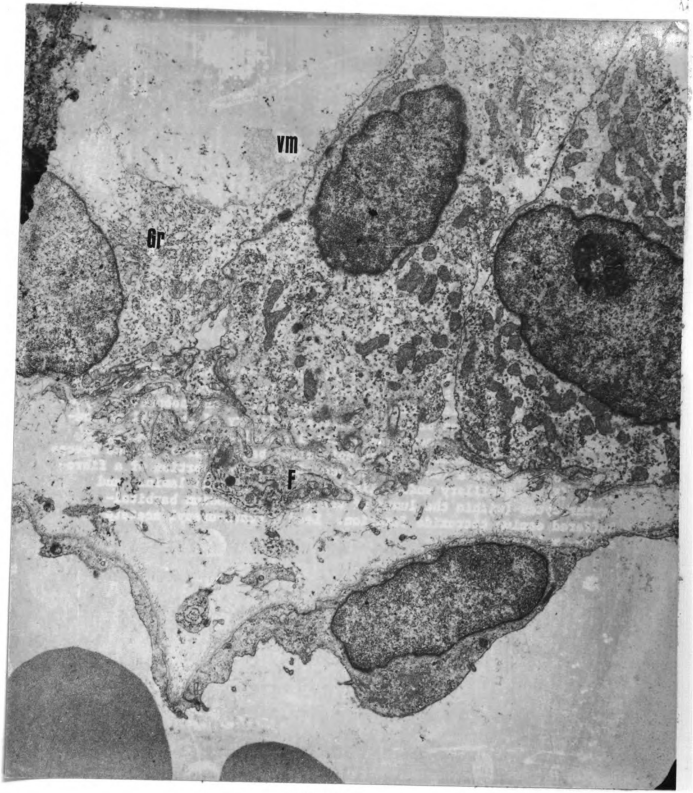


Figure 78

Figure 79. Pig 1001. Age 3-1/2 days. Terminal jejunum. Mid-villus. *E. coli* was introduced *per os* 24 hours before necropsy. From the same field as Figure 80. Two membrane-enclosed absorption vacuoles have displaced the organelles to the base of the cell. Apparent in the basal cytoplasm are normal mitochondria, occasional lysosomes, poly-ribosomes and glycogen rosettes. Within the intermural space are numer-ous fat droplets (f) recently passaged through the apical cytoplasm. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 36,000.

Figure 79

Figure 80. Pig 1001. Age 3-1/2 days. Terminal jejunum. Mid-villus. *E. coli* was introduced *per os* 24 hours before necropsy. A membrane-enclosed absorption vacuole has displaced the organelles to the periphery of the cell. Although from an infected animal, this cell was actively absorbing fluids. The apical tubule system is present. The cristae mitochondriales appear normal. There is active absorption of fat which can be seen as free droplets in the apical cytoplasm and as smaller droplets within the granular reticulum (rough endoplasmic reticulum). The basal absorption vacuole is membrane enclosed and appears to have mechanically restricted further movement of fat from the apical cytoplasm through the basal mural plasmalemma into the intermural space. There is, however, much fat deposited in this site. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 12,800.

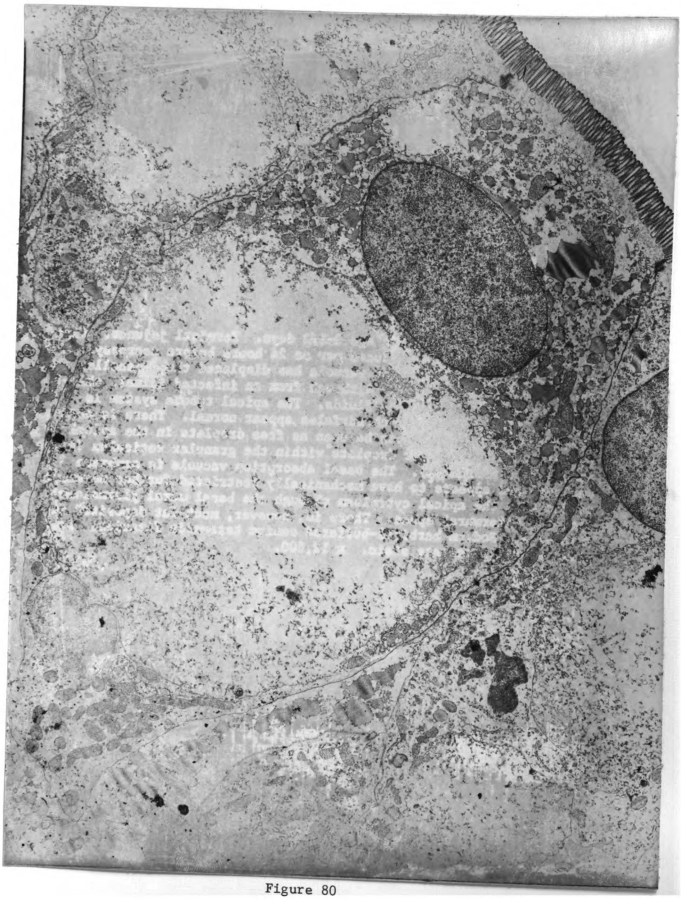


Figure 80

Figure 81. Pig 2251. Age 2-1/2 days. Terminal ileum. Basal third of villus. *E. coli* was introduced *per os* 14 hours before necropsy. Pinocytotic vacuoles and their continuity with elements of the apical tubule system are apparent. Polyribosomes and glycogen rosettes are present within the cytoplasm. The cristae mitochondriales of this cell are in the early stages of vacuolization. There are small nonmembranous vacuoles present in the apical cytoplasm (arrow). These are not clearly associated with either the apical tubule system or endoplasmic reticulum. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 28,000.

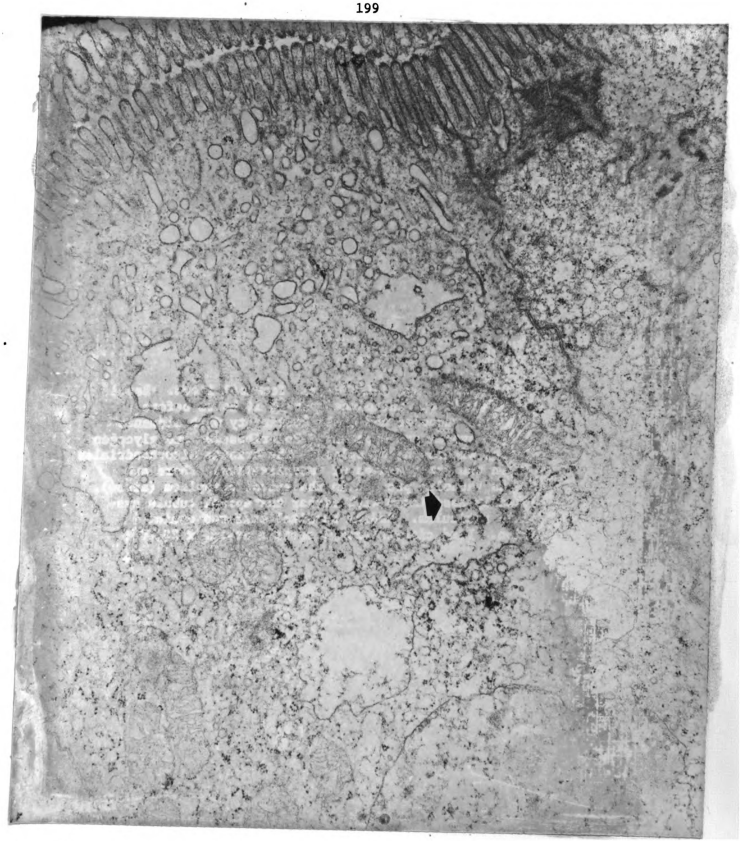


Figure 81

The basal plasmalemma of the villal epithelial cell was closely applied to the basal lamina. Beyond this, toward the lamina propria, interstitial tissue consisting of fluid-filled spaces and a few fine collagen strands separated the bases of the villal epithelial cells from the capillary endothelium, which was distinguished by the number of mitochondria, many micropinocytotic vesicles and its own slender basal lamina (Figures 69, 77 and 78).

Within the villal lamina propria of both jejunum and ileum of 2- to 5-day-old germfree pigs, the basal lamina was thin, of the order of 0.05 μ, and closely followed the basal plasmalemma of the epithelial cells (Figures 69 and 77). The interstices of the lamina propria consisted of fibroblasts, finely dispersed collagen strands in a fluid matrix, occasional inflammatory cells, and chylomicrons presumably traversing the fluid matrix en route to the lymphatic channels. The lacteals, lymphatic vessels and capillaries were invested with a thin basal lamina comparable with that of the villal epithelial cells (Figures 77 and 78).

The interstitial matrix was particularly free of polyribosomes, portions of membranes and other cell fragments. Lymphatic vessels were distinguished by their lumen contents, by the relative lack of endothelial cytoplasmic organelles compared to those of vascular endothelial cells (Figures 77 and 78), their relative lack of laminar folds in the luminal surface, their generally less active micropinocytosis, and the presence of delaminating membranes, not commonly seen within capillaries (Figures 77 and 78).

Eight hours after monocontamination with *E. coli* 0138:K81:NM, changes in the villal epithelial cells were not sufficiently distinct for positive identification of infected tissues. There was an apparent slight vacuolar swelling of the cristae mitochondriales of many cells that was not seen in otherwise comparable cells from germfree animals (Figure 73).

Often in other cells, the cytoplasmic matrix was seen to be uniformly much less dense than normal. There was in these cells relatively more membrane-lined vacuoles than normally seen in vacuolate cells from germfree animals. Otherwise, vacuoles were seen to be membrane-enclosed and generally no different in character from those in the cells of control pigs (Figure 73). In the light of subsequent observations it was apparent that these changes, though not pathognomonic of a diseased cell, were slight developmental changes in a process to be seen much more clearly in villal epithelial cells exposed to the test organism for longer periods of time.

Although villal epithelial cells from both the basal and midvillal regions of the ileum and jejunum were examined electron microscopically, no detectable differences were observed, related to tissue site or villal position, in the reaction of the villal epithelial cells to exposure to *E. coli* 0138:K81:NM. Generally it was noted that less mature cells at the base of the villus were minimally vacuolate and had fewer changes, of a less marked degree, than those from the midvillus.

Fourteen hours after exposure there were a number of changes in the villal epithelial cells. The cristae mitochondriales had become distinctly vacuolate (Figures 74, 81, 82, 83, 84, 85 and 86).

Figure 82. Pig 2251. Age 2-1/2 days. Terminal ileum. Basal third of villus. *E. coli* was introduced *per os* 14 hours before necropsy. The intricate interlocking of the mural plasmalemmae of neighboring cells is apparently not affected although vacuolization of the cristae mitochondriales, an early sign of degeneration, is present. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 28,000.

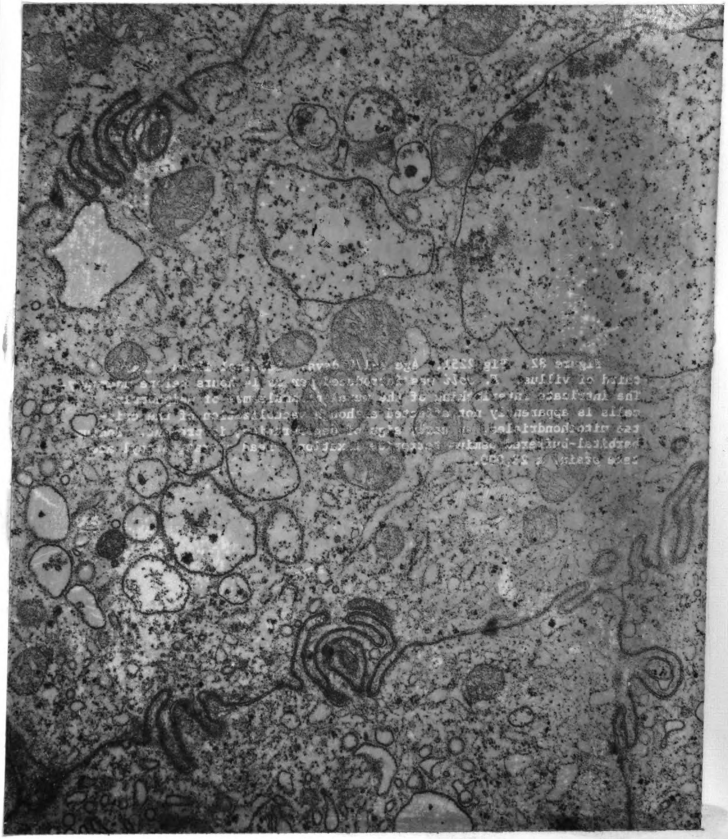


Figure 82

Figure 83. Pig 2251. Age 2-1/2 days. Terminal ileum. Midvillus. *E. coli* was introduced *per os* 14 hours before necropsy. There is early vacuolization of the cristae mitochondriales, otherwise these cells closely resemble actively absorbing cells from comparable tissues of germfree neonatal pigs. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 14,700.

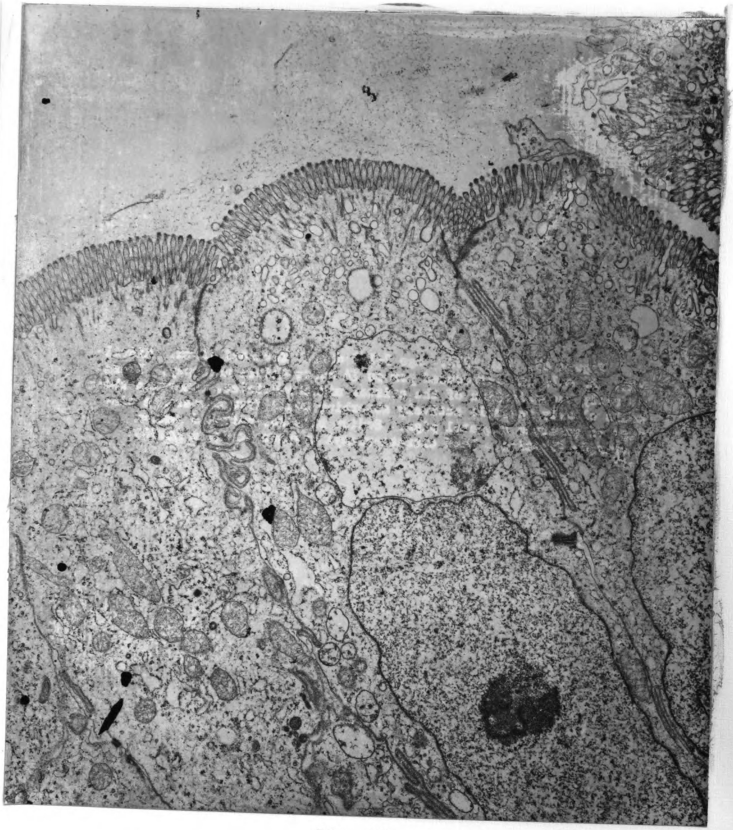


Figure 83

Figure 84. Pig 2251. Age 2-1/2 days. Terminal ileum. Midvillus. *E. coli* was introduced *per os* 14 hours before necropsy. There is mild, but distinct, vacuolization of the cristae mitochondriales associated with infection. Granular reticulum, Golgi cisternae, polyribosomes, glycogen rosettes and the nuclear structures appear normal, but the close approximation of foreign matter to the surface of the brush border is evidence of absence, or reduction in depth, of the glycocalyx. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 28,000.

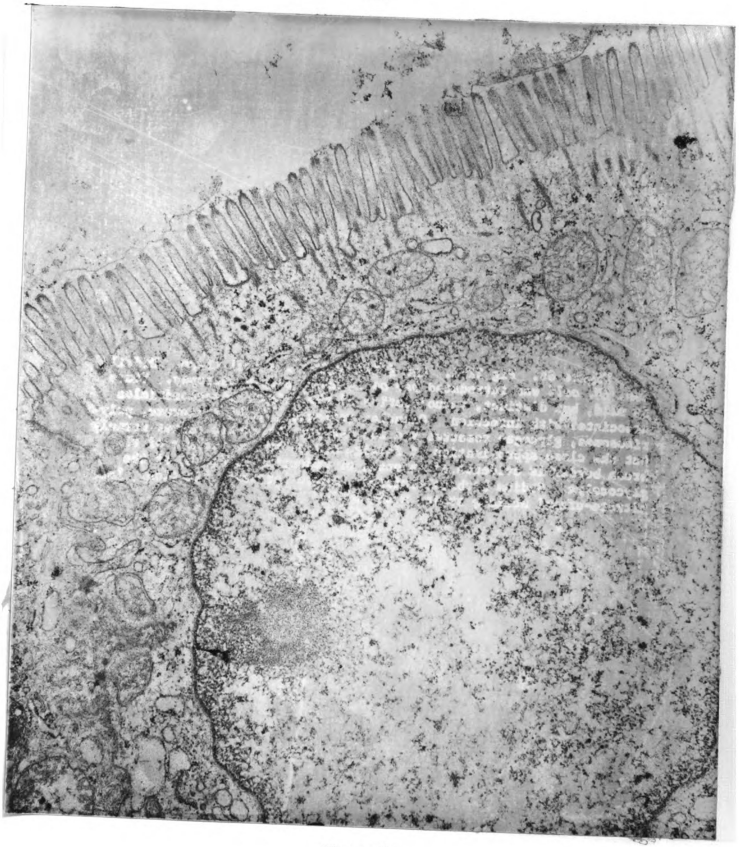


Figure 84

Figure 85. Pig 2251. Age 2-1/2 days. Terminal ileum. Midvillus. *E. coli* was introduced *per os* 14 hours before necropsy. There is slight but distinct vacuolization of the cristae mitochondriales. The microvilli appear normal, but the lack of stainable material between microvilli and the close approximation of a foreign particle to the tips of the microvilli are indicative of the absence or marked reduction in depth of the glycocalyx. The cytoskeleton is present, but its foundation in a terminal web is inapparent. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 46,200.

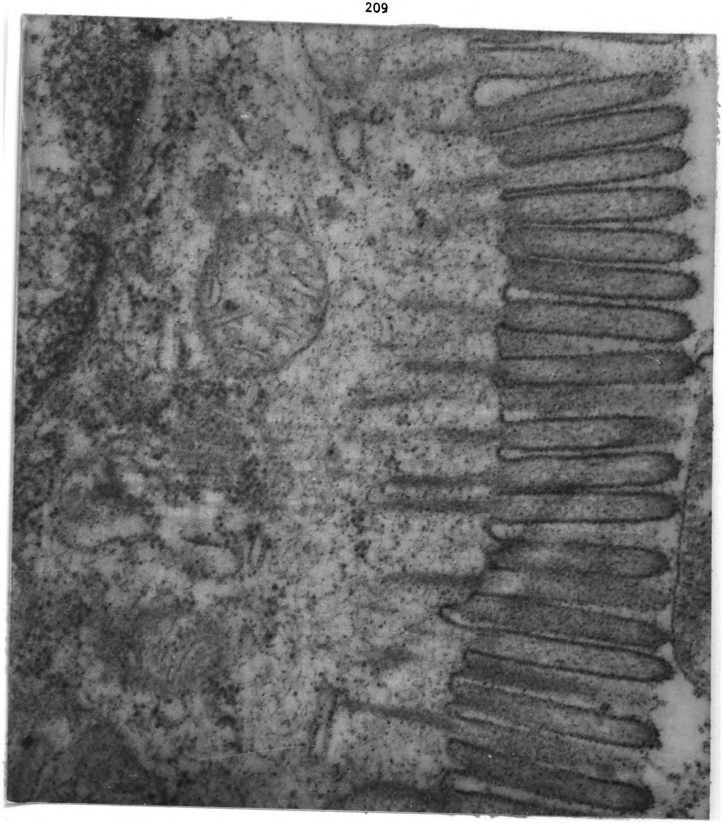


Figure 85

Figure 86. Pig 2252. Age 2-1/2 days. Terminal jejunum. Mid-villus. *E. coli* was introduced *per os* 14 hours before necropsy. There is slight but distinct vacuolization of the cristae mitochondriales. Small nonmembranous vacuoles, apparently not associated with endoplasmic reticulum, are present in the basal cytoplasm of the villal epithelial cells. Associated with these retrogressive changes is the presence of increased amounts of relatively clear interstitial fluid (f). Marked endothelial micropinocytosis (arrow) and internal laminar folding are characteristic of normal endothelial function. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 17,500.

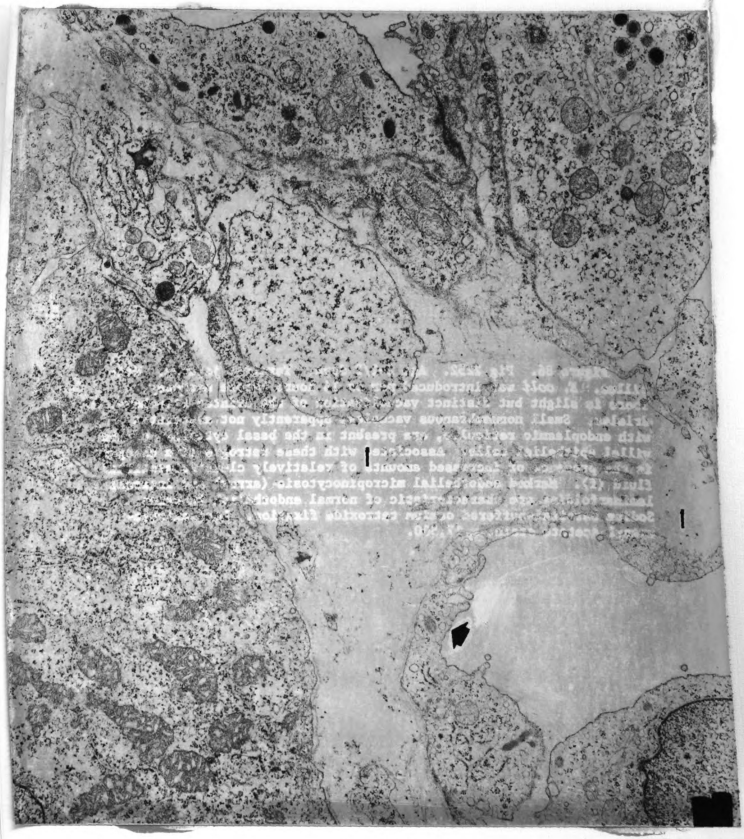


Figure 86

In addition to the presence of membrane-enclosed vacuoles, vacuoles not enclosed by a membrane, henceforth termed "nonmembranous vacuoles", were observed in the cytoplasm (Figures 81, 86, 87 and 88) of the villal epithelial cells. These became even more pronounced in cells exposed to *E. coli* for longer periods of time (Figures 89 and 90). In some cells they were so large that they included almost the entire cytoplasmic space, partly or completely enclosing membrane-lined vacuoles (Figure 91).

It was also at about 14 hours after inoculation that distinct evidence of rupture of the membranous vacuoles was seen (Figure 87) which in animals inoculated for even longer periods resulted in confluence of membranous vacuoles (Figure 92) and vacuolization of almost the entire cell (Figures 87, 88, 89, 91 and 92).

As was seen and recorded during histopathologic examination, it was possible to see in neighboring villi epithelial cells in many different stages of degeneration ranging from normalcy, to marked vacuolization but still associated with effective absorption (Figures 79 and 80) (as was characteristic of actively absorbing cells from germfree control neonates), to complete disruption of the cells (Figures 88, 89, 90 and 91).

In infected pigs there were villal epithelial cells in which there was positive evidence of active absorption, even though these cells were sometimes grossly distended with vacuoles. In these cells the intracytoplasmic vacuoles were membrane-lined, there was no extensive nonmembranous vacuolization, and the mitochondria were normal in appearance with evenly spaced, nonvacuolate cristae

Figure 87. Pig 2251. Age 2-1/2 days. Terminal jejunum. Midvillus. *E. coli* was introduced *per os* 14 hours before necropsy. The continuity of the bases of the microvilli with the apical tubule system is clearly demonstrated (arrow). Nonmembranous vacuolization is present in the apical cytoplasm and there is clear evidence of rupture of the membrane of the absorption vacuole so that the contents of the two vacuoles are confluent. Despite these signs of cell damage, the brush border is apparently normal and the interdigitations of the intermural plasmalemmae appear to be unaffected. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 12,800.

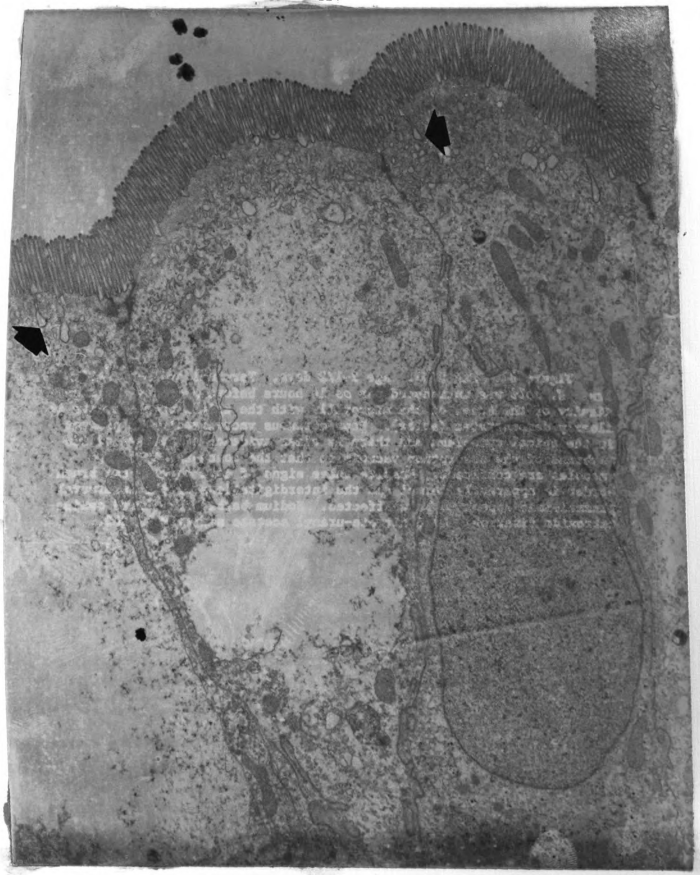


Figure 87

Figure 88. Pig 2251. Age 2-1/2 days. Terminal jejunum. Mid-villus. *E. coli* was introduced *per os* 14 hours before necropsy. Non-membranous vacuolization of almost the entire apical cytoplasm includes elements of the apical tubule system, mitochondria, and the endoplasmic reticulum. The brush border, on morphologic grounds alone, is incapacitated. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 18,400.

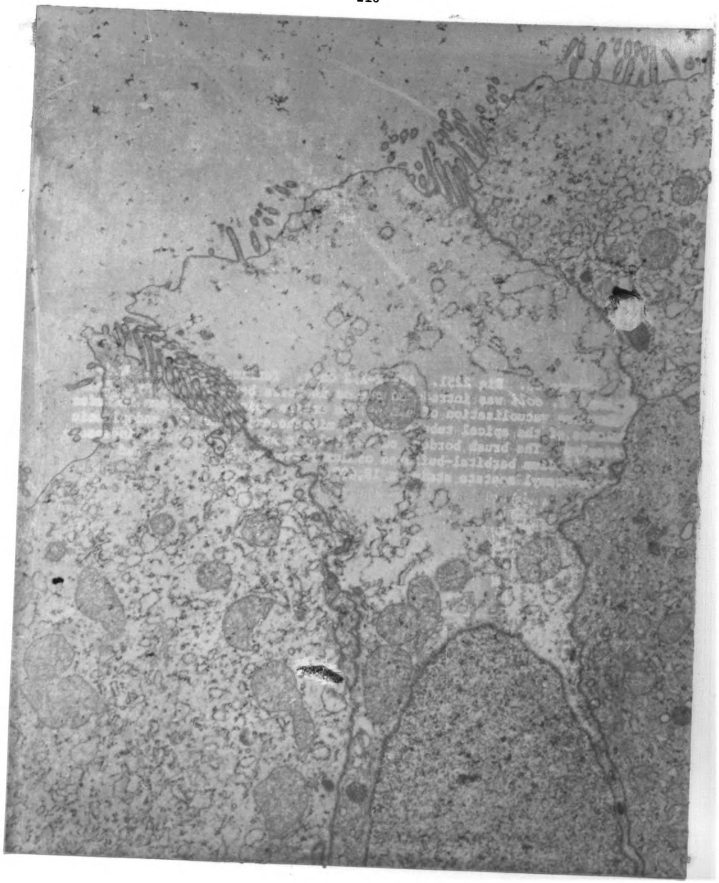


Figure 88

Figure 89. Pig 1001. Age 3-1/2 days. Terminal jejunum. Mid-villus. *E. coli* was introduced *per os* 24 hours before necropsy. Slight but distinct vacuolization of the cristae mitochondriales is apparent. Multiple nonmembranous vacuolization of the cytoplasm includes membrane lined vacuoles, organellar fragments, portions of disrupted endoplasmic reticulum and cell debris. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl actetate stain. x 9,100.

Figure 89

Figure 90. Pig 1001. From the same field as Figure 89. Detail of disruption of the cytostructure by membrane-lined, and nonmembranous, vacuoles. In a neighboring cell there is vacuolization of the cristae mitochondriales. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 17,500.

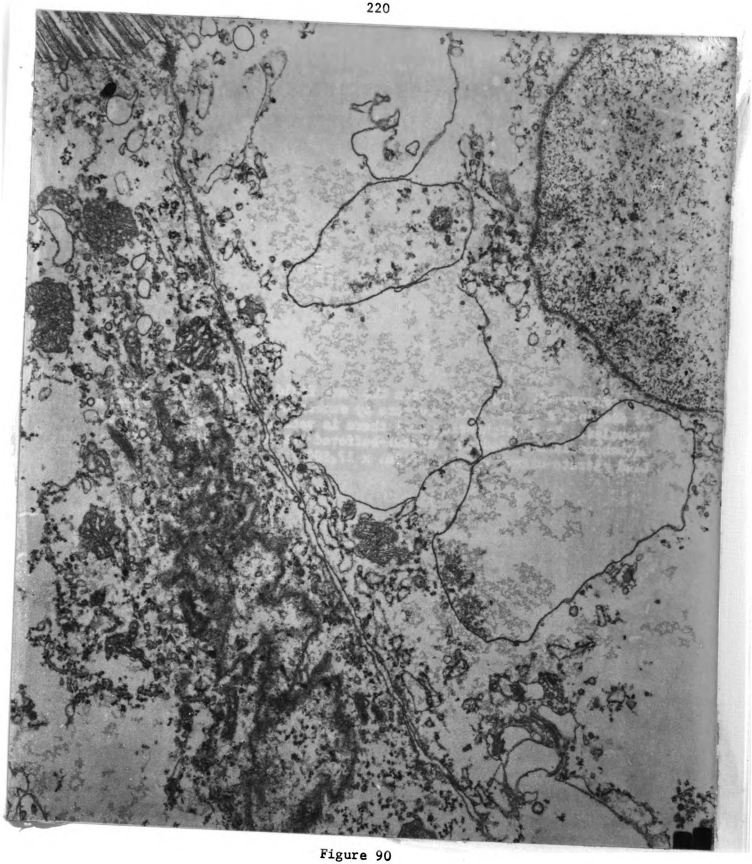


Figure 90

Figure 91. Pig 1001. Age 3-1/2 days. Terminal jejunum. Mid-villus. *E. coli* was introduced *per os* 24 hours before necropsy. Non-membranous vacuolization of almost the entire cytoplasmic space, includes cell debris, the nucleus and other organelles, together with many membrane lined vacuoles and tubular fragments. Marked internal vacuolization and disarray of the cristae of many of the mitochondria (M) is clearly apparent. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 14,400.

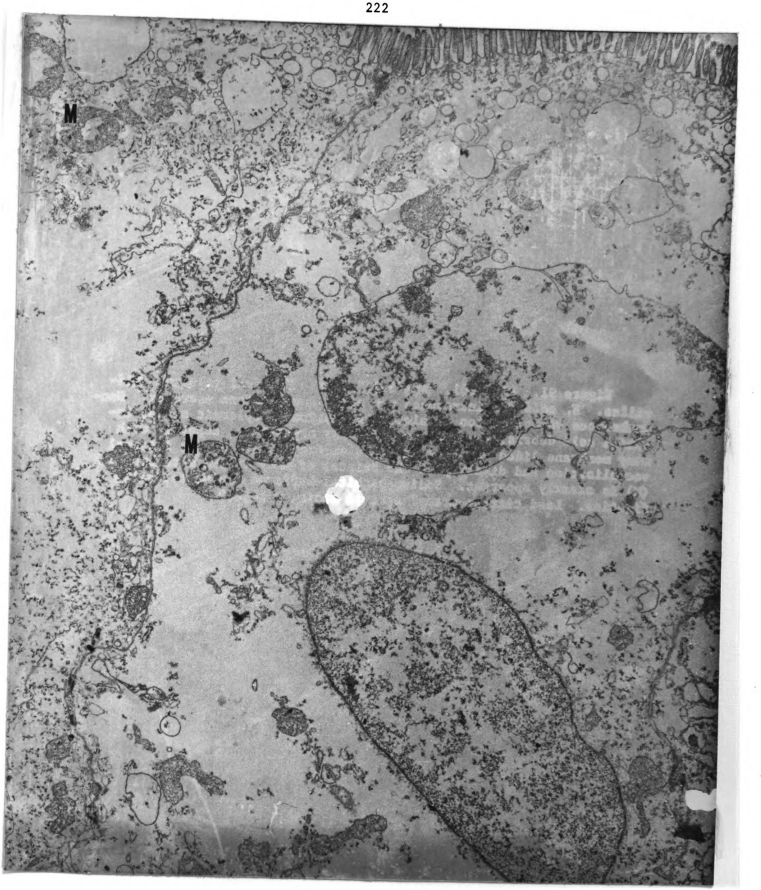


Figure 91

Figure 92. Pig 2258. Age 3 days. Terminal jejunum. Midvillus. *E. coli* was introduced *per os* 18 hours before necropsy. The contents of the multiple membranous vacuoles in the apices of these vilial epithelial cells become confluent as their separating membranes rupture. Eventually, most of the cytoplasmic space is occupied by the vacuoles. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 11,200.

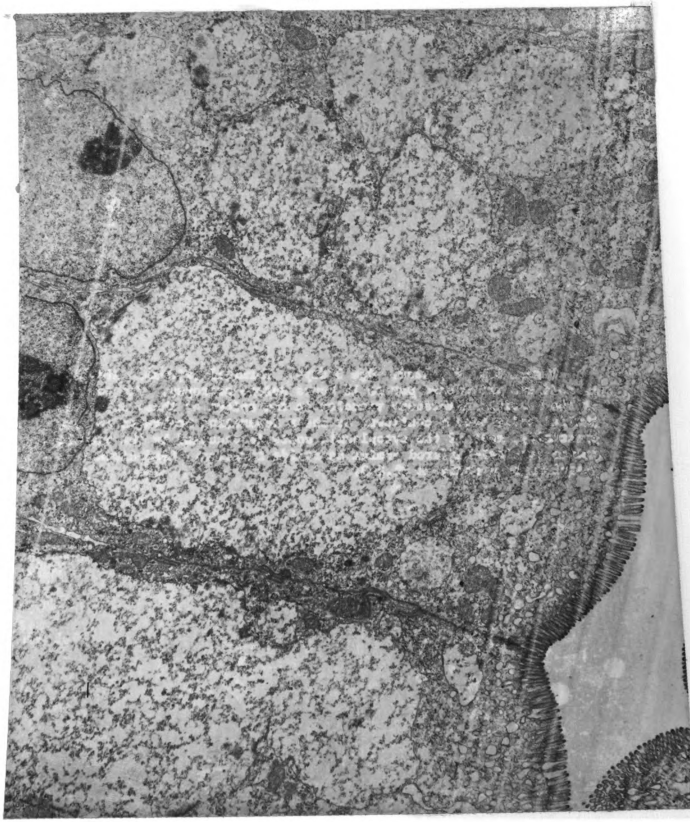


Figure 92

mitochondriales. Copious amounts of absorbed material such as lipid were present in the cytoplasmic matrix, within the endoplasmic reticulum, and in the basal intercellular (intermural) space (Figures 79 and 80).

In epithelial cells in which there were early signs of mitochondrial degeneration, but not extensive vacuolization, the interdigitations of the cell membrane with those of neighboring cells was apparently unaffected (Figures 82 and 87).

There was little change in the brush border that could be ascribed to the effects of exposure of this structure to *E. coli* for a period of 8 to 18 hours. In some cells with mitochondrial changes, the brush border appeared normal (Figures 81, 83 and 84), yet in some instances there was evidence (Figures 84 and 85) that the glycocalyx was very much reduced or missing. Variations in the height and density of the brush border were inconsistent, and only in grossly disrupted cells was it apparent that the brush border was morphologically incapable of function (Figure 88). In other cells undergoing early degenerative changes the brush border was remarkable for its density, depth and integrity (Figure 87).

It was observed that the microvilli were generally longer in the less mature cells and that, unless the degeneration associated with infection was very marked, the further along the villus that cells were sampled, the shorter were the microvilli (Figures 67 and 70).

Between 8 and 24 hours after infection there was observed to be an increase in the amount of interstitial fluid present in the

intramembranous space of the lamina propria of the villi. Sometimes this was clear fluid, and in these instances the associated capillary or lymphatic endothelial cells were micropinocytotically active (Figures 86 and 93). Such edema was associated with villal epithelial cells in which early retrogressive changes were apparent (Figure 86). At times the interstitial fluid contained free floating cytoplasmic membrane fragments, occasional chylomicrons and unidentified particulate matter (Figure 94). The basal laminae of the capillaries were seen to be much thicker at this stage than the basal lamina of the neighboring epithelium. Again, in other instances, the interstitium of the basement membrane was seen to be edematous but with additional collagen fibers present. In these instances the capillaries were less micropinocytotically active (Figure 95).

By 24 hours after inoculation, the contaminating organisms had become so plentiful in the intestinal tract that sections of the organisms were found during the electron microscopic examination. *Escherichia coli* 0138:K81:NM was seen to be nonfimbriated and of characteristic and readily identifiable structure (Figures 96, 97, 98 and 99).

At no stage of this investigation were organisms seen within the villal epithelial cells, nor was there evidence of *E. coli* organisms having entered the apical plasmalemma, apical tubule system, or any of the cytoplasmic substance of the villal epithelial cells.

Where the organisms were in close association with, and possibly attached to, the brush border, the microvilli were seen to be

Figure 93. Pig 1001. Age 3-1/2 days. Terminal jejunum. Mid-villus. *E. coli* was introduced *per os* 24 hours before necropsy. There are increased amounts of interstitial fluid containing suspended chylomicrons, flocculent material, and fine collagen fibrils. Slight micropinocytotic activity and relative lack of laminar folding is characteristic of normal lymphatic endothelium. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 11,200.

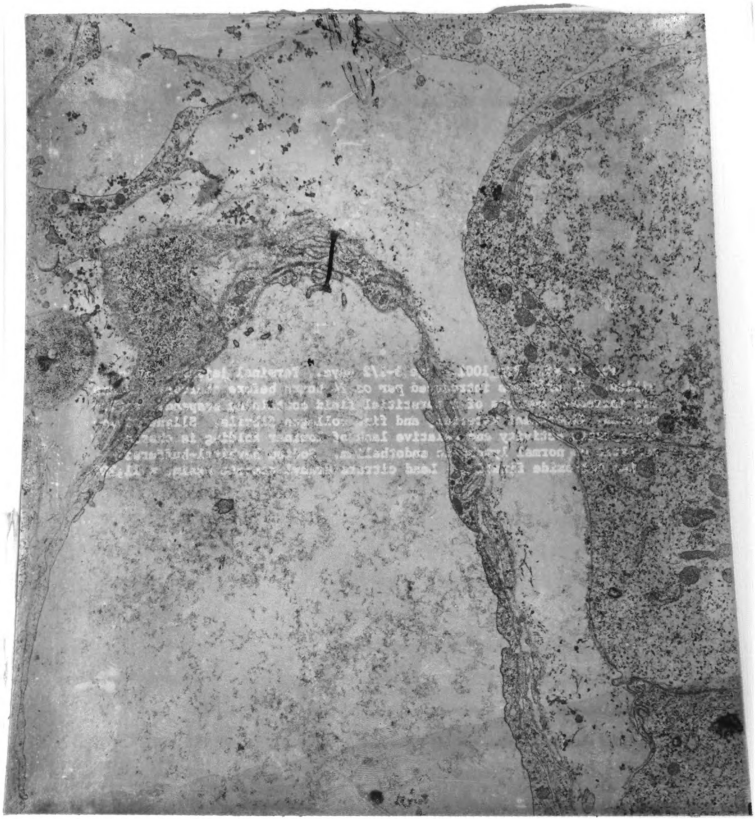


Figure 93

Figure 94. Pig 2258. Age 3 days. Terminal jejunum. Midvillus. *E. coli* was introduced *per os* 18 hours before necropsy. The interstitial tissue fluid of the lamina propria of this villus contains free, floating, cytoplasmic membrane fragments, occasional chylomicrons, collagen fibrils and unidentified flocculent material. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 23,800.

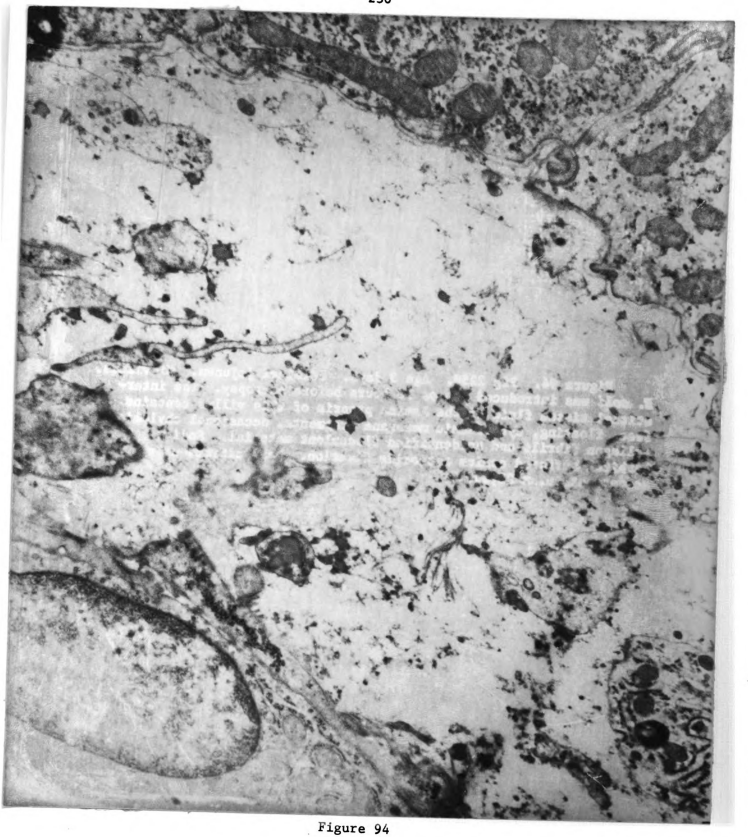


Figure 94

Figure 95. Pig 2251. Age 2-1/2 days. Terminal jejunum. Mid-villus. *E. coli* was introduced *per os* 14 hours before necropsy. There is marked interstitial thickening and edema of the lamina propria of this villus. Compared with other sections, there is extensive collagen production, increased thickness of the capillary basal laminae, and reduced endothelial micropinocytosis. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 11,200.

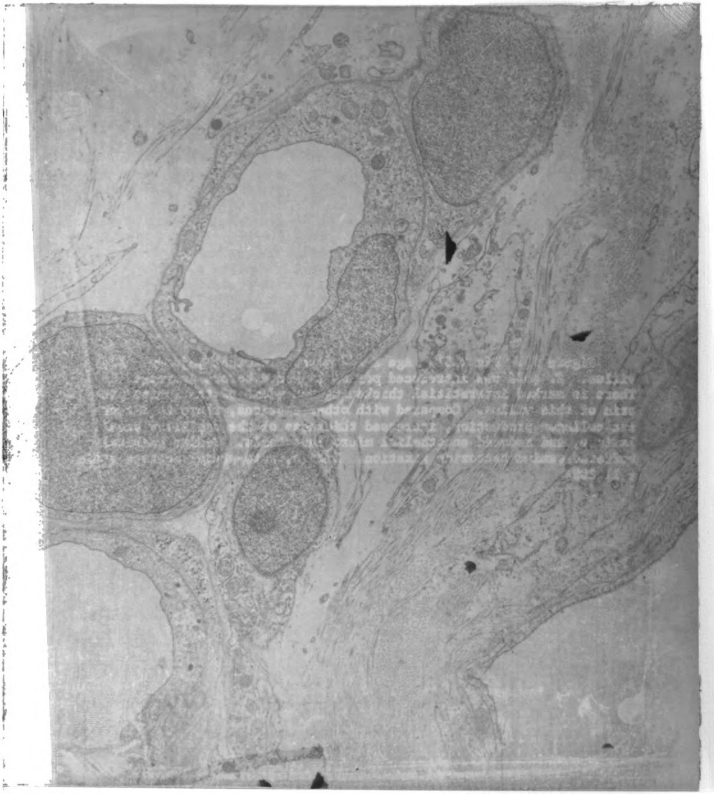


Figure 95

Figure 96. Pig 1001. Age 3-1/2 days. Terminal ileum. Basal third of the villus. *E. coli* was introduced *per os* 24 hours before necropsy. The monocontaminating organisms are of characteristic structure and are nonfimbriated. They are closely associated with atrophic microvilli, whereas it can be seen that the *E. coli* organisms are separated from the unaffected microvilli by a relatively uniform, nonstaining layer, presumably a glycocalyx. There are some slight changes in the cristae mitochondriales of the affected cell; however, the Golgi complex and its cisternae appear to be normal. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 28,000.

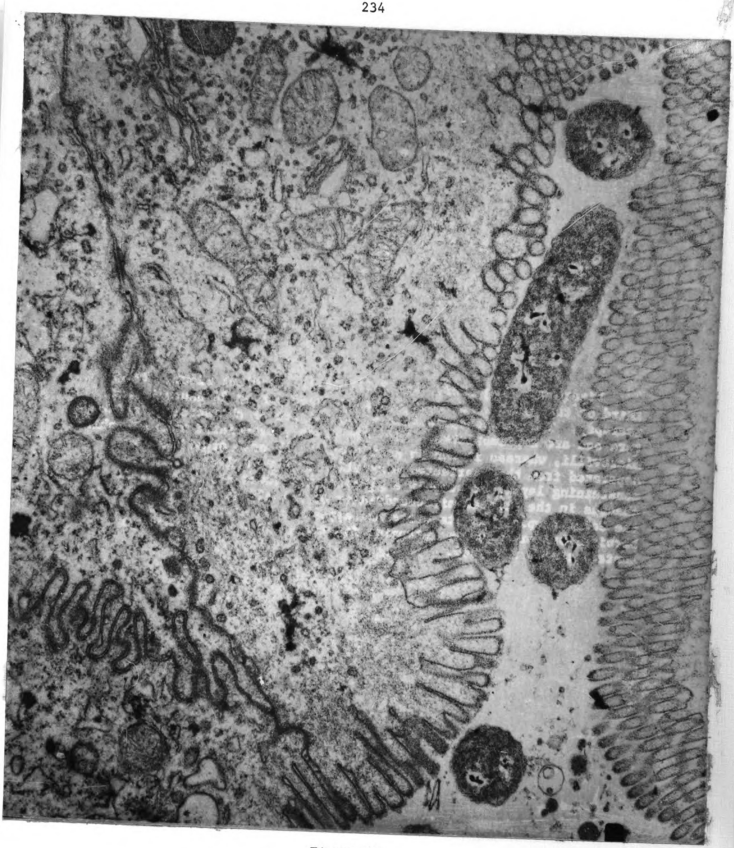


Figure 96

Figure 97. Pig 1001. Age 3-1/2 days. Terminal ileum. Basal third of the villus. *E. coli* was introduced *per os* 24 hours before necropsy. The organisms are nonfimbriated and are closely associated with atrophic microvilli of the apical plasmalemma of a mature affected cell (above), its maturity characterized by the presence of an apical tubule system. The microvilli of the younger villal epithelial cell (it has no apical tubule system) have had less contact with the *E. coli* organisms and are apparently unaffected. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 28,000.

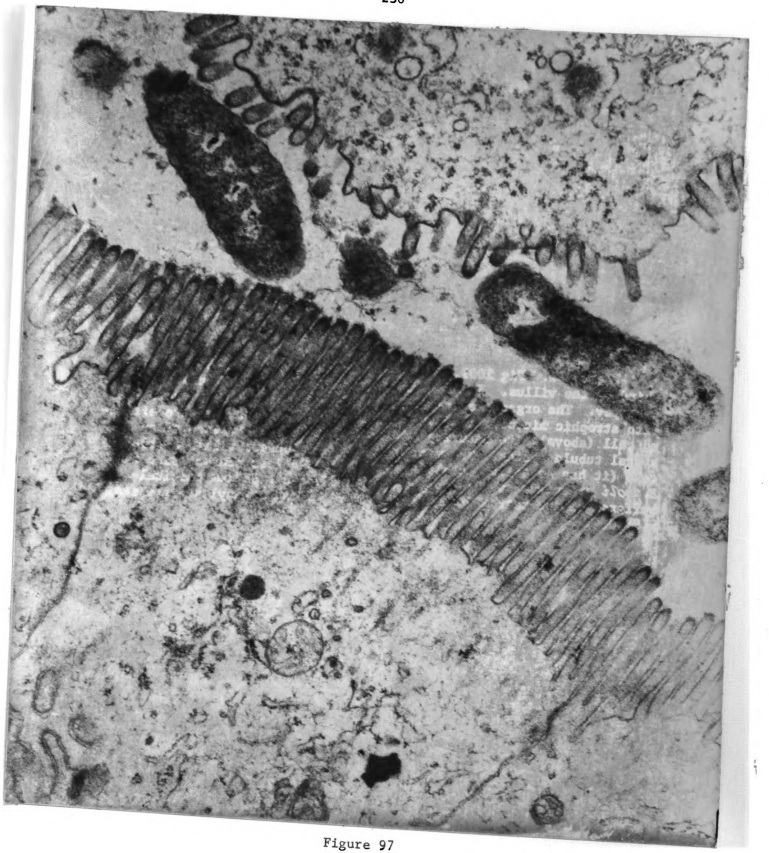


Figure 97

Figure 98. Pig 1001. Age 3-1/2 days. Terminal ileum. Basal third of villus. *E. coli* was introduced *per os* 24 hours before necropsy. Transverse sections of *E. coli* 0138:K81:NM reveal a characteristic internal structure. There is no evidence of flagella or fimbriae in the capsule of these organisms. Although the microvilli of the least mature cell in this field appear unaffected (there is some evidence of a meagre hirsute layer at the lumen surface) there is slight vacuolization of the cristae within the apical mitochondria. Sodium barbital-buffered osmium tetroxide fixation. Uranyl acetate stain. x 32,200.

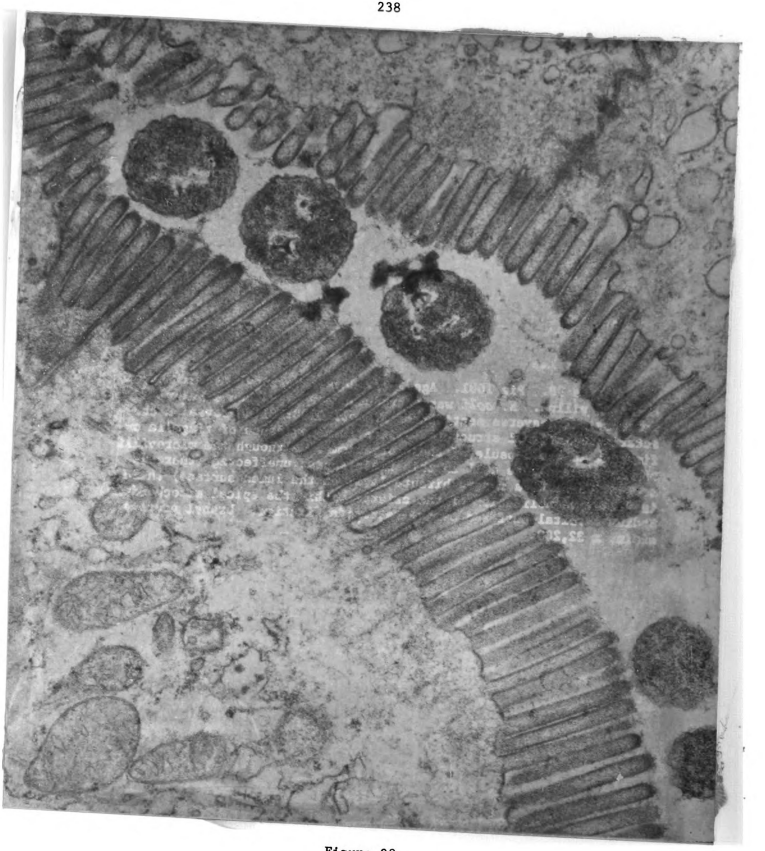


Figure 98

Figure 99. Pig 1001. Age 3-1/2 days. Terminal ileum. Basal third of villus. *E. coli* was introduced *per os* 24 hours before necropsy. The plasma membrane of *E. coli* 0138:K81:NM (arrow) is a trilaminar structure. The microvilli in close association with the bacterial wall are markedly atrophied, and cytoskeletal structure is not apparent. The trilaminar structure of the membrane of both the microvilli and the apical tubules can still be seen. There is presumptive evidence of either a thin electron-transparent bacterial capsule, or the remnants of an electron-transparent glycocalyx, between the tips of the microvilli and the external trilaminar membrane of the microorganism. There is no evidence of flagella or fimbriae. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 133,700.

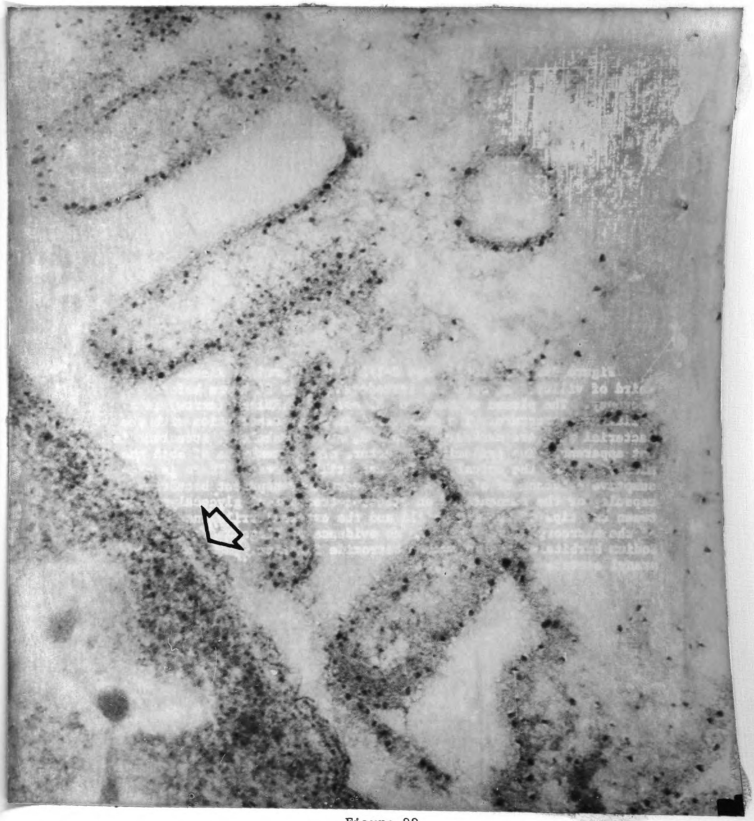


Figure 99

markedly foreshortened into stubby or globular, sometimes branched, forms in which the glycocalyx was reduced and in which neither the cytoskeleton nor its foundation in a terminal web could be detected (Figures 96, 97 and 99). Early vacuolar changes were detected in the cristae mitochondriales of nearby mitochondria (Figures 96 and 100) and at times these mitochondrial changes were observed in cells in which morphologic changes were not yet apparent in the microvilli (Figure 98).

Transverse sections of microvilli from comparably affected cells were examined. The proximity of neighboring microvilli was suggestive that the glycocalyx was reduced in depth (Figure 101). The lack of cytoskeletal detail in these microvilli compared to transverse sections of microvilli from villal epithelial cells of germfree control animals (Figure 71) was apparent and coincided with the observations made of longitudinal sections of microvilli from monocontaminated animals.

Twenty-four hours after inoculation, the vacuolar degeneration and disruption of organellar membranes was clearly apparent (Figures 89, 90 and 100). In more advanced degenerative states, the nuclei and membranous vacuoles were seen to be floating free in a matrix of fluid and suspended mitochondria, tubular fragments, membrane fragments, polyribosomes, and cytoplasmic debris (Figure 91).

By 32 hours after inoculation, in affected cells the mitochondria were swollen, with disruption of the cristae mitochondriales, the vacuolar degenerative changes were maximal, and only traces of normal cytostructure remained (Figure 102). Vacuolate cells became

Figure 100. Pig 1001. Age 3-1/2 days. Terminal ileum. Midvillus. *E. coli* was introduced *per os* 24 hours before necropsy. There is one markedly swollen mitochondrion, in others there is distinct vacuolization of the cristae mitochondriales; the microvilli appear foreshortened and their cytoskeletal structures are not apparent. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 28,000.

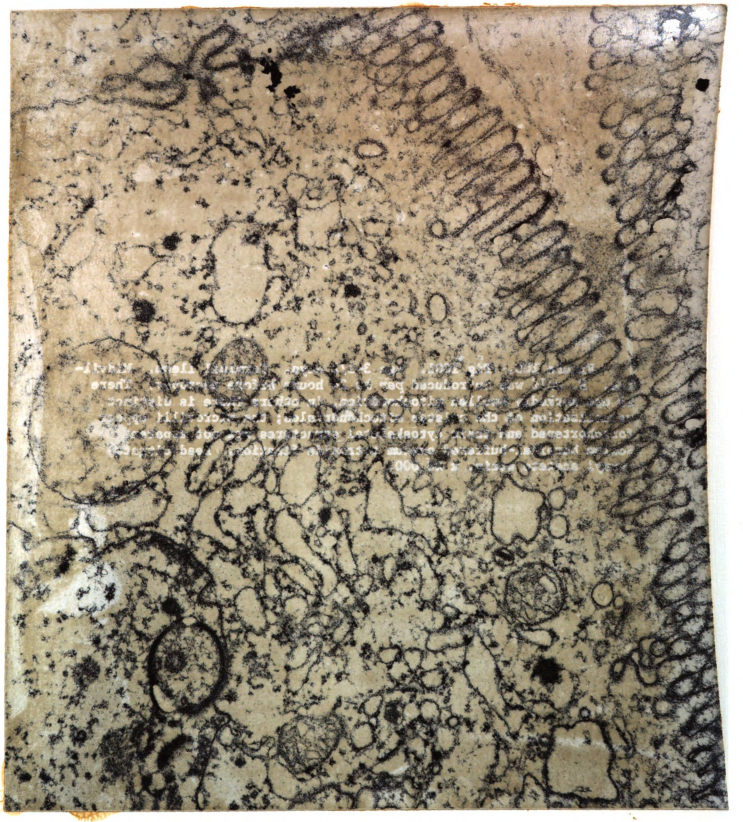


Figure 100

Figure 101. Pig 1001. Age 3-1/2 days. Terminal ileum. Midvillus. *E. coli* was introduced *per os* 24 hours before necropsy. Transverse section of microvilli from cells nearby to those shown in Figure 100. The proximity of neighboring microvilli is suggestive that the glycocalyx is reduced in depth, although in some areas a hirsute layer is present where the preparation is adequate for the representation of fine detail of the trilaminar membrane (arrow). Cytoskeletal detail is lacking. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 104,300.

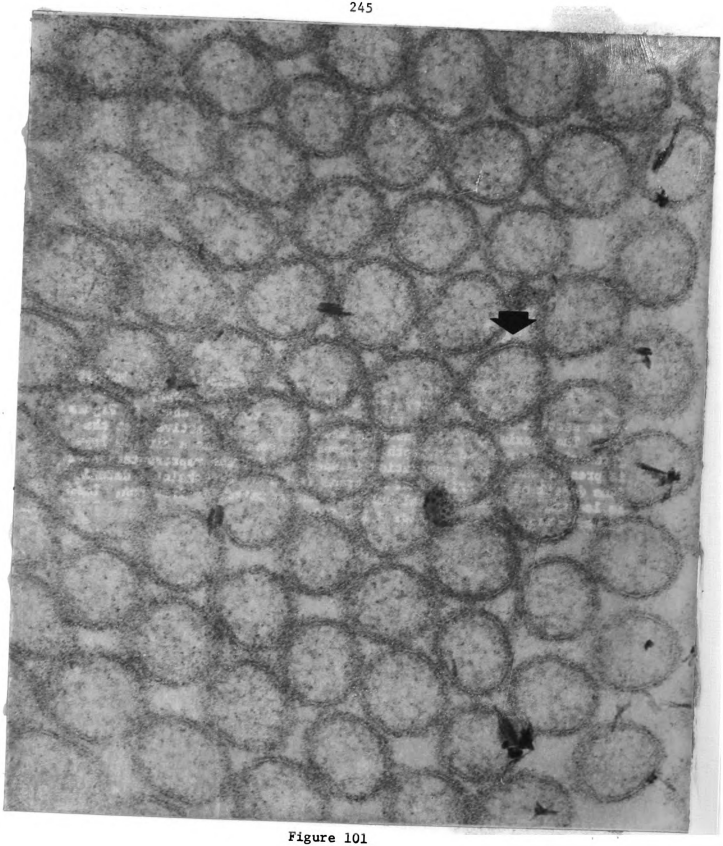


Figure 101

Figure 102. Pig 1006. Age 4 days. Terminal ileum. Midvillus. *E. coli* was introduced *per os* 32 hours before necropsy. Little of the normal cytostructure is retained in this vacuolate cell. There is marked disarray of the apical plasmalemma and microvilli; the mitochondria (M) are swollen and broken, and there is disruption and degeneration of the cristae mitochondriales. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 13,500.

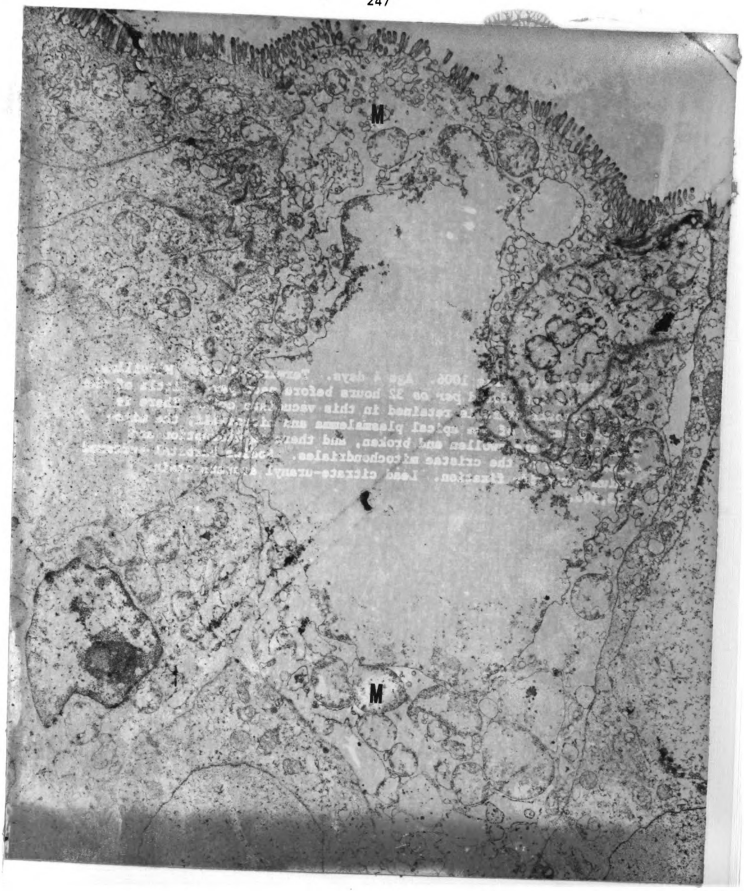


Figure 102

confluent with similarly degenerated neighboring cells, and the confluent cytoplasm consisted of broken membranes and organellar fragments floating in a fluid matrix (Figure 103). Eventually the apical plasmalemma was seen to be broken and the disrupted cell contents released to the lumen of the intestine (Figure 104).

By 96 hours after inoculation the only additional remarkable feature was that of the degree of collagen formation in the intramembranous space of the midvillal lamina propria (Figure 105). Such collagen formation was not a feature of the midvillal lamina propria of jejunal or ileal villi of germfree control neonatal pigs.

Figure 103. Pig 1006. Age 4 days. Terminal jejunum. Midvillus. *E. coli* was introduced *per os* 32 hours before necropsy. The confluent cytoplasm of neighboring degenerate cells consists of broken membranes and organellar fragments floating in a fluid matrix. The nucleus appears relatively unaffected. Although now separated from their attachment to the parent cells, the interdigitated mural plasmalemmae remain adherent as a unit (arrow). Sodium barbital-buffered osmium tetroxide fixation. Uranyl acetate stain. x 22,500.

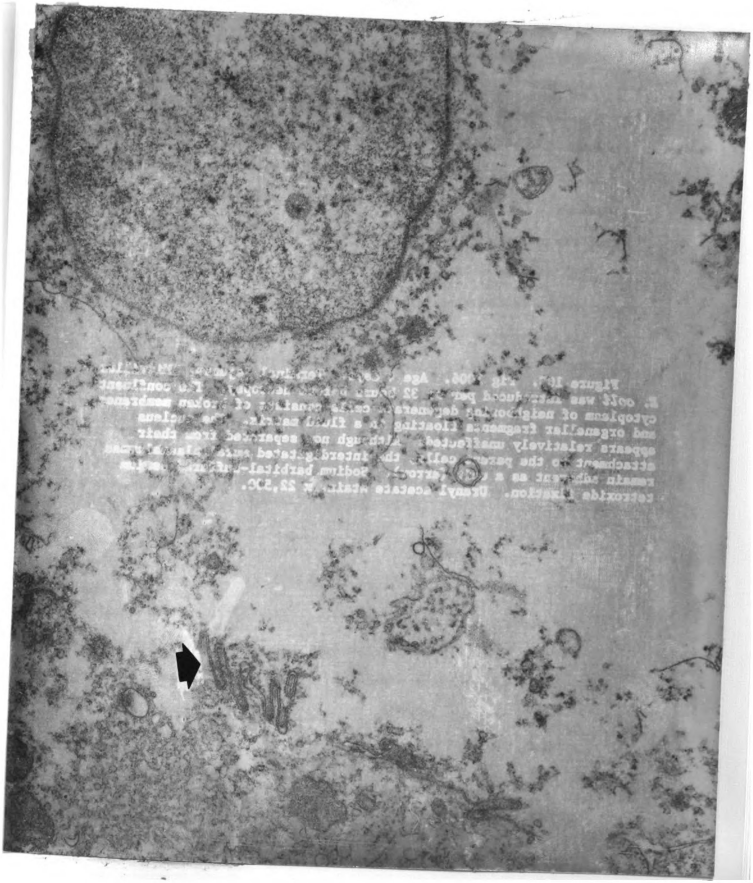


Figure 103

Figure 104. Pig 1006. Age 4 days. Terminal ileum. Midvillus. *E. coli* was introduced *per os* 32 hours before necropsy. Vacuolization and disruption of the cytoplasmic structure, associated with morphologic change in the apical plasmalemma, have resulted in rupture of the brush border and extrusion of the cell contents toward the lumen of the intestine. Sodium barbital-buffered osmium tetroxide fixation. Lead citrate-uranyl acetate stain. x 18,900.

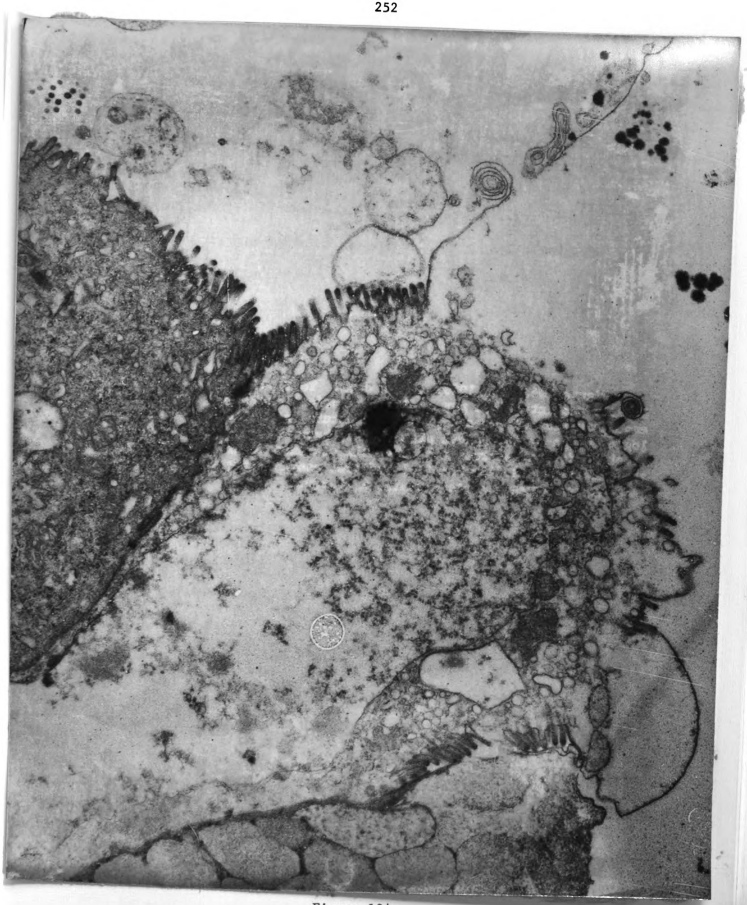


Figure 104

Figure 105. Pig 1009. Age 6-1/2 days. Terminal jejunum. Mid-villus. *E. coli* was introduced *per os* 96 hours before necropsy. With prolonged monocontamination the intramembranous space of the midvillal lamina propria is markedly infiltrated with collagen. Such collagen formation is not a feature of comparable tissues from germfree pigs but may well be present in the laminae propriae of conventional, clinically normal, neonatal pigs. Sodium barbital-buffered osmium tetroxide fixation. Uranyl acetate stain. x 9,100.

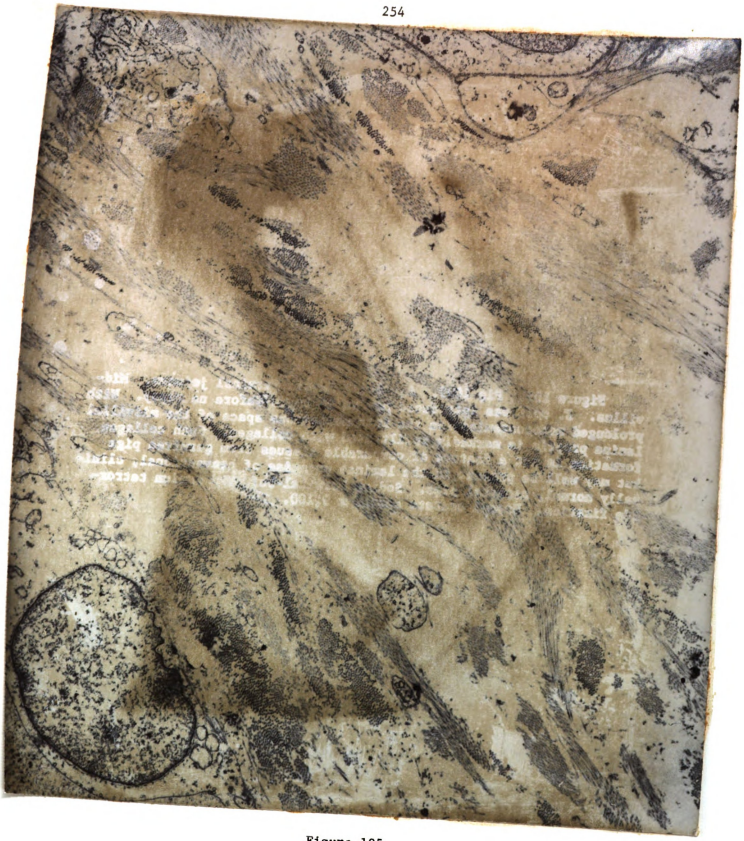


Figure 105

DISCUSSION

> "The other error...is that of thinking Science to consist in mere observation, and that the accumulation of records, without generalization, hypothesis, or the use of instruments which the understanding has forged, can ever be of the slightest advantage in the advancement of *knowledge*".
>
> F. G. Crookshank, 1922

The events leading to diarrhea in the neonate are interrelated and occur sequentially and often simultaneously. For the sake of clarity, and in relation to the voluminous literature that is available on the enteritides, malabsorption and diarrhea, these events will be discussed separately, somewhat along disciplinary lines.

The difficulties encountered by Barnum *et al.* (1967) in producing enteric colibacillosis under controlled experimental conditions and used by these authors as "...the single greatest argument against a primary causative role for *E. coli* in the disease" (colibacillary diarrhea) have not been encountered in this laboratory. The fact that, under gnotobiotic conditions, the administration of known numbers of organisms of a specific serotype of *E. coli* to neonatal gnotobiotic pigs is consistently followed some hours later by severe diarrhea in these animals; that consistently the same organism can be recovered in pure culture from these animals and not from clinically unaffected, germfree litter mates kept under identical conditions; and that the recovered organism will reinfect

experimental neonatal pigs, is demonstration of the primary causative role for *E. coli* in the disease, colibacillary diarrhea.

Clinical Findings

The single litter of older pigs used in these experiments (Pig numbers 0657 through 0663, Tables 1 and 2) were not so adversely affected clinically (see Table 4) by infection with the test organism, as were the gnotobiotic neonates in the remaining 4 litters (Tables 1 and 4). It was clearly evident that neonatal, colostrum-deprived pigs are readily infected by oral contamination with 3×10^6 *E. coli* 0138:K81:NM organisms and that severe diarrhea results. It is apparent that even a brief period of agalactia in a parturient sow, thus preventing pigs from ingesting colostrum, would permit the gastrointestinal tract of neonetal pigs exposed to pathogenic serotypes of *E. coli* to become heavily contaminated, thereby constituting a serious threat to the survival of the litter.

It is likely that the profuse diarrhea characterizing colibacillosis in gnotobiotic pigs due to *E. coli* 0138:K81:NM is dependent on the presence either in the mucosa of the intestinal tract or the lumen of this organ, of sufficient numbers of the organism, and a remarkably consistent latent period of 8 to 16 hours (Table 4). It is apparent that the oral dose of approximately 3×10^6 organisms of this serotype is sufficient to produce diarrhea in a gnotobiotic pig within 12 hours of the exposure.

In the gnotobiotic pigs systemically infected with this serotype of *E. coli* in a previous experiment (Christie, 1967), it was

observed that sufficient time must elapse for the organism to colonize the gastrointestinal tract, and in sufficient numbers, before the physiological activity of the intestine was so altered as to produce diarrhea.

Escherichia coli 0138:K81:NM consistently produced very pronounced diarrhea and dehydration and, under field conditions, one would anticipate infection of neonatal pigs with this particular serotype to result in a relatively high mortality rate.

With this serotype, 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, in earlier experiments with the same serotype, an occasional urocystitis, probably from an ascending infection of the female urethra, was recorded (Christie, 1967).

The clinical signs of the disease produced by oral exposure of gnotobiotic neonatal pigs with *E. coli* 0138:K81:NM 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 experiments reported here.

Other clinical signs of diarrhea and dehydration -- the physical nature of the fecal material, changes in the hair coat, erection of the hair shafts, and postural attitudes characteristic of scouring baby pigs, described by others in clinical reports of neonatal colibacillosis, were consistently present in those animals left alive for a sufficiently long period of time in these experiments.

Histopathologic Findings

Histopathologic Lesions in Tissues Other Than the Alimentary Tract

The histopathologic lesions of *E. coli* 0138:K81:NM infection of gnotobiotic neonatal pigs in tissues other than the alimentary tract have been described previously (Christie, 1967). Generally these changes were of a vascular nature, indicated by edema, and epithelial degenerations, such as hydropic degeneration, in the respiratory and urinary tracts and the aortic endothelium. The epithelial and endothelial changes were inconsistent and were possibly associated with incidental local concentrations of endotoxin or enterotoxic activity of the test organism.

Histopathologic Lesions in 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 "Histopathologic Findings" because the difference between the groups is distinctly one of degree.

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*. The presence of enterotoxin of *E. coli* so modifies these changes that they occur in cells situated nearer to the base of the villus, and at an earlier stage in the short lifetime of the cell, than in cells of comparable tissue from germfree neonates. It is in this way that the histologic changes become significant in a pathologic sense.

The Lamina Propria.

It is suggested that the hyperemic and inflammatory changes found in the infected pigs are manifestations of a response to *E. coli* endotoxins, whereas the less marked histologic changes such as cytoplasmic vacuolization and breakdown which probably account for the major clinical sign of diarrhea are possibly due to other components of enterotoxin (possibly an exotoxin) and resemble to a degree some of the physiologic changes that one can see in the digestive tract of the germfree pig.

Smith and Jones (1963) and Moon *et al.* (1966) 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* 0138:K81:NM in these experiments and those previously reported (Christie, 1967), it is clear that there are inflammatory changes associated with colibacillosis but that these changes are insufficiently pronounced to be readily apparent in tissues taken from field cases of the disease. There are apparently no lesions pathognomonic of the infection at an optical-microscopic level. Occasional foci of neutrophils and other inflammatory exudates were observed in sections from the lamina propria and

submucosa of the intestinal tract of these experimentally infected pigs. Endotoxins have long been known to induce neutrophilia (Carpenter, 1965), and it may be that, as organisms multiply and they or their products "spill over" into the lamina propria from various sites of phagocytosis or localization in the crypts, they or their endotoxins can produce transient microabscesses in these tissues (Figures 1 and 2).

Bacteria stimulate an inflammatory response in the "normal" intestine of the healthy conventional pig, and we have come to accept a constant state of acute inflammation (characterized by the presence of neutrophils and lymphocytes) as being the normal. It is now apparent that there is a difference between the "normal" inflammatory state of the intestine in which normal function proceeds in tissues that have developed a balance between cell formation, cell function, and cell destruction (and are reacting to the constant threat of invasion by these organisms) and actual tissue damage by the products of their existence such as enterotoxins.

The histopathologic changes in the intestinal tissues of the infected pigs from each of the experimental litters were essentially the same. The ileum and terminal jejunum were more severely affected than the duodenum and anterior jejunum as judged by villal epithelial changes and inflammatory changes in the lamina propria.

Varying degrees of subepithelial edema, involving the interstices of the lamina propria, were associated with two possible states within the villus -- active absorption and colibacillary enteritis. There were several poorly defined conditions apparent

within the laminae propriae which enabled an arbitrary decision to be made concerning which condition, absorption or malabsorption, was present. Confirmation of these somewhat subjective judgments was obtained during electron microscopic examination of infected and control tissues.

The optical microscopic features associated with active absorption were considered to be: moderate hyperemia, moderate distention of the lacteals, some interstitial edema (confined generally to the immediate vicinity of the capillaries and lacteals), and activity of the Breucke fibers -- indicated by their appearance in sections as short, blunt, contracted cells.

Those features considered indicative of absorptive inadequacy of the laminae propriae associated with colibacillosis were: relaxation (or paralysis) and elongation of the Breucke fibers; sometimes marked hyperemia, at other times diminished capillary activity (according to the stage of the condition and level of enterotoxin in the environment); pooling of lymph in the lacteals; and particularly collection of fluid in the immediate vicinity of the basal lamina of the epithelium. Sometimes the collection of fluid became so marked that the fluid-filled spaces were interlaced with strands of fibrous stroma, and the villal stalk was noticeably distended (Figures 15, 16 and 25). There were occasions when one or more of these evidences of villal dysfunction were seen in villi from control animals and hence could be misleading in defining colibacillary enteritis if not considered together with the other optical and electron microscopic lesions of the disease.

In discussing the possible pathogenesis of these changes in the lamina propria, some consideration has already been given to the likely role of the endotoxic fraction of *E. coli* enterotoxin (*vide supra*), but some further amplification is necessary.

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 are confined within the bacterial cell and are present in both pathogenic and nonpathogenic gram-negative bacteria. Endotoxins derived from the *Enterobacteriaceae* are weakly toxic, relatively stable products of bacterial destruction. They are weakly antigenic and cause well characterized clinical manifestations such as sweating, fever, prostration, diarrhea, and hyperglycemia followed by hypoglycemia. There is leukopenia, followed by leukocytosis, vascular malfunction (vasomotor shock, edema, and hemorrhage) and, sometimes, death. These so-called classical responses to endotoxins include altered response to epinephrine and tissue necrosis at sites of endotoxin concentration (Ribi *et al.*, 1964; Smith *et al.*, 1964). 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 hyper-reactive 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 series of experiments, and in previous studies (Christie, 1967), instances of these vascular changes were seen in the laminae propriae of the villi as hyperemia, edema and occasionally capillary hemorrhage and stasis. Edema of the intramembranous space and of the interstices of the lamina propria was so common in the villi of the monocontaminants that it could most likely be considered as 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. Christie (1967) considered that edema of the colon could in fact be an indication of the disease, but mild edema of the mesocolon was nonsignificant under experimental conditions in that it was commonly found in germfree neonatal pigs.

A rapid increase in number of *E. coli* organisms in the intestinal tract of a previously germfree pig would be associated with a corresponding increase in the amount of endotoxin present. The enterobacterial somatic O antigens are a portion of the endotoxic molecular complex that is a main constituent of the wall of the bacterial cell. The lipopolysaccharide portion of this endotoxic complex exerts endotoxic as well as O antigenic properties. Exposure of a host to sublethal amounts of the endotoxin can lead to sensitization and so increase the lethal effect of toxin absorbed from the lumen of the

intestine at a later time. Coliforms present in sterilized feed, though nonviable, could still sensitize an animal, germfree or otherwise, so that a subsequent exposure to viable coliforms could well produce an intensified endotoxic reaction (Barnum *et al.*, 1967). This could explain the variation in clinical response observed by Christie (1967) and the two recorded cases of hemorrhagic enteritis that he observed, but it is not likely to be a factor associated with the less overt clinical syndrome of persistent diarrhea seen in the infected animals in this experimental series.

The presence of circulating or intestinal-lumen antibody, a complicating factor in the expression of colibacillary diarrhea in "conventional" neonates of animals and man, was circumvented to a large extent in the neonatal gnotobiotic pigs used in these experiments. Comparable animals used in previous experiments and held under identical environmental and dietary conditions (Christie, 1967) were independently found to be agammaglobulinemic (Brooks, 1966). Again, the short periods of time between birth, monocontamination with *E. coli*, and necropsy of the test animals in these experiments (Table 2) precludes their significant sensitization by nonviable coliforms which might possibly be introduced with the sterile diet.

Endotoxic shock requires, for its full expression, endotoxin, endotoxin antibodies and a protein complex -- "complement". According to Barnum *et al.* (1967), it is the relative proportions of antibody and of endotoxin in an animal that determines whether the antibody will increase or decrease host resistance. Smooth strains of *E. coli* contain relatively more endotoxin than rough strains, but

it is not the endotoxin per se that determines virulence. The other two significant metabolic byproducts of *E. coli* metabolism are colicines (Heatley and Florey, 1946) and hemolysins. Although the property of hemolytic activity is a useful marker to a bacteriologist, hemolysis is no longer considered to be an indicator for pathogenicity or virulence (Barnum *et al.*, 1967).

It is accepted by some that the pathogenesis of the diarrhea of cholera is primarily that of altered vascular permeability of the lamina propria (Elliott *et al.*, 1968). Elliott *et al.* (1968) have reported experimental infection of dogs with *Vibrio sp.*, causing the canine cholera syndrome. Light microscopic and electron microscopic studies were made. Biopsy of control dogs and those with the untreated cholera syndrome led these workers to report minimal acute inflammation of the small intestine and slight capillary dilation in the lamina propria. Otherwise, no differences between the groups were detected. They postulated that the *Vibrio* organisms probably released toxic material that altered mucosal function but did not cause visible tissue damage even at the ultrastructural level.

The *Vibrio cholerae* toxin, choleragen, induced hyperemia and interstitial edema in the lamina propria of infant rabbit intestine with associated loss of fluid of low protein content through "histologically normal" epithelium into the lumen of the intestine (Finkelstein *et al.*, 1964).

Gordon *et al.* (1966) and Phillips (1966) found that the toxin of *V. coli* caused alteration of vascular and mucosal permeability in the intestine. Electron microscopic changes, probably associated

with increased permeability, were seen in the basal capillaries and numerous microvesicles were seen in the cytoplasm of their associated villal epithelial cells. It was hypothesized that, in cholera, increased capillary permeability would allow hydrodynamic movement of fluid from blood to the interstices of the lamina propria, through the basal lamina into the mucosa, and thence into the intestinal lumen.

From his own studies of the intestinal capillary pressure and filtration coefficients of the human small intestine, and citing the findings of Gordon *et al.* (1966) relating to fecal serum albumin content of human cholera patients, Fordtran (1967) presented strong arguments against basal capillary permeability's being a major factor in the pathogenesis of cholera diarrhea.

Despite this reinterpretation, it is possible that the pathogenetic mechanisms proposed by Gordon *et al.* (1966) might have some application in the study of neonatal colibacillary diarrhea, at least insofar as it could be a contributory factor to the outflow of fluid into the intestinal lumen. Fluid outflow will be considered briefly later in this discussion.

Villal Morphologic Change.

In the porcine viral disease transmissible gastroenteritis (TGE), Trapp *et al.* (1966), Hooper and Haelterman (1966) and Maronpot and Whitehair (1967) described shortening, blunting and at times fusing of the intestinal villi and morphologic alteration of the intestinal epithelium from simple columnar to cuboidal type. A similar morphologic change in the villi has been described for *E. coli* infection

in infants (Handforth and Sorger, 1961), *E. coli* infection in baby pigs (Barnum *et al.*, 1967), experimental *Shigella flexneri* infection in germfree guinea pigs (Sprinz *et al.*, 1961), celiac disease in man, tropical sprue in man, and untreated idiopathic steatorrhea in man (Shiner, 1967).

As pointed out by Christie (1962) and Rubin and Dobbins (1965) the more highly specialized an organ or tissue, the more limited is its range of morphologic response to different noxious factors. This concept was reiterated by Maronpot and Whitehair (1967) and seems to have been borne out by their observations with TGE, and yet it is of interest to note that in no papers reporting histopathology of colibacillosis in gnotobiotic pigs is shortening and blunting of the villi mentioned.

It is likely that, at a cellular level, the morphologic response to different noxious factors -- such as the TGE virus, certain enterotoxins of *E. coli*, the toxic byproducts normal both to the existence of the flora of the intestinal tract and to mucosal necrobiosis -- is the same or similar, although in any given case the actual biochemical site of toxin activity be different. At a tissue or structural level, however, the response may well be markedly different. In colibacillosis as seen in monocontaminated gnotobiotes, the effete villal epithelial cells are retained on the villal stalk for almost the entire period of time normally required for cell movement along the villus. With TGE, the diseased cells are lost from the villus, leaving a denuded lamina propria which retracts to a level close to the surface of the crypts. In the latter case, within a few hours,

cuboidal epithelial cells grow out over the stump, and the short blunt villi that result are considered the "lesion" of TGE. Hypothetical reasons for this main difference in manifestation of cell damage will be proposed later in this discussion.

It is reasonable to assume that, should colibacillosis be complicated by the presence of other elements of the intestinal flora and their metabolic byproducts, by malnutrition, or by any other of a number of factors, these additional factors could contribute to the sloughing of cells (made effete by the action of enterotoxin of *E. coli*), resulting in premature loss of epithelium and a resulting histologic picture of foreshortened, blunt, stubby villi, reminiscent of those seen in TGE, shigellosis, celiac disease and idiopathic steatorrhea. So-called "conventional" hosts, that is, those bearing a bacterial flora in addition to *E. coli*, were described as being characterized by stunted, clubbed and occasionally fused villi (Kenworthy and Allen, 1966). Gnotobiotic pigs contaminated with two serotypes of *E. coli* in the same experimental series had intestinal villi with considerable variation in shape, including some branching forms, but no stunting or clubbing of the villi was reported (Kenworthy and Allen, 1966).

In fatal cases of epidemic infantile gastroenteritis associated with *E. coli* O111, Handforth and Sorger (1961) observed markedly foreshortened villi covered with flattened epithelial cells, and the histologic picture they describe does indeed closely resemble the lesions of TGE in pigs (Trapp *et al.*, 1966; Maronpot and Whitehair, 1967). However, these five human cases of diarrheal disease attributed

to enteric colibacillosis (Handforth and Sorger, 1961) were of long duration -- between two and five weeks -- and, according to the authors, presented pathologic changes markedly different from those seen in acute epidemic infantile gastroenteritis. It would be a misrepresentation of Handforth and Sorger (1961) to claim on their evidence, without regard to the severe malnutrition and other predisposing factors that were present, that the villal atrophy and abnormal thinning of the small-intestinal wall they described was characteristic of "...*E. coli* infection in infants" as is stated by Maronpot and Whitehair (1967).

Vacuolization of the Villal Epithelial Cell.

Preeminent in the small intestinal mucosa of clinically affected neonates are large intracytoplasmic vacuoles which are also present to a considerable extent in the intestinal mucosa of germfree neonatal pigs. The determination by histochemical means of the nature of the contents of these vacuoles was relatively unsuccessful.

The use of dilute solutions of Alcian blue 8GS was introduced by Steedman (1950) as a rapid, easily applied specific stain for mucins. In practice, Alcian blue does not stain the majority of mucoproteins, but will specifically stain acid mucopolysaccharides of both epithelial and connective tissue mucin (Pearse, 1961). None of the material contained within the vacuoles was Alcian blue-positive, thereby excluding the possibility of its being acid mucopolysaccharide.

Similarly excluded by negative response of the vacuole contents to the periodic acid-Schiff reaction were all other types of known

epithelial mucins, connective tissue mucins, enterochromaffin (Thompson and Hunt, 1966), plasma and serum, the dystrophic substances observed in the so-called glycogen and lipid storage diseases of man (Lillie, 1951), and the "serous coagula" -- presumably serofibrinous material, fibrin, neutral polysaccharides, glycogen and glycoprotein (Thompson and Hunt, 1966).

The vacuole contents were negative for Mayer's mucicarmine stain, Best's carmine stain, and oil red O, thereby excluding epithelial mucin, glycogen, and fat, respectively.

From these results, and from the appearance of the vacuoles on electron microscopic examination, it was concluded that the fluid contents of the vacuoles were of a serous nature. The fact that these contents were of low fat, protein, and carbohydrate content (significantly lower than serum levels of these substances) suggests that the material was similar to interstitial fluid.

Microbiologic Aspects

Unlike *Shigella spp.* and *Vibrio coli*, *E. coli* is considered a normal resident of the intestinal tract of man and animals -- hence its common name "the colon bacillus" -- and its adaptation to a commensal life in the intestinal tract, even of the pathogenic serotypes, will have a marked effect on its pathogenicity and pathogenesis.

The ecology of the "normal" intestine is complex and has been the subject of study for many years. One criticism of the use of gnotobiotic animals in research into intestinal disease is that account cannot be made of the effects of interacting microorganisms and their roles in the establishment or maintenance of such disease

processes. Herein lie both the advantages and disadvantages of gnotobiotic animal experimentation.

Recent experiments concerned with colibacillosis in neonatal pigs have involved oral inoculation of experimental animals with prescribed doses of organisms and immunologic, histopathologic and electron microscopic studies of the organism in its intestinal habitat. Some variation in results, probably associated with difference in infective serotype, dose of organisms, and experimental animals, has been observed.

Staley *et al.* (1968d) examined tissues submitted to Gram's stain and saw bacteria in the lumen, attached to the distal half of the villi of both jejunum and ileum. They found bacteria occasionally in the "core" of the villi.

In the experiments described in this thesis, sections of all small-intestinal tissues sampled were stained with the Brown and Brenn stain for bacteria. Although these were examined carefully, there were no organisms detected in the lumen, or within either the mucosa or lamina propria, or in the intracytoplasmic vacuoles.

Staley *et al.* (1968d) used a very high dose of organisms to infect their experimental animals (100×10^9 organisms) and a fimbriated serotype of *E. coli* 055:B5:H7, which may account for this difference.

According to Barnum *et al.* (1967), in order to reproduce enteric colibacillosis it is necessary that a susceptible host be "infected" with large numbers of an enteropathogenic strain of *E. coli*. The ambiguity of this statement is illustrated by Saunders *et al.* (1963),

who observed diarrhea in a group of colostrum-deprived pigs after feeding as few as 1000 living *E. coli* organisms. It is obvious that with 100 x 10^9 organisms present in the intestine, as was used by Staley *et al.* (1968d), there will be many organisms found in the tissues that will represent those experimentally administered and not necessarily organisms that have reproduced and colonized within the intestine.

It was for this reason that an infective dose of approximately 3 x 10^6 organisms was used in each of the experiments reported here. It was hoped to introduce sufficient young organisms that an infection would be established but insufficient that the disease syndrome would be caused by the organisms actually introduced. With a sufficiently low dose, it was considered more probable that the disease would be associated with multiplication and growth of the organisms within the host.

The presence of fimbriae on *E. coli* 055:B5,H7 might be a major factor in the visualization of the organism with Gram's stain in infected tissues as described by Staley *et al.* (1968d). The staining of fimbriae with the Gram's counterstain would assist somewhat the visualization of fimbriated serotypes compared to nonfimbriated serotypes such as *E. coli* 0138:K81:NM and might explain in this experiment -- and other experiments with this serotype (Christie, 1967) -- this consistent inability to see organisms in tissues stained either with hematoxylin and eosin or the Brown and Brenn stain for bacteria.

Staley *et al.* (1968d) examined sections of duodenum, jejunum and ileum with the electron microscope 2, 6 and 20 hours after dosing

hysterotomy-derived neonatal pigs with 100 x 10^9 *E. coli* 055:B5,H7 organisms. They saw neither intracellular organisms nor cellular damage in any of the duodenal or jejunal tissues, but they did detect bacterial invasion in the villal epithelial cells of the 20-hour-exposed ileum. The organisms were attached to the cell surface, and attachment sites were characterized by microulcers and increased density of the terminal web immediately beneath the attachment site. They observed that *E. coli* 055:B5,H7 entered the ileal villal epithelial cell by invagination of the plasma membrane where the terminal web was least dense. Once within the cell, the invaginated plasma membrane enveloped the bacteria in a membrane lined vacuole (Staley *et al.*, 1968d).

Although comparable tissues (jejunum and ileum) were examined during these experiments, at no stage were organisms seen either free or membrane-enclosed within the villal epithelial cells. There were many instances (for example Figures 96, 97, 98 and 99) where *E. coli* 0138:K81:NM was in close association with the brush border. Although it was apparent that, in the vicinity of the organisms, the microvilli were foreshortened and somewhat atrophied, there was clear evidence that the trilaminar structure of the apical plasmalemma and the apical tubules nearby was retained (Figure 99). There was no evidence to suggest that microerosion had occurred, or was about to occur, nor was there any evidence of invagination of the apical plasmalemma or engulfment of the organism by the apical cytoplasm. One could explain these differences by taking into account the different chemical and physical natures of the enveloping coat of the bacterial serotypes used in these various experiments.

In a normal intestinal tract there are occasional disruptions in the integrity of the mucosal surface (microulcers) where an organism, although ill-equipped for epithelial invasion, can enter subepithelial tissues and enter the circulation via capillaries or lymphatic channels. If this site, for example a tonsillar crypt, be anterior to the stomach, then this occasional contamination could effectively enable an organism such as *E. coli* to bypass the threat of destruction in the acid contents of the stomach and re-enter the gastrointestinal tract in the region of the jejunum or ileum where environmental circumstances are more favorable. This possible mechanism for infection has been established for *E. coli* (Fey *et al.*, 1962) and for *E. coli* 0138:K81:NM in gnotobiotic pigs (Christie, 1967; Christie, unpublished data).

There is no doubt that *E. coli* 0138:K81:NM has the ability to enter the epithelial barrier and produce a bacteremia very early in an infection, but it may be that in most circumstances, with this organism at least, this phenomenon is entirely irrelevant to the establishment of a colibacillary diarrhea, provided sufficient numbers of the organism can enter the intestinal tract.

"Positive" results have been obtained in intestinal-loop studies both with viable organisms -- including *E. coli* 0138:K81:NM -- and sterile preparations of enterotoxin from pathogenic serotypes of *E. coli* (Barnum, personal communication). The development of diarrhea, associated with typical microscopic lesions, in gnotobiotic neonatal pigs orally dosed with sterile samples of *E. coli* enterotoxin from pathogenic serotypes has also been observed (Christie, unpublished data).

With these results in mind, it is clear that an essential factor in the production of colibacillary diarrhea is not the ability of the organism to enter the villal epithelial cell or even invade the lamina propria (although these capabilities may directly or indirectly assist the organism in the colonization of the intestine) but to populate the lumen, the intervillal spaces and perhaps the crypts, thereby maintaining a level of enterotoxin in the chyme bathing, and being absorbed by, the mucosal surface.

Not only does the apical tubule system absorb colostrum, but probably also products of coliform colonization and metabolism, specifically enterotoxin. There was no evidence that the infectious agent itself was absorbed. It is no longer sufficient to say that *E. coli* is "readily absorbed by neonatal intestinal epithelium" (Staley, 1969) in that it is clear that some serotypes of *E. coli* are readily invasive of the epithelial mucosa whereas, with other serotypes, such as *E. coli* 0138:K81:NM, this evidence was not found despite intensive search.

Quite apart from the above considerations, it is likely that the ingestion of *E. coli in toto* by the villal epithelial cell is not an effective means of reducing infection nor of removing bacteria. In view of the rapid turnover rate of the villal epithelial cells of the small intestine, the intracytoplasmic sojourn of *E. coli* is likely to be short, soon to be ended by the necrobiosis of the villal epithelial cell and the early release of the microorganism into the lumen.

Alternatively, it may be that the encapsulated bacteria are brought into association with a lysosome to form a compound lysosome (deDuve, personal communication), and in this way the bacteria is perhaps lysed and digested, although Staley *et al.* (1968d) did not see evidence of lysosomal destruction of the encapsulated organisms.

As in these experiments, so also Staley *et al.* (1968d) did not observe in their electron microscopic evidence of bacteria in the lamina propria of the villus; neither were they observed in the lateral intercellular spaces, nor was there any conclusive evidence of intracellular digestion of the enveloped bacteria.

In the case of an organism exerting its toxic effect by the production of enterotoxin, factors which assist in the maintenance of the organisms or growth of colonies, which maintain a suitable level of contact between the toxic material and its site of action, and which inhibit the production or distribution of antibiotic, antitoxic, and protective humoral or cellular factors will increase the pathogenicity of the organism.

Losos (1964) speculated that the fimbriae of *E. coli* acted as a mechanism for attaching the microorganism to the epithelium of the small intestine. Smith and Halls (1967) considered that *E. coli* which cling to the mucosal surface, or those that populate the crypts, would be more pathogenic than those that do not, in that they would resist the "washing out" effect of the chyme as it moved through the intestinal lumen.

The absence of fimbriae from *E. coli* 0138:K81:NM might be a major factor in the apparent inability of this microorganism to

erode and enter the brush border of a normal epithelial cell such that it would be in a position favorable for pinocytosis by the apical plasmalemma.

In these experiments, *E. coli* 0138:K81:NM, although not fimbriated, did actively migrate within the lumen to the base of the villi, and possibly even further (although sections of the crypts were not examined under the electron microscope). From a study of the structure of the intestinal wall it is not difficult to imagine that, even in instances of diarrhea, particulate matter at the center of the lumen would be swept along rapidly, whereas particulate matter near the base of the villi would move relatively more slowly, and possibly at no more rapid pace than in clinically normal hosts. It is likely that the rate of movement of particulate matter from the bases of the villi would depend more on the compression and relaxation of individual villi on neighboring villi during "pumping" movements than on the axial flow of chyme associated with peristalsis. If the degree of vacuolization of the villal epithelial cells is marked, and the level of endotoxin present in the intestine is such that the Breucke fibers are relaxed (or paralyzed), yet the muscular coats are stimulated to hyperactivity (by endotoxic sensitization of smooth muscle to exogenous and endogenous stimuli) then, theoretically, conditions exist that would allow a rapid axial flow of chyme yet permit a relatively stable particulate population to exist at the level of the crypt openings, trapped there by the villi themselves. The histologic picture seen many times during this series of experiments gave support to this speculation.

If, in addition to these hypothetical mechanical factors, this particular serotype had the ability to cling to the mucosal surface by either chemical or physical means, then as Smith and Halls (1967) suggest, pathogenicity would probably be increased.

Although it has been demonstrated that serotypes of *E. coli* can enter the circulation of a host animal, which then undergoes intermittent bacteremic episodes (Christie, 1967), and that certain serotypes can regularly invade and establish infections in extra-enteric sites such as the pleural and peritoneal cavities (Britt, 1967), the most common manifestation of colibacillosis in neonates is its enteric form. It is more likely that *E. coli* 0138:K81:NM, and perhaps other serotypes, exert their pathologic effects primarily on the gastrointestinal tract because that environment is more favorable in terms of multiplication and survival and because the organism itself is basically noninvasive and tends to stay where it has successfully colonized. Indeed, there is no ecological need for *E. coli* 0138:K81:NM to invade the mucosa or lamina propria, and such invasion could be just as hazardous a process for the microorganism as it would be for the host.

Unlike enteric serotypes of *E. coli*, the pantropic serotype 083:K.:NM (Britt, 1967) is invasive for gnotobiotic pigs, rapidly colonizing serous surfaces and producing toxins which are retained and can only be absorbed by their environment. It is understandable that this infection kills rapidly, and in so doing limits the microorganism's own continued existence and its potential for infecting other susceptible hosts.

Those more commonly known pathogens such as *E. coli* with the 0138 and the K81 antigens are less invasive, predominantly enteric in habitat, and except for neonatal pigs (or older pigs in adverse environmental or physical circumstances) they produce a high morbitidy but low mortality, thereby promoting their own widespread distribution among susceptible hosts and their continued successful existence.

Ligated Intestinal-loop Preparations

Barnum *et al.* (1967) described experiments with ligated intestinal loops of susceptible pigs inoculated with *E. coli* in which they demonstrated that the net outflow of water into the "positive" loops took place through the intact epithelial membrane "as it apparently does in enteric colibacillosis".

In ligated intestinal-loop preparations in 21-day-old conventional pigs it has been found that, in common with a number of other serotypes known to be pathogenic for swine, the organism used in these experiments is "strongly reactive" (D. A. Barnum, personal communication). A "strongly reactive" or "positive" ligated intestinal-loop preparation is one in which ligated pig intestine has been injected with the enteropathogen 12 to 14 hours previous and has become markedly distended with fluid. "Positive loops are a clear demonstration of the net flow of water into the intestinal lumen which occurs in response to certain enteropathogenic bacteria" (Barnum *et al.*, 1967).

This biological preparation, although made in the host animal, does constitute an artificial and abnormal situation. The intraluminal fluid is retained, distending and flattening the intestinal wall beyond the limits found in pigs with clinical diarrhea and submits the vessels and mucosae to extraordinary pressures. Despite these limitations, it has been described as a pathogenicity test by a number of authors (Nielsen, 1953; Moon, 1965; Gyles and Barnum, 1965).

Any lesion of the small intestine which interferes with orderly intestinal peristalsis and causes stasis of intestinal contents eventually results in intraluminal bacterial overgrowth (Donaldson, 1967). This fact confirms the importance of normal peristalsis in limiting bacterial growth in the small intestine. Those lesions associated with stasis and massive proliferation of enteric microorganisms include surgical blind loops (Doig and Girdwood, 1960) and strictures (Seyderhelmen *et al.*, 1924; Halsted *et al.*, 1956).

The real significance of a positive ligated intestinal-loop preparation is that the ligated segment of intestine distends without their being any significant amount of chyme in the lumen of the intestine prior to inoculation with the test organism. The fluid collecting in the loop must therefore be derived from the circulation, and its production cannot be associated with the main pathogenetic theories of diarrhea such as inhibition of active sodium transport, impairment of chloride and bicarbonate absorption, hypokalemia, osmotic diarrhea and deranged intestinal motility. The intestinal-loop preparation does not, at present, contribute significantly to our understanding of the pathogenesis of neonatal colibacillary diarrhea.

Histochemistry

The term "enzyme activity" has been used in this thesis to describe the observed tissue staining associated with a reaction, even though histochemists generally agree that this staining process might not represent the true functional capabilities of the particular enzyme. The enzyme activity was qualitatively assessed individually and comparatively within litters and within tissue groups preserved the same way prior to processing. Melnyk *et al.* (1966) agree that qualitative tissue and cellular localization is the most reliable parameter for evaluating enzyme distribution and activity.

Assuming proper technique and appropriate tissue and enzyme controls, decreased intensity of histochemical staining of enzyme catalyzed reaction products is an indication of the deficiency of the enzyme in a particular tissue or cell.

> "Alteration of the brush border and decrease of specific functional enzymes in intestinal epithelial cells bears a significant relationship to nutrient absorption".

(Maronpot and Whitehair, 1967).

It has been argued that enzymic complement is reduced where villal epithelial cell turnover rate is increased in that the cell is still acquiring its full enzymic load while it occupies the normal position of an adult cell on the surface of the villus (Creamer, 1967). Although from these experiments and a previous report (Christie, 1967), it is clear that in colibacillosis due to *E. coli* 0138:K81:NM there is considerable damage to individual villal epithelial cells, there is no clear histopathologic evidence of a marked increase or decrease in cell turnover rate.

Creamer (1964b), with his own findings and those of Padykula *et al.* (1961), presented convincing evidence that, within a species, villal shape and length of crypt to villus ratio are relative indications of cell turnover rate. Villal length and shape in tissues from monocontaminated pigs were not noticeably altered from those of the germfree control pigs in these experiments. It is apparent that any difference of turnover rate between the groups in these experiments was not marked. One can reasonably conclude that the alteration in intracellular enzyme concentration, activity, and distribution in these experimental tissues is more likely to be due to a direct effect of the microorganism, or its enterotoxin, than secondarily through cell destruction and replacement with biochemically and functionally immature epithelial cells.

Apart from the functional and biochemical deductions to be made from histochemical studies, it was suggested by Pearse and Riecken (1967), but not yet established, that the demonstration of enzyme groups bound to membranes would provide an indicator of the structural integrity of the particular membranes to which they are bound.

These experiments constituted an opportunity to study cells histochemically, predict functional and morphologic change, and then confirm or refute such predictions by electron microscopic examination of comparable cells.

Alkaline Phosphatase

Biochemical studies of the brush border separated by microdissection have revealed that alkaline phosphatase (as well as leucine aminopeptidase and the disaccharidases) are associated with the

microvilli (Eichholz and Crane, 1965; Crane, 1966). Earlier, Miller and Crane (1961) found that the brush border contained all of the cellular activity of the terminal digestive enzymes including the alkaline phosphatases, and yet in a more recent publication (Maronpot and Whitehair, 1967) alkaline phosphatase activity was depicted as being characteristically present in the brush border and concentrated in the apical cytoplasm of villal epithelial cells in 3- to 5-week-old conventional pigs.

In the germfree neonatal pig, alkaline phosphatase activity was restricted to the epithelial cells of the villi and, by the techniques used, was not demonstrated in the epithelial cells of the crypts. Within the individual cell, there was a high concentration in the brush border. At times there was a narrow reaction-free zone at the apex of the cell that would correspond to the terminal web and the apical tubule region. The rest of the alkaline phosphatase activity was concentrated in the apical third of the cell and in a narrow zone close to the basement membrane. Since the latter two sites for enzyme activity were not demonstrable in the tissues preserved in cold formalin, it is apparent that such staining was associated with nonspecific phosphatases and should be discounted as indicative of alkaline phosphatase activity.

Localization of alkaline phosphatase at the brush border of the villal epithelial cell in neonatal germfree pigs corresponds with localization of this enzyme at the apical border of mucosal cells in conventional baby pigs (Maronpot and Whitehair, 1967; Thake, 1968) and in other species (Shnitka, 1960; Padykula *et al.*, 1961; Miller and Crane, 1961; Eichholz and Crane, 1965; Crane, 1966; Ito, 1969).

It is apparent that the variation in amount of nonspecific phosphatase activity seen in the stained tissues in this experiment was due to the differing preservation procedures. The most satisfactory preparations were those preserved in cold formalin prior to staining, the method advocated by Pearse (1961). The very specific localization of the enzyme with a minimum of enzyme diffusion artifact and the excellent histologic detail was in sharp contrast with the lack of specificity and histologic detail of tissues fixed in cold acetone or of frozen unfixed tissues. It was possible to increase the specific localization of alkaline phosphatase in frozen unfixed tissues by reducing the substrate incubation time markedly, but the result was less satisfactory than that obtained with formalin-fixed tissue.

In the diagrammatic representations of enzyme distributions in the jejunum and ileum of their control group pigs, Maronpot and Whitehair (1967) have depicted alkaline phosphatase (and leucine aminopeptidase) in both the brush border and the apical third of the villal epithelial cells. With improved preservation methods and localization techniques used in the processing of tissues from germ-free control pigs in these epxeriments, it was observed that these enzymes were in fact localized in the region of the brush border. These results are in agreement with those of Barrnett (1959), Shnitka (1960), Padykula *et al.* (1961), Eichholz and Crane (1965), Crane (1966), Pearse and Riecken (1967) and Ito (1969). It is likely that the results of Maronpot and Whitehair (1967) were in part due to nonspecific phosphatase activity in the apical cytoplasm and partly to the diffusion of alkaline phosphatase (and leucine aminopeptidase)

from the brush border into the apical cytoplasm during cold preservation and tissue processing.

The glycocalyx is a hirsute layer comprising the filamentous structure closely associated with the unit membrane of the microvilli (Ito, 1965). Ito suggested that the glycocalyx had a protective function and Johnson (1966) described the localization of enzymatic activity in this structure. Staley *et al.* (1968b) suggested that the early appearance (by 48 hours) of the glycocalyx might reflect the precocious development of alkaline phosphatase activity in the pig. Some confirmation of this hypothesis is borne out in alkaline phosphatase studies in this experiment.

In monocontaminated pigs, there was a loss of alkaline phosphatase activity starting 12 to 14 hours after exposure, and this loss was more apparent in the epithelium more distally placed along the villi. The degree of staining was also less intense in sections taken further along the small intestine.

By 93 hours after exposure there was renewed intensity of staining for alkaline phosphatase activity throughout the small intestine.

The loss of alkaline phosphatase activity associated with enteric disease and diarrhea in this experiment agrees with the findings of Maronpot and Whitehair (1967) and Thake (1968), who observed loss of alkaline phosphatase activity from the villal epithelial cells of baby pigs clinically affected with TGE.

It is clear that, whether primarily or secondarily, alkaline phosphatase activity of the brush border of villal epithelial cells is reduced or lost for a period during enteric colibacillosis and

that such loss will impede terminal digestion and the process of absorption in the affected cell. Whether loss of alkaline phosphatase activity is a sign of a direct toxic effect of *E. coli* enterotoxin on the enzyme or its precursors, or whether it is a sign of ultrastructural disorientation of the glycocalyx or brush border, will be discussed later in this thesis. It can be forecast, however, that the enterotoxin of *E. coli* 0138:K81:NM exerts an effect at the level of the brush border, which is significant in terms of the processes of digestion and absorption.

Acid Phosphatase

As described in man (Fric and Lojda, 1964; Riecken *et al.*, 1966), the rat and the mouse (Pearse and Riecken, 1967) and baby pigs (Maronpot and Whitehair, 1967; Thake, 1968), acid phosphatase was seen to be distributed throughout the cytoplasm of the intestinal absorptive cells of germfree neonatal pigs, particularly in the apical cytoplasm. This probably corresponds to the distribution of lysosomes within the cytoplasm of these cells.

In germfree neonatal pigs the clear perinuclear zone seen in villal epithelial cells stained for acid phosphatase corresponds to the zone in the normal cell that is occupied by the Golgi complex and the endoplasmic reticulum. Lysosomes are generally in the periphery of the cell, and the normal cell, when stained for acid phosphatase activity, would be expected to have a clear perinuclear zone of minimal acid phosphatase activity.

Twelve or more hours after monocontamination with *E. coli* 0138:K81:NM, there was histochemical evidence of a decrease in acid

phosphatase activity, and there was a replacement of the granular appearing stain reaction with a smoother, more homogeneous staining of the cytoplasm of villal epithelial cells. It is speculated that these changes are indicative of reduced lysosomal activity, possibly associated with disruption of lysosomes, either by direct action of enterotoxin or by indirect action of the enterotoxin on the metabolic activities or on the membrane-transport activities of the lysosomes. It would be expected that disruption of a number of lysosomes within a particular cell would result in loss of specific site location of the stain reaction products and release of formerly confined lytic enzymes (including acid phosphatase) so that the stain reaction was seen to be homogeneous throughout the cell. As the cell contents were lysed by the released lytic enzymes (including acid phosphatase), general loss of intensity of the staining reaction would be experienced.

Considering these speculations and the results obtained for acid phosphatase activity in this experiment, it could be reasonably anticipated that 12 to 14 hours after exposure of gnotobiotic neonatal pigs to *E. coli* 0138:K81:NM, there would be disruption of lysosomes and beginning cytoplasmolysis of villal epithelial cells. Subsequent dissolution of the cell, probably associated with rupture of the apical plasmalemma and discharge of the fluid contents of the cell into the intestinal lumen, could be expected.

Adenosine Triphosphatase

As described in man (Pearse and Riecken, 1967) and in conventional baby pigs (Maronpot and Whitehair, 1967; Thake, 1968), adenosine triphosphatase was seen to be distributed throughout the cytoplasm but particularly localized in the apical cytoplasm and the brush border of the villal epithelial cells of germfree neonatal pigs. Thake (1968) considered that the slightly more intense reaction for adenosine triphosphatase activity seen in the apical cytoplasm may have been due to alkaline phosphatase since the reactions for demonstration of both enzymes are carried out at pH 9.4.

The most distinctive change in adenosine triphosphatase distribution that was associated with colibacillosis was the partial loss of enzyme activity from the brush border of cells, particularly from the jejunum and ileum, within 12 hours of monocontamination with the microorganism.

The second detectable change in the infected pigs was that of loss of intensity of the staining for adenosine triphosphatase activity within the cytoplasm of the villal epithelial cells. It is only in erythrocytes that a correlation has been shown between the rates of adenosine triphosphate hydrolysis and rates of membrane transport of fluids (Whittam and Ager, 1965). Since adenosine triphosphatase plays many roles in cell metabolism, apart from fluid transport, it is not yet possible to specify those stages of the absorptive process blocked by loss or destruction of adenosine triphosphatase in neonatal pigs with colibacillary diarrhea.

The losses of adenosine triphosphatase described above do, however, constitute events of significance because of the very nature of the role of this enzyme in biochemistry.

Loss of adenosine triphosphatase from the brush border presents a significant block to the first stage of membrane transport at the apical plasmalemma, in that the source of energy for such a process is dependent on the proper function of adenosine triphosphatase. Within the mitochondrion, adenosine triphosphatase is an integral part of the essential functions of this organelle -- the oxidation of carbohydrates, fats and amino acids, the formation of high energy yielding adenosine triphosphate, active intracellular transport of ions, and intramitochondrial water metabolism (Lehninger, 1964). Disruption and loss of functional adenosine triphosphatase would most likely result first in the inability of the mitochondrion to excrete metabolic water. Collection of fluid within the mitochondrion would be seen first as vacuolization of the mitochondrial matrix, then as distention of the mitochondrion to the point of eventual rupture.

The disruption of mitochondria would be a terminal lesion in a cell of such diverse metabolic activity as the villal epithelial cell, and this lesion alone would be sufficient to explain all other changes observed within the intestinal cells of neonatal gnotobiotic pigs monocontaminated with *E. coli* 0138:K81:NM.

Leucine Aminopeptidase

As described in the columnar cells of the rat, human, and monkey small intestine (Nachlas *et al.*, 1957, 1960) and within the brush

border and supranuclear cytoplasm of villal epithelial cells of 3- to 5-week-old conventional pigs (Maronpot and Whitehair, 1967), leucine aminopeptidase was also found in the villal epithelial cells of neonatal germfree pigs. In these animals, as in man (Pearse and Riecken, 1967), the enzyme was apparently localized within the brush border, with only slight traces of enzyme activity in other sites. The enzyme was not concentrated in the supranuclear cytoplasm to any significant extent.

The loss of leucine aminopeptidase activity from the brush border of villal epithelial cells of the jejunum and ileum 16 to 18 hours after monocontamination with the test serotype of *E. coli* was distinctive. Since leucine aminopeptidase is a "membrane associated" enzyme (Pearse and Ricken, 1967), this loss of cytochemical activity, when considered with the loss of alkaline phosphatase activity, presents convincing evidence of a biochemical lesion in the brush border.

It is reasonable to forecast that such a lesion might be associated with morphologic change in the microvilli and glycocalyx of villal epithelial cells from neonatal pigs with colibacillary diarrhea.

Succinate Dehydrogenase

As described in the mucosa throughout the small intestine of rats and dogs (Nachlas *et al.*, 1957), man (Pearse and Riecken, 1967), 3- to 5-week-old conventional pigs (Maronpot and Whitehair, 1967) and 1- to 2-week-old conventional pigs (Thake, 1968), succinate dehydrogenase activity was also observed in the cytoplasm of mucosal cells of both villi and crypts, at all levels of the small intestine, of neonatal germfree pigs.

The distribution of the enzyme corresponded closely with the intracytoplasmic distribution of mitochondria, as seen in electron micrographs of duplicate tissues from these experimental animals. Considering the role of succinate dehydrogenase in mitochondrial metabolism, such distrubtion was to be expected.

The reaction for succinate dehydrogenase results in the intramitochondrial deposition of formazan as small dots with an average diameter of 0.2 to 0.3 micron (Scarpelli and Pearse, 1958). Pearse (1958, 1961) has emphasized the sensitivity of methods for, and the accuracy of localization of, succinate dehydrogenase, in which

> "it is probable that the amount of formazan deposited is directly related to enzyme activity. We can now see and record small alterations...in single cells and we can thus detect changes of cellular function such as that from normal growth to neoplasia".

(Pearse, 1961).

The particular value of the demonstration of succinate dehydrogenase in these experimental animals lies in the specificity of the stain reaction and its increased reaction velocity in swollen but metabolically active mitochondria. Succinate is oxidized much faster by swollen mitochondria (Raaflaub, 1953) and this observation was confirmed histochemically by Scarpelli and Pearse (1958), who found that the velocity of the succinate dehydrogenase reaction, but not of the DPNH-diaphorase reaction, was markedly increased in swollen mitochondria.

The increased intensity and definition of the stain reaction within a few hours of monocontamination that was observed in some instances in these experiments (Figure 58) was probably due to

swelling of the mitochondria as a preliminary step in the eventual distention and rupture of these organelles.

Within 12 hours of monocontamination, there was marked loss of succinate dehydrogenase activity from the mucosal cells, and this loss was more marked in more caudal sites of the jejunum and ileum and was observed in tissues from the pigs with all clinical signs of acute colibacillary diarrhea. The loss of enzyme activity was still apparent 32 hours after monocontamination.

It is considered that this knowledge of the succinate dehydrogenase activity and distribution, when considered with that of adenosine triphosphatase and lactate dehydrogenase activity, provides reasonable evidence of mitochondrial dysfunction associated with colibacillosis.

Lactate Dehydrogenase

As described in the mucosal epithelium of rats (Nachlas *et al.*, 1958; Hess *et al.*, 1958), lactate dehydrogenase was observed to be present in both the apical and basal cytoplasm of intestinal absorptive cells at all levels of the small intestine of the neonatal germfree pigs used in these experiments. The most marked enzyme activity was observed in tissues from the more anterior sites, duodenum and anterior jejunum, and this activity might be related to the higher energy requirements of the more intense digestive-absorptive activity claimed for the anterior small intestine by some authors (Nielsen *et al.*, 1968).

The binding of lactate dehydrogenase with mitochondrial membranes, and with those of the endoplasmic reticulum, provides an explanation for the described distribution of this enzyme in the intestinal absorptive cells of the neonatal germfree pig.

The loss of lactate dehydrogenase activity observed to be associated with monocontamination of the experimental animals with *E. coli* 0138:K81:NM was much less marked than the observed loss of activity of the other enzymes (*vide supra*), although such a comparison is quite invalid in terms of assessment of function or metabolic activity. The distribution of lactate dehydrogenase in both mitochondria and endoplasmic reticulum would explain the rather more persistent staining for activity of this enzyme if it was that either endoplasmic reticulum or mitochondria persisted relatively unaltered for a longer period of time in a cell experiencing enterotoxic stress.

Twenty-eight hours after monocontamination, by which time diarrhea is very well established in gnotobiotic neonatal pigs, there is a distinct loss of lactate dehydrogenase activity, which is indicative of dysfunction or disruption of either the mitochondria or endoplasmic reticulum, or both.

Lactate dehydrogenase is very widely distributed in a high proportion of the mitochondria of most tissues (Pearse, 1961), and it is tempting to speculate that the subjective assessment of increased staining for lactate dehydrogenase of the laminae propriae of villi from infected pigs is a sign of the release of enzyme following disruption of its organellar relationships and its escape with tissue

fluid into the laminae propriae. If this were the case, however, one would expect that one of the major sources of serum lactate dehydrogenase would be that amount of enzyme released from the continuing extensive necrobiosis of alimentary tract mucosa, known to be part of normal intestinal activity. Again, in diseases involving widespread damage to the intestinal mucosa, such as TGE or colibacillary enteritis, one would expect to see elevated levels of serum lactate dehydrogenase. Such events have not been reported.

Considering the lesions of colibacillosis already described in these experimental animals, it is more likely that enzymes released from organellar membranes are lost, first to the cytoplasm generally, and then through the ruptured apical plasmalemmae to the lumen of the intestine. A second consideration is that the enzyme itself is a large protein molecule and would not be absorbed, even by intact functional villal epithelial cells, without considerable proteolysis, which would in turn most likely destroy its enzymic function and render it undetectable by histochemical means.

Function of intramitochondrial enzymes is most likely to be dependent on specific spatial arrangement, in sequence, with other enzymes associated with a particular metabolic cycle (Lehninger, 1964), and disruption of this lattice would certainly remove or markedly diminish their enzymic activity. The spatial arrangement of the endoplasmic reticulum in parallel arrays, separated by a remarkably uniform distance which varies according to cell type and function (Fawcett, 1966), is strongly suggestive of a similar functional structure. Presumably, distortion of the parallel array of these

membranes, or chemical breakdown of the membrane components, would effectively block the function of lactate dehydrogenase in cell metabolism, and the demonstration of lactate dehydrogenase activity in histochemical tests.

In summary it is probable that the staining for lactate dehydrogenase activity seen in the laminae propriae of the villi of both germfree and gnotobiotic neonatal pigs is associated with the activity of mitochondria and endoplasmic reticulum of cells constituting the lamina propria, and not the consequence of enteric colibacillosis.

Electron Microscopy

The role of the intracellular membrane as a means of delineating and defining cytoplasmic spaces is apparent but of relatively little importance. It is no longer considered to be simply a bimolecular layer of phospholipid with nonlipid (usually protein) molecules bound to its polar surface by ionic bonds, thereby acting as an inert barrier to free diffusion of specific substances, with a degree of relative permissiveness to lipid-soluble solutes. It is increasingly clear that membranes are "specific arrangements of catalytic proteins" (Korn, 1969), and with this concept in mind, much of the behavior of neonatal porcine intestinal cells in absorption and malabsorption associated with experimental colibacillosis becomes explicable.

The Brush Border

> "With increasing knowledge regarding the spatial order and relationships within the brush border and the microvillus, it seems probable that close anatomical relationships within

> this structure will carry with them important significance for the digestive and absorptive properties of this organelle".

(Eichholz, 1969). Crane (1969) has stated that it was only in the last 8 years that the brush border was recognized to be a digestive-absorptive surface organelle, less complex perhaps than the mitochondria, but a structurally integrated, subcellular organelle controlling and interacting with the internal environment of the villal epithelial cell as well as forming its luminal surface.

Enzymes catalyzing digestive and absorptive functions seem to be spatially arranged in the brush border so as to offer a kinetic advantage for absorption (Crane, 1968; Crane, 1969) not only of digested foodstuffs, but of sodium and possibly other ions, by a mechanism of cooperative interaction between these and other absorbed molecules in the mobile carrier transport system (Crane, R. K., personal communication).

The Glycocalyx.

Ito (1969) has summarized the role of the glycocalyx as being responsible for, or contributing to, "absorption, transport, electrical characteristics, immunological behavior, and adhesive nature" of the apical plasmalemma. The glycocalyx is most probably structurally layered, with a surface component of intrinsic hydrolases closely applied to the trilaminar membrane (Crane, 1969) and required primarily for the terminal digestion of carbohydrates and proteins. Ugolev (1965) has described this region of the plasma membrane as the most important site for terminal digestion. Adenosine

triphosphatase and alkaline phosphatase have been shown by many to be localized in or near the brush border, and this localization has been confirmed for neonatal germfree porcine villal epithelial cells in these experiments.

Staley *et al.* (1968a) stated that the glycocalyx was not present in the neonatal pig villal epithelial cells and suggested that it was synthesized after whole protein absorption ceased. Yet in the present experiments, there was ample evidence of a glycocalyx, albeit meagre yet clearly present, in many animals from 3 days of age and older. It is likely that the staining methods used by Staley *et al.* (1968a) were less satisfactory for the demonstration of the glycocalyx than was the osmium tetroxide fixation and Reynold's lead citrate-uranyl acetate staining used for the experimental tissues in these experiments. Other evidence is also provided in these experiments for the presence of the glycocalyx in neonatal pigs. The strongly positive staining of the brush border of all control pig tissues with Best's carmine stain and the strongly positive periodic acid-Schiff reaction were indicative of the presence of the glycocalyx in pigs within, at most, 48 hours of surgical delivery. Further evidence for the presence of a functional glycocalyx in germfree neonatal pigs was the demonstration of the presence of alkaline phosphatase and leucine aminopeptidase in the brush border, and the partial loss, or absence, of the activity of these enzymes from the brush border of the monocontaminated pigs.

Ito (1969) has demonstrated, in the cat intestinal absorptive cell, that alkaline phosphatase activity is localized in the deeper

layers of the glycocalyx, immediately adjacent to the plasma membrane, and on the external and internal surfaces of the trilaminar membrane. Our present concepts of the biochemistry of carbohydrate digestion and absorption require an arrangement such as this for the successful passage of a molecule such as glucose through the trilaminar membrane. The exact chemical and structural nature of the mobile carrier transport system within the lipoidal membrane is not yet known, but there is strong evidence to suggest that the common carrier for both sodium, and a limited number of specific carbohydrates (such as glucose and galactose), is most likely protein, obeys the kinetic laws characteristic of enzyme binding, and may be a "transport-enzyme" (Crane, R. K., personal communication). If such "transport enzymes" were in fact proteins, and integral parts of the membrane fabric with its transport "acceptor sites" exposed to both inner and outer surfaces of the membrane, the only requirement necessary for movement of an "accepted" solute molecule or molecules from one side of the membrane to the other would be an allosteric transformation of the carrier molecule (Jardetsky, 1966). Sodium-potassium-magnesium adenosine triphosphatase probably transports cations by this process (Skou, 1965).

Ito (1969) found that the glycocalyx of the cat intestinal absorptive cell is more labile when the underlying cell is moribund, and he concluded that this component might be a dynamic surface component "requiring an intact cell for its synthesis and maintenance". His autoradiographic investigations have clearly shown that the glycocalyx is continuously synthesized by the villal epithelial

cell and moved to the surface, the whole process in the cat requiring 30 to 60 minutes, their being a constant replacement of the surface coat with a minimum turnover time of about 4 hours and an upper limit of 8 to 10 hours.

In the experiments described in this thesis, it has been shown histochemically and microscopically that there is some loss, both in form and function, of the glycocalyx of villal epithelial cells from neonatal gnotobiotic pigs monocontaminated with *E. coli* 0138:K81:NM. Whether this loss is a primary effect of the enterotoxin or secondary to enterotoxic damage of the intracytoplasmic structures responsible for the production of the glycocalyx, is equivocal.

The early concentration of autoradiographic components of the glycocalyx in the Golgi complex, and their subsequent appearance in the glycocalyx, considered together with the accepted role of the Golgi complex as a major intracellular site for synthesis of cell products, is presumptive evidence of the role of the Golgi complex in the continuous formation of the glycocalyx. Assuming that the glycocalyx of the neonatal pig intestine is derived in a similar manner to that of the cat, it is clear that the internal cellular disruption associated with monocontamination with *E. coli* 0138:K81:NM would immediately lead to cessation of synthesis and transport of the materials required for the continuous replenishment of the glycocalyx.

Although Ito (1969) has speculated that the glycocalyx might be

> "...characteristic for each species and for each cell type, and furthermore that it may vary in its structure and function in the same cell under different physiological conditions..."

there is little doubt that the glycocalyx is an integral part of the complex sequence of events occurring in the brush border, and that alteration in, or loss of, the glycocalyx will result in loss of essential sequential steps in the digestive and absorptive process. Whether of a primary or secondary nature, the partial or complete loss of the glycocalyx from villal epithelial cells observed in neonatal pigs monocontaminated with *E. coli* 0138:K81:NM would be expected to contribute materially to inadequate digestion and malabsorption.

The Apical Trilaminar Membrane.

This membrane, to which the glycocalyx is closely applied, is continuous over the microvillus, the support of the whole structure apparently depending on the integrity of the cytoskeleton. The cytoskeleton, or core, of the microvillus consists of groups of rods, each rod composed of "...a single strand of dense material helically wound upon itself to form a compact rod" (Eichholz, 1969). The design of the arrangement of these rods as described in this thesis (see Results) will no doubt be found to be more complex as improved stained preparations are available for examination, and indeed this has already been found to be so (Drees, D. T., personal communication).

The effect on microvilli of the presence of *E. coli* 0138:K81:NM, or its enterotoxin, is not immediately apparent. Staley *et al.*

(1968c, 1968d) described "exfoliation" of the microvilli to form microerosions in the apical plasmalemma prior to entry of the microorganisms into the villal epithelial cell. Although in the present experiments many instances were seen in which *E. coli* 0138:K81:NM organisms were closely approximated to the brush border, no discrete erosions of the microvilli directly associated with these microorganisms were seen. Compared with the intracytoplasmic organelles, microvilli are relatively resistant to the toxic principle associated with *E. coli* 0138:K81:NM, although in areas of high concentration of the organism or its enterotoxin, such as close proximity to the viable bacteria, the microvilli are affected.

It was observed that, where bacteria were adherent to the brush border, there was the formation of bizarre forms, foreshortening and stunting of the microvilli (Figures 96 and 99) extending over the entire apical surface of a particular cell and, at times, extending to neighboring cells. It was further observed, however, that in both germfree and monocontaminated pigs, as cells matured and moved along the basal lamina toward the tip of the villus, the microvilli became generally shorter and broader. One reason for this change can be proposed. There are no ribosomes or granular endoplasmic reticulum associated with the membranes constituting the apical plasmalemma, and there is no apparent ability of the apical trilaminar membrane to repair itself in case of injury or to replace used apical membrane. The regularity with which pinocytotic vesicles are formed from the apical plasmalemma during the short life of the villal epithelial cell is remarkable. It is reasonable to speculate

that, as pinocytotic vesicles form and are pinched off at the bases of the microvilli, so the trilaminar membrane of the apical plasmalemma is drawn from the walls of the microvilli to accommodate depletion of the trilaminar membrane. As the cell reaches the end of its life at the apex of the villus, so according to the amount of pinocytotic activity it has accomplished, it will have either long or foreshortened microvilli.

In those cells altered by intracytoplasmic vacuolization and organellar disruption, the brush borders were seen to have undergone marked degrees of degeneration.

In summary, in these experiments, three basic forms of microvillal change were observed: first, foreshortening and the formation of abnormal forms associated with proximity to *E. coli* 0138:K81:NM and presumably due to high concentration of enterotoxin in the immediate vicinity; second, frank disruption of the microvilli associated with intracytoplasmic disruption and disorganization of the cell with which the microvilli are associated; and third, a normal process of foreshortening apparently associated with maturation of the cell.

The lack of cytoskeletal detail, seen in sections of microvilli from monocontaminated animals, was not a consistent finding, and too few examinations of this particular feature of diseased cells were made for valid conclusions to be drawn.

The Terminal Web.

McNabb and Sandborn (1964) considered that the terminal web provided a structural basis for the cytoskeletal rootlets of the

microvilli. This structure was not seen in tissues from baby pigs according to Staley *et al.* (1968a), who used glutaraldehyde fixation for their experimental animal tissues. It may be that this structure is more easily stained and demonstrated after osmium tetroxide fixation since there was observed slightly more densely staining material in the region of the terminal web in villal epithelial cells of neonatal animals used in these experiments. It is understandable that, in those cells in which there is an extensive apical tubule system, the terminal web will be somewhat attenuated and perhaps made indistinct by the presence of the apical tubules.

There were no apparent changes observed in the terminal web of cells from monocontaminated pigs that could be attributed to the primary effects of the enterotoxin. Changes that were observed were inconsistent and were associated secondarily with disruption of the apical plasmalemma in diseased cells.

The Subapical Zone

Unlike the situation reported by Staley *et al.* (1968a), organelles were commonly seen in the subapical zone of the intestinal absorptive cells of both germfree and monocontaminated neonatal pigs (Figures 70, 72, 74, 76, 80, 84, 85, 96, 100, and 102).

The Apical Tubule System.

The presence of the apical tubule system as described by Staley *et al.* (1968a) was consistently observed in the mature villal epithelial cells of germfree and monocontaminated neonatal pigs. According to Staley *et al.* (1968a), the similarity of the membrane structure

of the apical tubules, the vacuoles, and the plasmalemma of the villal epithelial cell leads one to conclude that the vacuoles might be derived from, or be extensions of, the plasmalemmal membrane structure extending from the cell surface in the form of tubules into the apical cytoplasm. There were many instances in the experiments reported herein where it was clearly apparent that the laminae of the apical tubule system were continuous with (and possibly derived from) both the trilaminar membrane of the brush border and the membranes of the apical endoplasmic reticulum.

As was hypothesized in the discussion of the apical trilaminar membrane (*vide supra*), it might be that this membrane, in a process somewhat resembling pinocytosis and conjointly with the endoplasmic reticulum, contribute laminar material to the apical tubule system. Some evidence for this speculation lies in the observation that the microvilli are relatively longer in those more recently produced villal epithelial cells, than they are in cells that have an extensive apical tubule system.

Changes in the apical tubule system specifically associated with monocontamination with *E. coli* 0138:K81:NM were not apparent in these experiments. It was not possible to determine whether the disruption of the apical tubule system in the monocontaminated animals was secondary to general disorganization and disruption of the cells or if it was primarily affected by enterotoxin. The possible role of the apical tubule system in the absorption of enterotoxin is discussed elsewhere in this thesis.

Endoplasmic Reticulum and the Apical Vacuoles

There was morphologic evidence that the membrane-enclosed vacuoles observed in the cytoplasm of both germfree and monocontaminated neonatal pigs were probably distended elements of the endoplasmic reticulum. In view of the widespread gross vacuolization seen in both germfree and monocontaminated animals it seems that the most likely explanation for the presence of these vacuoles is that they are associated with absorption. Electron microscopically it was apparent that the vacuolization associated with *E. coli* infection was different from that of the germfree neonates in that, in infected animals, the membranes enclosing the vacuoles eventually fragmented, allowing escape of the vacuolar fluid into the extramembranous cytoplasm.

In sharp contrast, the vacuoles of the villal epithelial cells from germfree control pigs were seen to remain surrounded by intact membranes, despite instances of extreme distention with absorbed fluids. So-called "nonmembranous vacuoles" were present in the villal epithelial cells of both the germfree and monocontaminated animals, and these were considered to be localized areas of fluid containing little or no particulate matter which had been absorbed through the apical plasmalemma into the cytoplasmic matrix but had not yet entered the endoplasmic reticulum. Such vacuoles are apparent in Figure 72 and can be compared with membrane-enclosed vacuoles in the same electron micrograph.

The spine-like structures observed in the membrane-enclosed tubules of the subapical cytoplasm of the intestinal absorptive cell

have been postulated by Staley *et al.* (1968a) to be possible receptor sites or possible sites for enzyme activity. The presence of phosphatases in the plasmalemma (Goldfischer *et al.*, 1964) and their association with pinocytotic vesicle formation (Marchesi, 1965) has been cited by Staley *et al.* (1968a) as evidence of the probability of their postulate.

The endoplasmic reticulum has a number of metabolic activities which are characterized by the enzymes associated with its membrane component. Although an electron histochemical study of the changes in the endoplasmic reticulum associated with colibacillosis was not made in these experiments, the changes in vacuolar membrane structure and function, when considered with the histochemical findings (*vide supra*) of a general reduction within the cytoplasm of both adenosine triphosphatase and lactate dehydrogenase activity, provides additional support for the concept of a biochemical lesion in the membranes of the endoplasmic reticulum's being associated with enterotoxin activity. It is reasonable to expect that such lesions would cause a failure in membrane function, expressed either as disruption of the membrane or as an inability of the membrane to transport fluids in one or both directions. If this be true, the distention and rupture of the membrane-enclosed vacuoles observed in the infected cells could be expected to occur.

The major electron microscopic difference between the granular and agranular endoplasmic reticulum is the presence of ribosomes in the limiting membranes of the former structure. Indeed, it is likely that the two major types of endoplasmic reticulum are a continuous

system of planar membranes and tubules, their respective functions and enzyme content varying according to whether simple transport, synthesis of protein "packaging" materials (in which ribosomes are required), specialized transport enzymes, and/or synthetase mechanisms, etc., are present.

In those markedly vacuolate cells from control animals it can be hypothesized that there is a lag in the transport of fluids from the vacuoles of villal epithelial cells of neonatal germfree pigs but that this removal is successfully accomplished with the passage of time. It may be that such vacuolate cells are incapable of further absorption until the fluids that have already been absorbed are successfully evacuated by the endoplasmic reticulum. The fact that the membranes remain intact around these vacuoles, despite marked distention, is probably indicative of their health and is in very strong contrast with the extensive disruption of membranes within the cells of animals monocontaminated with *E. coli* 0138:K81:NM.

The vacuolization so characteristic of villal epithelial cells from the more terminal sites of the small intestine of neonatal germfree pigs is probably a sign of either increased absorptive efficiency of the apical plasmalemma or alternatively (and more likely) relative deficiency or inefficiency of one or more enzymes of the endoplasmic reticulum that are required for the rapid and efficient transport of absorbed fluids within the cisternae of the endoplasmic reticulum away from the cell and into the lamina propria.

Whether this postulated enzyme deficiency is characteristic of germfree pigs and is not made up until a normal intestinal flora

is established is not known. Certainly, the feces of germfree pigs are more fluid than those of conventional animals for at least 4 weeks from birth, and the villal epithelial cells from germfree pigs retain a markedly vacuolate appearance throughout this time (Christie, unpublished data). It is apparent that, in germfree animals, despite vacuolization of these cells, absorption proceeds at an adequate level, whereas in neonatal pigs monocontaminated with *E. coli* 0138:K81:NM, the vacuolization and eventual disruption of the villal epithelial cell is such that the cell fails as an integral part of the absorptive mechanism.

There was no evidence that the vacuolization of the villal epithelial cells of either the control animals or the monocontaminates was due to the collection of fat within the cytoplasm.

The presence of stained fat in the apical cytoplasm of villal epithelial cells in germfree and some monocontaminates agrees with the concept of lipid absorption in micellar phase through the apical plasmalemma (Isselbacher, 1967), and by the apical pinocytotic system of tubules and vacuoles (Mattisson and Karlsson, 1964; 1965; 1966). Once inside the cell, the finely dispersed lipid aggregated just below the terminal web, where droplets could be seen with no surrounding membrane. Deeper within the cell, the droplets were seen to be surrounded by both granular and agranular endoplasmic reticulum.

According to Dawson (1967), the droplets leave through the base of the cell into the intercellular space, pass into the extracellular fluid and then proceed through pores into lymphatics. Some

evidence in confirmation of this chain of events was seen in electron micrographs of a villal epithelial cell from a 24-hour monocontaminated pig which was actively absorbing fat.

It is clear that impairment of digestion of fat within the lumen will occur if bile salts or pancreatic enzymes are deficient or in some way made ineffective. There is no evidence to suggest that *E. coli* 0138:K81:NM infection adversely affects either the pancreas or the liver in neonatal gnotobiotic pigs (Christie, 1967). However, in view of the marked derangement of membranous intracellular structures of the villal epithelial cells that is associated with this infection, one might expect that the derangement would also lead to deranged function of membrane-bound enzymes. It was apparent that stainable fat had entered the apical cytoplasm of villal epithelial cells of the duodenum and early jejunum in monocontaminated and clinically affected pigs, whereas in tissues more distal in the small intestine, no oil-red-O-positive material was observed in the laminae propriae.

Since fat in the form of triglycerides or chylomicrons could be demonstrated in electron micrographs of affected cells, and since the entry of the micellar lipid is a nonenergy process not mediated by enzyme, it may well be that fatty acid and monoglyceride in micellar form can enter a diseased villal epithelial cell through a patent brush border, yet not be further absorbed because of disruption of the triglyceride synthetase mechanism.

Enterotoxin of *E. coli* could disrupt this mechanism in many ways -- either directly or indirectly. This could occur directly

by chemical destruction of triglyceride synthetase in the agranular reticulum or by combination with one or more of the enzyme or substrate molecules of either the biochemical energy-producing cycles or protein-synthesis mechanism of the granular reticulum. This could occur indirectly by physical disruption of these or other interdependent systems. Electron microscopic evidence of specific injury to either the granular or agranular endoplasmic reticulum could be considered presumptive evidence of this aspect of pathogenesis of fat malabsorption.

The villal epithelial cells from the jejunum and ileum of the clinically affected animals were generally markedly vacuolate, in some instances to the point of complete disruption. It is more likely that deficiency of the triglyceride synthetase mechanism, or failure of the protein synthesis required in the manufacture of chylomicrons, is a phenomenon secondary to more generalized organellar disruption that contributes to the overall malfunction of the absorption mechanism in colibacillosis.

The Nucleus

Staley *et al.* (1968a) described the position of the nucleus within the cell and related it to the position of the epithelial cell on the villus. At the base of the villus they described the nucleus as being closer to the basement membrane, whereas toward the tip of the villus the nucleus "...occupied the apical portion of the cytoplasm".

In contrast, it was seen in these experiments and in those previously reported (Christie, 1967) that the position of the nucleus within vacuolate villal epithelial cells was not consistent according to position of the cell on the villal stalk. A degree of consistency of nuclear position in neighboring villi was observed, in that on a single villus, and often in neighboring villi, cells would generally have basally situated nuclei, whereas sometimes in the same section of the tissue, but at a little distance from that site, villi and their immediate neighbors would generally have apically situated nuclei.

It is unlikely that this positioning of the nucleus indicates a basic difference in the nature of the endoplasmic reticulum of the subapical and the basal cytoplasm. Whether the nucleus is pushed to the apex of the cell or to the base of the cell will depend on which of the sites of endoplasmic reticulum activity is less efficient in transport of absorbed fluids and hence becomes vacuolate.

It is tempting to accept the observation of Staley *et al.* (1968a) and postulate that the change is one of enzyme maturation, aging, or senility, according to the position of the cell on the villal stalk, but their observation was not confirmed by this author.

An alternative hypothesis, that the vacuolization and therefore the nuclear position depends on the relative content of the chyme -- carbohydrate, fat, protein and water -- bathing the villi at that particular time, is proposed. It was of interest to observe that in electron micrographs of cells in which absorbed fat was readily detected, the nucleus was displaced into the apex of the cell (Figure

80), whereas in those vacuolate cells in which fat was not readily observed, the vacuolization was apical and tended to push the nuclei towards the base of the cells.

No relationship was detected between infection with *E. coli* and the position of the nucleus within affected cells from the tissues examined in these experiments. At no stage in these tissues were the nuclei of the affected cells seen to undergo the classic changes described for necrosis -- pyknosis, karyoschisis, karyorrhexis or karyolysis.

The Mitochondria

The mitochondrion is composed of two discrete membranes, "...aggregates of controlled, directional metabolic processes" (Korn, 1969), which catalyze the biochemically integrated reaction sequences of fatty acid oxidation, the tricarboxylic acid cycle, oxidative phosphorylation and electron transport. Its functions include selective permeability, organization of the electron transport and adenosinetriphosphate-generating cycles, conversion and storage of chemically derived energy, and loci for specific antigens (Johnson, 1969). The enzymes of these processes are not soluble nor are they floating freely in the matrices of the cristae mitochondriales but are most probably membrane-bound in a lattice or mosaic of integral subunits constituting the membrane itself and arranged in a spatial configuration that favors the sequential reactions of the particular metabolic cycle.

As was forecast speculatively in the interpretation of the histochemical tests for adenosine triphosphatase and succinate dehydrogenase and confirmed morphologically during electron microscopic examination of comparable tissues, vacuolization of the cristae mitochondriales, followed successively by swelling, internal disruption, and eventually rupture of the mitochondria, was associated with monocontamination of the villal epithelial cells of neonatal pigs with *E. coli* 0138:K81:NM.

The early changes, primarily moderate vacuolization of the cristae mitochondriales, were consistent and easily detected; more advanced degenerative changes were such that at times it was difficult to recognize the disrupted mitochondria, and they were likely to be confused at this stage of degeneration with membrane-enclosed vacuoles containing membranous and particulate material.

Again, it is not possible to state from these results whether the mitochondrial changes are primarily the result of enterotoxin activity on their constituent enzymes or secondarily the result of generalized disruption of the cytoplasmic constituents. However, considering the basic biochemical role of adenosine triphosphatase both in the mitochondria and other organelles, it is tempting to speculate that *E. coli* 0138:K81:NM enterotoxin has a specific toxic effect on adenosine triphosphatase and, through this effect, has the ability to produce the spectrum of ultrastructural changes observed in the tissues from these experimental animals.

The Golgi Complex

In mature cells, lytic enzymes are transferred from the Golgi complex by coated vesicles to digestive vacuoles where degradation of some intracytoplasmic protein occurs (Friend and Farquhar, 1967), and it may be that this is one of the functions of the Golgi complex in the villal epithelial cells of the neonatal pig. The probable role of the Golgi complex in the production of the glycocalyx has already been discussed (*vide supra*). Some vacuolization of the archoplasm was observed in cells from germfree control pigs and in the cells from monocontaminated pigs.

The general reduction of acid phosphatase activity observed in the villal epithelial cells from monocontaminates could indicate loss of lysosomal activity or failure of the Golgi complex in its lytic enzyme-producing activity.

Again, the reduction in amount of detectable glycocalyx associated with monocontamination with *E. coli* could be the result of failing activity of the Golgi complex, either due to organellar disruption or direct toxic activity of the *E. coli* enterotoxin. Other reasons accounting for this observation suggest themselves -- inability of the cell transport system to carry the preformed elements of the glycocalyx from the Golgi complex to the brush border, disruption of the transport route, changes in the brush border preventing the deposition or replenishment of the glycocalyx, or direct lytic or toxic activity of the enterotoxin upon the glycocalyx.

The precise effect of enterotoxin on the Golgin complex was not determined in these experiments.

Maturation and Necrobiosis of Villal Epithelial Cells

The high turnover rate of villal epithelial cells theoretically necessitates a high level of reproduction of epithelial cells within the crypts and requires that the same number of cells be lost, or shed, from the tips of the villi. Even if the villal epithelial cell turnover time for neonatal pigs is as long as that recorded for man, it is obvious that some hundreds of cells per day are lost from each villus. The exact nature of this necrobiosis has not yet been determined despite the work reported by Leblond and Messier (1958), Deschner *et al.* (1963), Abrams *et al.* (1963) and Lesher *et al.* (1964).

It is clear that, with this level of necrobiosis occurring, two major considerations need to be made in interpretation of epithelial lesions associated with colibacillosis in neonatal gnotobiotes.

First, it is likely that epithelial cells near the tip of the villus are already undergoing changes which will soon lead to necrobiosis. If this be true, then cells at the tip of the villus observed under both optical and electron microscopes will have structural changes not only associated with any specific or experimental condition (such as colibacillosis) but will also exhibit changes associated with incipient necrobiosis. Such changes may lead to confusion in interpretation of ultrastructural changes associated with the intended stress.

Second, since there is ultrastructural evidence of limited ability of the villal epithelial cell for biosynthesis of macromolecules (except for the function of the granular endoplasmic

reticulum), the changes leading to necrobiosis may well be simply the complete utilization of the existing stores of enzymes and organellar components and an inability to repair or replace these deficiencies. Hence, after a short life span, the cell becomes effete and is lost from the villus. Since highly specialized cells, such as the villal epithelial cells, have a limited range of response to stress, it can be readily imagined that an effete villal epithelial cell, no longer replete with its normal enzyme complement and other components, will probably react morphologically with vacuolization and breakdown of organelles, hydropic degeneration, and rupture of the cell. Those cells not so expended are presumably pushed from the basal lamina at the tip of the villus by the stream of ascending cells following behind.

It is postulated that the ultrastructural changes within the villal epithelial cells associated with colibacillosis due to *E. coli* 0138:K81:NM resemble, to a degree, the ultrastructural changes associated with necrobiosis. The most likely difference is that most of the villal epithelium is affected in enteric colibacillosis and that such epithelium is retained on the villus (see later), whereas in conventional or germfree neonates the cells undergoing necrobiosis are restricted to the tips of the villi and are rapidly shed when no longer functional.

In order to avoid the possibility of confusion between ultrastructural changes in villal epithelial cells due to *E. coli* enterotoxin and those due to necrobiosis in experiments reported in this thesis, only villal epithelial cells from the midvillus and basal third of the villus were examined.

Kenworthy *et al.* (1967) reported an electron microscopic study of the epithelium from the tips of the villi of pigs contaminated with specific serotypes of *E. coli*. Their description of ultrastructural changes in these cells closely resembled those changes one would expect to find in effete villal epithelial cells, and there was apparently no way these authors differentiated between the pathologic effects of their test organisms and ultrastructural changes associated with necrobiosis.

In tissues from pigs 42 hours old, Staley *et al.* (1968a) recorded finding on the villi long columns of degenerating cells "...stacked on their tips". The figure purporting to display this phenomenon does not bear out their conclusion. It is apparent that the villus depicted had curved out of the plane of the tissue section and they had in fact photographed a sagittal section of a portion of the villus. The alleged change in cell type depicted in their photograph, from columnar to cuboidal, and the apparent loss of the brush border, is entirely compatible with change in the plane of the cells from longitudinal section, as they depict in midvillus, to transverse section as is depicted toward the tip of the villus. There is little doubt that some of the cells they observed were degenerating. Their description of the intracellular changes of swelling and fragmentation of the microvilli, swelling, vacuolization, and loss of the cristae mitochondriales, are what one would anticipate in effete villal epithelial cells; and their electron microscopic confirmation of this concept constitutes a valuable contribution to the literature. It is of interest to note that an additional "degenerative"

change described was the presence of intracytoplasmic membrane-enclosed vacuoles possibly originating from degenerating apical tubules and mitochondria.

There are alternative conclusions that can be drawn from their evidence (*vide supra*) which tend to support the hypothesis that these authors (Staley *et al.*, 1968a) have possibly confused the intracellular signs of normal function of the jejunal villal epithelial cell with the signs of cellular degeneration.

In interpreting Vodovar's conclusion that the pig intestinal epithelial cell must elongate during maturation (Vodovar, 1964) Staley *et al.* (1968a) stated that the villi reach apparently mature length before 42 hours because, in their experimental pigs killed 42 hours after birth, degenerating cells were evident at the tip of the villus. Although the latter statement has already been questioned (*vide supra*) it is also an unlikely assumption that the process of growth, maturation and necrobiosis of villal epithelial cells does not begin and continue until after the birth of the fetus. It is more reasonable to assume that from the moment of the embryological development of the specialized epithelium of the small intestine these cells multiply, mature, die and are shed during the intrauterine life of the fetus. In this case one would expect to find mature cells undergoing necrobiosis in the villal epithelium of pigs at the moment of birth.

In relation to the position of the Golgi complex in the mature cell, Staley *et al.* (1968a) intimated that in the neonatal pig the Golgi complex is in a subnuclear position whereas

> "...the Golgi is located in a supranuclear position in the three week old piglet, indicating a change in polarity of the cell as the cell matures".

Arguing along similar lines as before, one could reasonably expect to find "mature" cells immediately prior to the stage of necrobiosis at the tip of the villus in the neonate, and if the hypothesis of Staley *et al.* (1968a) is correct, one would see cells in that position with supranuclear Golgi apparatus.

This is generally not so, hence another explanation can be proposed, and that is a process of reorganization rather than maturation. Payne and Marsh (1962) reported that pigs maintained on a protein-free diet up to 106 hours after birth still actively absorbed immunoglobulins, whereas contact with colostrum and/or protein initiates the phenomenon of "closure" (Lecce *et al.*, 1964). Lecce *et al.* (1964) were also able to "close" the epithelium to globulin absorption with a heat-stable low-molecular-weight component dialyzed from colostrum or skim milk. It may be that the reorganization of the cell associated with "closure" not only involves the loss of the apical tubular system and decline in pinocytotic activity but also relocation of the Golgi complex and elongation of the entire villal epithelial cell.

Mural Interdigitations

It is morphologically evident that the villal epithelial cells are attached laterally to each other by tight junctions called "junctional complexes" (Farquhar and Palade, 1963) or, more commonly, desmosomal attachments or terminal bars. These unions generally

extend for a short distance below the brush border. The remainder of the cell surface, laterally and in contact with the basal lamina should be considered the base of the cell through which water and solutes leave. There was, however, a remarkable degree of interdigitation of the neighboring cell walls, sometimes associated with desmosomes and sometimes not. Again, there were occasions when desmosomes were found low on the cell walls, close to the basal lamina.

Kenworthy *et al.* (1967), in their paper describing ultrastructural changes of villal epithelial cells associated with *E. coli* infection, depicted the alleged degeneration and disappearance of cell membranes on either side of desmosomal plaques. Reinterpreting their electron micrographs in the light of the findings of the experiments described herein, and considering the ultrastructural appearance and behavior of broken or ruptured membranes, it seems more likely that the phenomenon of intermittent disappearance of the cell membrane on either side of the desmosomal plaque that they depict is artefactual in that the membrane is present but is simply not resolved by the electron microscope at that particular point. Numerous instances of this phenomenon were observed during the study described herein and were readily distinguishable from those instances where membranes were in fact ruptured or disintegrating.

Electron microscopically, it was seen that, even in cells in an advanced state of disruption, there were few, if any, of the "classical" nuclear signs of necrosis that are familiar to the optical microscopist. It is not possible to say that the cells affected

by *E. coli* enterotoxin are necrotic in the classical sense. Although the villal epithelial cells are disrupted and effete, the mural interdigitations are retained. As a consequence, the affected cell is not lost or sloughed from the villus -- as it is in TGE -- hence the lamina propria is not left denuded and does not retract, or shorten, as it does in TGE. In enteric colibacillosis the villus retains a covering of effete villal epithelial cells which, although relatively or completely ineffective in absorption (and possibly contributing to loss of tissue fluid from the edematous lamina propria) do serve to maintain the long villi that are characteristic of primary colibacillary enteritis.

Staley *et al.* (1968a) stated that

> "...despite apparent degeneration and instability of the cell membrane, the desmosomal attachments were intact and held the cells together. There was no evidence of nuclear degeneration as indicated by shrinking, serration of the nuclear membrane, or abnormal clumping of the chromatin".

After examination of their electron micrographs, and in consideration of the findings of the experiments described herein, it is likely that Staley *et al.* (1968a) had, in fact, been examining absorptive cells in a sagittal section of the villus. The lack of nuclear changes normally associated with degeneration and necrosis indicates that the cells they were describing were not, in fact, primarily degenerating cells but mature villal epithelial cells carrying on their functional activity. Such activity of necessity involves intracytoplasmic changes, some of which are found in a degenerating cell, and which in the final stages of necrobiosis will be associated

with the disruption of the organelles and extrusion of the nucleus and cytoplasmic contents, or else shedding of the intact cell from the extreme tip of the villus.

The Lamina Propria

In the germfree neonatal pig the lamina propria of the villus was seen to be relatively free of excess interstitial fluid (Figures 67, 69 and 77) and consisted primarily of lymphatic and vascular endothelium, Breucke fibers, some histiocytes, and occasional fibroblasts in a fluid matrix in which some fine collagen strands, chylomicrons and fat droplets were suspended (Figures 69, 77 and 78). The endothelial cells of capillaries were characterized by the presence of mitochondria and many micropinocytotic vesicles within the cytoplasm, marginal folding of the internal membrane, and the presence of erythrocytes in the lumen (Figure 78).

Lymphatic endothelium was characterized by fewer mitochondria, fewer micropinocytotic vesicles and marginal folds, and the presence of particulate matter and delaminating membranes within the lumen (Figure 77).

It was apparent that monocontamination with *E. coli* 0138:K81:NM was associated with increased amounts of interstitial fluid (edema) containing membranous fragments, collagen fibrils, and unidentified flocculent material, but these changes were not accompanied by distinctive evidence of endothelial damage or malfunction of either capillaries or lymphatics.

It was observed in the laminae propriae of villi from neonates monocontaminated 14 or more hours beforehand that there were increased amounts of fluid of edema and collagenous fibrils. There was apparently reduced micropinocytotic activity of capillary endothelial cells and increased thickness of the basal laminae surrounding the capillaries (Figure 95).

By 96 hours after monocontamination with *E. coli* 0138:K81:NM the lamina propria was densely infiltrated with collagenous fibrils suspended in tissue fluid. Such collagen formation was not a feature of comparable tissues from germfree pigs, but may well be present in the laminae propriae of conventional, clinically normal, neonatal pigs.

Zweifach (1964), reviewing *E. coli* endotoxin activity, recorded that it did not appear to have a direct cytotoxic action, but he did 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, at an increased rate, then at higher levels of endotoxin, at a decreased rate of sequential reaction of the vasculature due to endotoxic sensitization of the muscular vessels to exogenous and endogenous stimuli. There was no evidence of damage to the endothelium of the capillaries and venules. There was increased capillary permeability, and *E. coli* endotoxin -- a lipopolysaccharide -- did produce stasis of the capillary bed by a mechanism of narrowing of the lumina of the draining vessels (Zweifach, 1964).

Insufficient numbers of tissues from the laminae propriae were examined in these experiments to enable valid conclusions to be

drawn. In the tissues that were examined, however, the impression gained was not that of epithelial breakdown and leakage, but rather that of capillary contraction and pericyte contraction which agrees in part with Zweifach's findings. There was also an impression of impermeability and inactivity of the vessels of the lamina propria that would probably lead to inefficient drainage of fluids from the lamina propria, through the vessels, to the general circulation. This comment is somewhat contradictory to the observations made for the effects of *E. coli* endotoxin (Zweifach, 1964) and of *Vibrio sp.* toxin (Elliott *et al.*, 1968) on the vasculature.

A similarity between human intestinal lymphangiectasia, as described by Dobbins (1966), and certain aspects of colibacillosis of neonatal gnotobiotic swine can be seen in that there is apparent partial "closing down" of capillaries, retarded emptying of lacteals, and collection of fluid of edema in the intramembranous space and the interstices of the lamina propria. As it is for human intestinal lymphangeictasia in which there is closure of the interendothelial cell spaces and "...unusually prominent intracellular filaments", there are some detectable differences between the intestinal lymphatic endothelial cells of the germfree and the monocontaminated experimental pigs. It is not likely that the mechanism of "exit block" that is responsible for fluid collection in the villal epithelial cells of lymphangiectasia patients is a major factor in the pathogenesis of fluid retention in the villi of the monocontaminated neonatal pigs, although the role of this phenomenon as a contributory factor to colibacillary diarrhea should be kept in mind.

Absorption and Malabsorption

In the state of absorption the net flow of fluid is from the intestinal lumen into the lamina propria and thence to the vessels of the villus. In the diarrheal state, the net flow is from the vessels to the intestinal lumen. In the latter case the net flow might be due either to increase in the amount of fluid excreted or lost from the villus to the lumen, the absorption component remaining unchanged; a diminished rate of absorption, the vessel to lumen flux remaining at a normal level; or else both diminished absorption and excessive fluid excretion or loss. This bidirectional flux holds for water and its electrolytes -- chloride, sodium, potassium, bicarbonate and others whose net flow varies markedly in diarrhea.

The present state of knowledge is such that it is not yet possible to relate specific ultrastructural alterations of the villal epithelial cell to a particular biochemical defect. It would be fortuitous if it were found that the enterotoxin of *E. coli* 0138:K81:NM produced its effects through a single biochemical lesion, but it is clear from the clinical, histopathologic and ultrastructural manifestations that this is most unlikely.

From the information abstracted in the Literature Review (*vide supra*) and the histochemical and ultrastructural changes reported in this thesis (at almost every step, from the terminal digestion of foodstuffs in the superficial layers of the glycocalyx to the eventual removal of metabolites by the vessels of the lamina propria), there are indications of disturbed function. Which of these absorptive systems, singly or in combination, is the specific target of

E. coli 0138:K81:NM enterotoxin, and which are secondarily disrupted, could not be determined from the results of these experiments.

It is clear that *E. coli* 0138:K81:NM adversely affects the brush border morphologically and enzymatically, so that the terminal digestive processes, particularly of proteins and sugars, essential for their initial absorption through the apical plasmalemma (Holt and Miller, 1961; Miller and Crane, 1961; Gray, 1967) will be markedly reduced or prevented.

Those processes described for amino acid transport (Wiseman, 1953; Fisher, 1967; Newey, 1967), sodium and glucose transport (Lifson and Parsons, 1957; Parsons, 1967; Crane, R. K., personal communication), the transport of water (Fordtran, 1967), reciprocal-anion movement in ileum and colon (Fordtran, 1967), micellar fat absorption (Hoffman and Borgstrom, 1962; Isselbacher, 1967), and many others, less clearly defined, require an intact functional plasmalemma for optimal function. Although much is yet to be discovered of the absorptive process at the level of the brush border, it is apparent that the histochemical and morphologic changes in the brush border that are associated with colibacillary diarrhea will be significant factors in malabsorption.

Again, energy dependent reactions involved in absorption, such as those for the sodium and potassium pumps (Robinson, 1960, Fisher, 1967; Newey, 1967; Parsons, 1967), absorption of basic carbohydrate molecules (Lifson and Parsons, 1957; Parsons, 1967; Crane, 1968), the postulated processes of "chemi-osmotic coupling" (Mitchell, 1961)

and the outward sodium pump at the brush border (Parsons, 1967), and reesterification of triglyceride (Senior, 1964; Porter, 1969) will depend on functional mitochondria, endoplasmic reticulum, and possibly other cellular energy sources and utilization points. Mitochondrial degeneration, and the diminution of succinate dehydrogenase and adenosine triphosphatase activity, are strongly suggestive of the likely failure of the energy-dependent essential absorptive processes in the intestinal mucosa of neonates monocontaminated with *E. coli* 0138:K81:NM.

The intracellular processes concerned with absorption, carbohydrate metabolism (Aurrichio *et al.*, 1963; Dahlqvist *et al.*, 1963; Levin, 1967) and synthesis of chylomicrons from protein, cholesterol and phospholipid (Zilversmit, 1965) require the integrity of intracytoplasmic membranes for normal function, are enzyme mediated, and will be similarly disrupted in cells undergoing the degenerative changes observed in these experiments.

Flow of water in absorption is so much a matter of controversy at this time that it is likely to be both an active and a passive process. Osmotic changes of the lumen contents have a marked effect on net water flow (Fordtran, 1967). Water absorption is known to depend on the active absorption of an ion such as sodium, but water also follows sodium down an osmotic gradient. On an osmotic basis, to postulate net flow of water from the vessels of the villus to the lumen requires that there be an increased number of osmotically active particles in the lumen. Theoretically, the source of these particles could be from vessels of the lamina propria subsequent to

endothelial damage, allowing escape of large proteins and colloids to the lumen, from the action of bacteria in manufacture of osmotically active metabolites, or from the failure of osmotically active particles, such as sodium, to be absorbed.

From the results of these experiments, endothelial damage of vessels in the lamina propria, leading to escape of osmotically active substances, is not likely to be a factor in the enteritis associated with monocontamination with *E. coli* 0138:K81:NM, although further study of this aspect of pathogenesis for this and other serotypes of *E. coli* is necessary.

Barnum *et al.* (1967) suggest that *E. coli* splits macromolecules normally present in the lumen into smaller molecules of increased osmotic activity. If this be true, should the presence of *E. coli* not alter the capacity of the intestinal epithelial membrane as a semipermeable membrane -- thereby permitting osmosis -- but so change the membrane as to prevent the proper absorption of the digested particles, then a net outflow of fluid from the intestinal wall to the lumen will result.

Fordtran (1967) has referred to the ease of diffusion of sodium ions back into the lumen through the large aqueous pores of the apical plasmalemma of the villal epithelial cells in the duodenum and anterior jejunum. This same "leak back" is prevented in the terminal jejunum and ileum because of the relatively smaller size of the aqueous pores in that region of the intestine. Darrow (1946) considered that intestinal hypermotility was a major cause of inadequate sodium absorption in infantile diarrhea.

Considering all of the above factors mediating against absorption and the general ultrastructural disruption of the villal mucosa -- particularly in the terminal jejunum and ileum -- it is likely that sodium loss will be an important factor in neonatal colibacillary diarrhea.

Factors external to the villus, and possibly of importance in the pathogenesis of colibacillary diarrhea have been discussed in detail elsewhere (Christie, 1967).

CONCLUSION

The need for future research will be at the ultrastructural and biochemical level if a more complete understanding of the pathogenesis of colibacillosis is to be achieved.

The more detailed characterization of the ultrastructural and histochemical lesions, some of which are described in this thesis, awaits only the application (and in some instances the development) of appropriate electron histochemical procedures. The determination of which lesions are primary and which are secondary should remove much of the speculation about pathogenesis that is presented in this thesis.

Obviously, important lesions to be further investigated are those of the basic energy-producing processes involving adenosine triphosphatase and its related organelles -- the mitochondria, possibly the endoplasmic reticulum, and the brush border. The alterations of the glycocalyx are also obvious targets for intensive study and are likely to be important contributory factors in the pathogenesis of colibacillary diarrhea.

Of equal importance are membrane transport studies, but these studies will require the techniques and attention of a number of scientific disciplines.

It seems appropriate that future experimental work in neonatal colibacillary diarrhea should include analysis of intestinal contents, and ligated intestinal-loop contents, at all levels of the tract,

for sodium, potassium, bicarbonate, chloride, glucose and amino acid content. Suitable experimental treatment groups might well be conventional and germfree neonatal pigs, monocontaminated with *E. coli* 0138:K81:NM or inoculated with sterile preparations of enterotoxin from such an organism.

The role of the colon in water absorption and ion exchange has long been known. Recently Levitan *et al.* (1962) and Fordtran (1967) have reported that in the normal animal only about 20% of the colon's capacity to absorb water and electrolytes is used. It is clear that future studies of colibacillary diarrhea should include consideration of the factors of secretion and absorption in the colon, and the motility of the large intestine, in addition to such changes that are known to exist in the small intestine.

Although the experiments and observations described in this thesis are not comparable in some respects with the clinical syndrome of infantile colibacillary diarrhea or with colibacillary diarrhea of conventional baby pigs, at least two of the unique characteristics of the neonatal gnotobiotic pig -- lack of placental transfer of immune globulins and relative freedom from bacterial antigens -- enables one to examine in much more elementary fashion the basic lesions of enteric colibacillosis.

Such a study, uncomplicated by the manifestations of sensitization, immune phenomena, antigen-antibody responses, malnutrition, and the multiple histologic, physiologic and immunologic effects associated with the presence of other intestinal flora, is open to criticism from the viewpoint that infantile diarrhea and the neonatal

diarrheas of animals are never seen clinically in such an uncomplicated state. If these factors are not forgotten, but put aside temporarily whilst the basic lesion is considered, then we might more easily understand the pathogenesis of colibacillary diarrhea.

With a basic concept of the pathogenesis, it should then be possible, according to our knowledge of the modifying superimposed and complicating factors present in particular clinical situations, to develop the means of prophylaxis, alleviation or cure of the disease, in an individual, or as an entity.

SUMMARY

Forty-eight gnotobiotic pigs were used in experiments in which 34 animals were fed between 1.8 and 3.0 x 10^6 *E. coli* 0138:K81:NM organisms. Observations were made of the clinical effects of the infection at varying times from 4 to 96 hours after monocontamination. The animals were killed and observations were made of the macroscopic lesions of disease. Tissues were taken from the mucosa at 5 levels of the small intestine and submitted to optical microscopic and electron microscopic examination, and histochemical staining for alkaline phosphatase, acid phosphatase, leucine aminopeptidase, succinate dehydrogenase, and lactate dehydrogenase activity.

Diarrhea occurred between 8 and 12 hours after monocontamination with *E. coli*. Raised hair coat, projectile watery diarrhea and dehydration were also recorded within 18 hours of monocontamination.

At necropsy, the intestines were at times flaccid and fluid filled, and varying degrees of edema and hyperemia of the gastric and intestinal walls were observed. Occasionally ascites was present and in those animals scouring for longer periods, erythema venenatum was seen.

The optical microscopic lesions ranged from those of a mild acute enteritis to a histologic picture, in persistently scouring pigs, similar to that seen in clinically normal germfree control animals. The most common lesion observed in the villal epithelial cells from monocontaminated animals was marked vacuolization and

hydropic degeneration. This lesion was microscopically indistinguishable from the vacuolization associated with absorption which was seen in both monocontaminated and germfree neonates.

Electron microscopic examination revealed the basic difference between these two types of vacuolization. So-called absorption vacuoles were membrane-enclosed, probably within the endoplasmic reticulum, and were associated with normal function, normal cytologic enzyme activity and distribution, and absence of diarrhea in host animals. Vacuolization of the mucosa of infected neonates was at first similar to that seen in the control animals, but within 12 to 20 hours of monocontamination with *E. coli* 0138:K81:NM, there was observed diminution in the form and histochemical function of the glycocalyx, loss of alkaline phosphatase and leucine aminopeptidase activity from the brush border, and degeneration of the microvilli. There was vacuolization of the cristae mitochondriales leading to swelling, gross enlargement and rupture of the mitochondria, vacuolization then lysis of the endoplasmic reticulum and Golgi complex, and eventual complete disruption of the internal cytoplasmic structure. The cell then became a fluid-filled sac with its nucleus floating in a fluid matrix of membrane fragments and unidentified particulate matter. Associated with these changes were loss of activity of alkaline phosphatase, acid phosphatase, adenosine triphosphatase, leucine aminopeptidase, succinate dehydrogenase and lactate dehydrogenase.

These effete cells generally were retained on the villal stalk by the persistence of the external cell membrane and the preservation

of the mural interdigitations with neighboring cells. At times rupture of the apical plasmalemma and extrusion of the cell contents into the intestinal lumen were seen.

Edema of the lamina propria was observed in the monocontaminated neonates, followed by infiltration of the interstices with collagen fibrils. Some evidence of contraction and functional inactivity of the vasculature of the lamina propria was observed.

It is speculated (1) that the enterotoxin of *E. coli* 0138:K81:NM is readily absorbed by the villal epithelial cell and is toxic for membrane-bound enzymes, (2) that it exerts its toxic effects primarily on those energy-producing systems involving adenosine triphosphatase -- the mitochondria, the endoplasmic reticulum and the Golgi complex, (3) that it is primarily toxic for the glycocalyx and secondarily toxic for this entity by its effect on the Golgi complex, and (4) that it is secondarily disruptive to the laminar organelles of the intestinal absorptive cell.

Persistence of mural interdigitations permitting continued intercellular adhesion with retention of the effete cells on the villal stalk is hypothesized as an explanation for the major difference in the histologic appearance of the small intestine in colibacillary diarrhea and the enteric diseases involving villal atrophy in man and animals.

It is speculated that the endotoxic fraction of *E. coli* 0138:K81:NM enterotoxin is responsible for the inflammatory responses observed in the lamina propria and the basic changes in villal function.

It is considered that neonatal colibacillary diarrhea is primarily a disease of malabsorption.

LIST OF REFERENCES

LIST OF REFERENCES

Abrams, G. D., Bauer, H., and Sprinz, H.: Influence of the normal flora on mucosal morphology and cellular renewal in the ileum. A comparison of germ-free and conventional mice. Lab. Invest., 12,(1963): 355-364.

Adam, A.: *Dyspepsiekoli. Zur Frage der bakteriellen Ätiologie der sogen. alimentären Intoxikation.* Jb. Kinderheilk. phys. Erzieh., 116,(1927): 8-40.

Albersheim, P., and Killias, U.: The use of bismuth as an electron stain for nucleic acids. J. Cell Biol., 17,(1963): 93-103.

Anderson, J. W.: Ultrastructural correlates of intestinal protein absorption. Report of the Ross Conferences on Pediatric Research, 1963-1965, Ross Laboratories, Columbus, Ohio, 50,(1965): 28-39.

Anon.: Summary of reports on the health conditions in the Americas, 1950-1953. (Scientific Publication No. 25) Pan American Sanitary Bureau, Washington, D.C. (1956).

Anon.: Proceedings of the National Serminar on *Enterobacteriaceae*, June 8/9, 1961. Kasauli, Punjab. Indian J. med. Res., 50,(1962): 640-659.

Armed Forces Institute of Pathology: *Manual of Histologic and Special Staining Technics*, 2nd edn, Washington, D.C. (1960).

Auricchio, S. A., Rubino, A., Landolt, M., Semenza, G., and Prader, A.: Isolated intestinal lactase deficiency in the adult. Lancet, 2,(1963): 324-326.

Badenoch, J.: The blind loop syndrome. In *Modern Trends in Gastroenterology Second Series*, F. Avery Jones, ed. Paul B. Hoeber, Inc., New York, N.Y. (1958): 231-242.

Bahr, L. von,: Zentbl. Bakt. Parasitkde., 66,(1912): 335. cited by Adam (1927).

Barka, T.: On the acid phosphatases of liver and RE-cells. J. Histochem. Cytochem., 8,(1960): 320-321.

Barka, T.: Studies of acid phosphatase. I. Electrophoretic separation of acid phosphatases of rat liver on polyacrylamide gels. J. Histochem. Cytochem., 9,(1961): 542-547.

II. Chromatographic separation of acid phosphatases of rat liver. J. Histochem. Cytochem., 9,(1961): 564-571.

Barka, T.: Electrophoretic and chromatographic heterogeneity of acid phosphatases of rat liver. J. Histochem. Cytochem., 9,(1961): 630-631.

Barnum, D.A.: Personal communication, Michigan State University, July 24, 1968.

Barnum, D.A., Glantz, P.J., and Moon, H.W.: *Colibacillosis*. Ciba Pharmaceutical Co., Summit, New Jersey (1967).

Barrnett, R.J.: The demonstration with the electron microscope of the end-products of histochemical reactions in relation to the fine structure of cells. Expl Cell Res., Suppl. No. 7, (1959): 65-89.

Bennett, P.C.: Edema Disease. In *Diseases of Swine*, 2nd edn, H.W. Dunne, ed., The Iowa State Univ. Press, Ames, Iowa (1964).

Bessis, M.: Studies on cell agony and death: An attempt at classification. In *Cellular Injury* (Ciba Foundation Symposium) Symposium on Cellular Injury. A.V.S. de Reuck and Julie Knight, eds., J. and A. Churchill, Ltd., London (1964): 299.

Bizzozero, G.: *Uber die Regeneration der Elemente der schlauchformigen Drusen und des Epithels des Magendarmkanals*. Anat. Anz., 3,(1888): 781-784.

Black, D.A.K.: Body-fluid depletion. Lancet, 1,(1953): 353-360.

Brambell, F.W.R.: The passive immunity of the young mammal. Biol. Rev., 33,(1958): 488-531.

Bray, J.: Isolation of antigenically homogeneous strains of *Bact. coli neapolitanum* from summer diarrhoea of infants. J. Path. Bact., 57,(1945): 239-247.

Bray, J., and Beavan, T.: Slide agglutination of *Bacterium coli* var. *Neapolitanum* in summer diarrhoea. J. Path. Bact., 60,(1948): 395-401.

Britt, A. L.: Pathologic effects of *Escherichia coli* 083:K.:NM in gnotobiotic pigs. Ph.D. Thesis, Michigan State University, East Lansing (1967).

Britt, A. L., and Waxler, G.L.: *Escherichia coli* in gnotobiotic pigs. Proceedings, Gnotobiotic Symposium and Workshop, Michigan State University, East Lansing, Mich., June 7 (1964). Abstract.

Brooks, R. A.: Serum proteins and antibodies in the germfree and gnotobiotic pig. M.S. Thesis, Michigan State University (1966).

Burgos, M. A., Deane, H. W., and Barrnett, R. J.: Sites of reaction products in alkaline phosphatase preparations incubated with two classes of organic phosphate esters. J. Histochem. Cytochem., 2,(1954): 442-442.

Burgos, M. A., Deane, H. W., and Karnovsky, M. L.: Histochemical and chemical evidence for more than one alkaline phosphomonoesterase. J. Histochem. Cytochem., 3,(1955): 103-115.

Bustad, L. K.: Pigs in the laboratory. Scient. Am., 214(6),(1966): 94-100.

Bustad, L. K., and McClellan, R. O.: Swine in biomedical research. Frayne Printing Co., Seattle, Washington, U.S.A. (1966).

Butler, A. M., McKhann, C. F., Gamble, J. L., and March, P.: Intracellular fluid loss in diarrheal disease. J. Pediat., 3, (1933): 84-92.

Caro, L.: Electron microscopy radioautography: Golgi zone as a site of protein concentration in pancreatic acinar cells. Biochem. Cytol., 10,(1961): 37-43.

Carpenter, P. L.: *Immunology and Serology*, 2nd edn W. B. Saunders Co., Philadelphia, Pa. (1965).

Carter, F. S.: Paediatrics in Freetown, Sierra Leone. Archs Dis. Childh., 36,(1961): 190-199.

Christie, A. C.: A study of the argentaffin cells with the electron microscope. Q. Jl. microsc. Sci., 96,(1955): 295-301.

Christie, B. R.: A muscular dystrophy of Merino lambs in Western Victoria. Vict. vet. Proc., 20,(1961-62): 30-32.

Christie, B. R.: Experimental colibacillosis in gnotobiotic pigs. M.S. Thesis, Michigan State University, East Lansing (1967).

Clark, S. L.: The ingestion of proteins and colloidal materials by columnar absorptive cells of the small intestine in suckling rats and mice. J. biophys. biochem. Cytol., 5,(1959): 41-49.

Code, C. F.: The semantics of the process of absorption. Perspect. Biol. Med., 3,(1960): 560-562.

Code, C. F.: Proceedings of the cholera research symposium. U.S. Department of Health, Education and Welfare, Washington, D.C. (1965): 87.

Cooke, W. T.: Water and electrolyte upsets in the steatorrhea syndrome. J. Mt. Sinai Hosp. N.Y., 24,(1957): 221-231.

Cori, C.F.: The fate of sugar in the animal body. I. The rate of absorption of hexoses and pentoses from the intestinal tract. J. biol. Chem., 66,(1925): 691-715.

Crane, R. K.: Hypothesis for mechanism of intestinal active transport of sugars. Fedn Proc. Fedn Am. Socs exp. Biol., 21,(1962): 891-895.

Crane, R. K.: Enzymes and malabsorption: A concept of brush border membrane diseases. Gastroenterology, 50,(1966): 254-262.

Crane, R. K.: A concept of the digestive absorptive surface of the small intestine. In *Handbook of Physiology. Alimentary Canal*, C. F. Code ed., American Physiological Society, Washington, D.C. (1968).

Crane, R. K.: Introductory remarks. Fedn Proc. Fedn Am. Socs exp. Biol., 28,(1969): 5-5.

Crane, R. K.: Personal communication, Michigan State University, February 19, 1969.

Creamer, B.: Variation in small-intestinal villous shape and mucosal dynamics. Br. med. J., 2,(1964a): 1371-1373.

Creamer, B.: Small-intestinal mucosal dynamics and the environment. Br. med. J., 2,(1964b): 1373-1375.

Creamer, B.: The turnover of the epithelium of the small intestine. Br. med. Bull., 23,(1967): 226-230.

Crookshank, F. G.: (1922) cited by W. I. Card: Br. med. Bull., 23,(1967): 294.

Cruickshank, R.: Diarrhoeal diseases in under-developed countries. In *The Theory and Practice of Public Health*, W. Hobson ed., Oxford University Press, London (1961): 139.

Dahlqvist, A., Hammond, J. B., Crane, R. K., Dunphy, J. V., and Littman, A.: Intestinal lactase deficiency and lactose intolerance in adults. Gastroenterology, 45,(1963): 488-491.

Darrow, D. C.: Body-fluid physiology: the relation of tissue composition to problems of water and electrolyte balance. New Engl. J. Med., 233,(1945): 91-97.

Darrow, D. C.: The retention of electrolyte during recovery from severe dehydration due to diarrhea. J. Pediat., 28,(1946): 515-540.

Dawson, A. M.: Absorption of fats. Br. med. Bull., 23,(1967): 247-251.

De Duve, C.: The lysosome concept. In *Lysosomes*, Ciba Foundation Symposium, A.V.S. deReuck and M. P. Cameron, eds., J. and A. Churchill, London (1963).

Dempsey, E. W.: Electron microscopy of the visceral yolk sac epithelium of the guinea pig. Am. J. Anat., 93,(1953): 331-363.

Dent, C. E., and Schilling, J.A.: Studies on the absorption of proteins: the amino-acid pattern in the portal blood. Biochem. J., 44,(1949): 318-333.

Deschner, E., Lewis, C. M., and Lipkin, M.: *In vitro* study of human rectal epithelial cells. I. Atypical zone of H^3 thymidine incorporation in mucosa of multiple polyposis. J. clin. Invest., 42,(1963): 1922-1928.

Diamond, J. M., and Tormey, J. M.: Studies on the structural basis of water transport across epithelial membranes. Fedn Proc. Fedn Am. Socs exp. Biol., 25,(1966): 1458-1463.

Dich, J., and Nielsen, K.: Passage of I^{131} - albumin and I^{125} - gamma globulin into the small intestine of the pig. Can. J. comp. Med., 28,(1964): 257-262.

Dixon, J. M. S.: The fate of bacteria in the small intestine. J. Path. Bact., 79,(1960): 131-140.

Dobbins, W. O.: An ultrastructural study of the intestinal mucosa in congenital β-lipoprotein deficiency with particular emphasis upon the intestinal absorptive cell. Gastroenterology, 50,(1966): 195-210.

Dobbins, W. O.: Electron microscopic study of the intestinal mucosa in intestinal lymphangiectasia. Gastroenterology, 51,(1966): 1004-1017.

Doig, A., and Girdwood, R. H.: The absorption of folic acid and labelled cyanocobalamin in intestinal malabsorption. Q. Jl. Med., 29,(1960): 333-374.

Donaldson, R. M.: Normal bacterial populations of the intestine and their relation to intestinal function. New Eng. Jl Med., 270,(1964): 938-945.

Donaldson, R. M.: Studies on the pathogenesis of steatorrhea in the blind loop syndrome. J. clin. Invest., 44,(1965): 1815-1825.

Donaldson, R. M.: Role of enteric microorganisms in malabsorption. Fedn Proc. Fedn Am. Socs exp. Biol., 26,(1967): 1426-1431.

Doniach, I., and Shiner, M.: Duodenal and jejunal biopsies. II. Histology. Gastroenterology, 33,(1957): 71-86.

Donnellan, W. L.: The structure of the colonic mucosa. The epithelium and subepithelial reticulohistiocytic complex. Gastroenterology, 49,(1965): 496-514.

Drees, D. T.: Personal communication, Michigan State University, April 30, 1969.

Dubos, R.: The microbiota of the gastrointestinal tract. Gastroenterology, 51,(1966): 868-874.

Dubos, R., and Schaedler, R. W.: Some biological effects of the digestive flora. Am. J. med. Sci., 244,(1962): 35/265-41/271.

Dunne, H. W.: Streptococcosis, colibacillosis and bordetellosis. In *Diseases of Swine*, 2nd edn, H. W. Dunne, ed., The Iowa State University Press, Ames, Iowa (1964).

Duthie, H. L.: Methods for studies of intestinal absorption in man. Br. med. Bull., 23,(1967): 213-216.

Edwards, P. R., and Ewing, N. H.: *Identification of* Enterobacteriaceae, 3rd edn, Burgess Pub. Co., Minneapolis, Minn. (1962).

Eichholz, A.: Fractions of the brush border. Fedn Proc. Fedn Am. Socs exp. Biol., 28,(1969): 30-34.

Eichholz, A., and Crane, R. K.: Studies on the organization of the brush border in intestinal epithelial cells. I. Tris disruption of isolated hamster brush borders and density gradient separation of fractions. J. Cell. Biol., 26,(1965): 687-691.

Elkinton, J. R., Winkler, A. W., and Danowski, T. S.: Transfers of cell sodium and potassium in experimental and clinical conditions. J. clin. Invest., 27,(1948): 74-81.

Elliott, H. L., Carpenter, C. C. J., Sack, R. B., and Yardley, J. H.: Small bowel morphology in experimental canine cholera. A light and electron microscopic study. In *Program and Abstracts of the Sixty-fifth Annual Meeting.* The American Association of Pathologists and Bacteriologists. Chicago, Ill. (1968).

Emmel, V. M.: Effects of HCl on alkaline phosphatase in kidney and intestine: Histochemical and quantitative study. Proc. Soc. exp. Biol. Med., 75,(1950): 114-117.

Evanson, J. M., and Stanbury, S. W.: Congenital chloridorrhea or so-called congenital alkalosis with diarrhoea. Gut, 6,(1965): 29-38.

Ewing, W. H., Tanner, K. E., and Tatum, H. W.: A new serotype of *Escherichia coli* associated with infantile diarrhea. Pub. Health Rep., 70,(1955): 107-114.

Farquhar, M. G., and Palade, G. E.: Junctional complexes in various epithelia. J. Cell Biol., 17,(1963): 375-412.

Fawcett, D. W.: *An Atlas of Fine Structure. The Cell. Its Organelles and Inclusions.* W. B. Saunders Co., Philadelphia, Pa. (1966).

Feigin, I., and Wolfe, A.: The phosphatase of the nervous system. J. Neuropath. exp. Neurol., 14,(1955): 11-22.

Ferguson, W. W.: Experimental diarrheal disease of human volunteers due to *Escherichia coli*. Ann. N. Y. Acad. Sci., 66,(1956): 71-77.

Ferguson, W. W., and June, R. C.: Experiments on feeding adult volunteers with *Escherichia coli* 111, B4, a coliform organism associated with infant diarrhea. Am. J. Hyg., 55,(1952): 155-169.

Fey, H.: *Neuere Untersuchungen über die Colisepsis des Kalbes.* Schweiz. Arch. Tierheilk., 104,(1962): 1-12.

Fey, H., and Margadant, A.: *Zur Pathogenese der Kälber-Colisepsis. I. Verteilung des Sepsistyps in den Organen.* Zentbl. Bakt. Parasitkde., 182,(1961): 71-79.

Fey, H., Lanz, E., Margadant, A., and Nicolet, J.: *Zur Pathogenese der Kälbercolisepsis.* Dtsch. tierärztl. Wschr., 69,(1962): 581-586.

Finkelstein, R. A., Norris, H. T., and Dutta, N. K.: Pathogenesis of experimental cholera in infant rabbits. I. Observations on the intra intestinal infection and experimental cholera produced with cell-free products. J. infect. Dis., 114,(1964): 203-216.

Fisher, R. B.: Absorption of proteins. Br. med. Bull., 23,(1967): 241-246.

Fordtran, J. S.: Speculations on the pathogenesis of diarrhea. Fedn Proc. Fedn Am. Socs exp. Biol., 26,(1967): 1405-1414.

Fordtran, J. S., and Locklear, T. W.: Ionic constituents and osmolality of gastric and small-intestinal fluids after eating. Am. J. dig. Dis., 11,(1966): 503-521.

Fordtran, J. S., Rector, F. C., Locklear, T. W., and Ewton, M. F.: Water and solute movement in the small intestine of patients with sprue. J. clin. Invest., 46,(1967): 287-298.

Fric, P., and Lojda, Z.: Enzymes of the human jejunal mucosa. Acta gastro-enter. belg., 27,(1964): 526-530.

Friedenwald, J. S., Gryder, R. M., and Gryder, J. W.: The substrate specificity of alkaline phosphatase of rat kidney. Archs Biochem. Biophys., 73,(1958): 262-272.

Friend, D. S., and Farquhar, M. G.: Functions of coated vesicles during protein absorption in the rat vas deferens. J. Cell Biol., 35,(1967): 357-376.

Fujimoto, Y., and Olson, C.: The fine structure of the bovine wart. Path. vet., 3,(1966): 659-684.

Gay, G. C.: *Escherichia coli* and neonatal disease of calves. Bact. Rev., 29,(1965): 75-101.

Giles, C., Sangster, G., and Smith, J.: Epidemic gastro-enteritis of infants in Aberdeen during 1947. Archs Dis. Childh., 24,(1949): 45-53.

Gitter, M.: The enteropathogenic effect of *E. coli* in rabbits and pigs. Br. vet. J., 123,(1967): 403-409.

Glässer, K., Hupka, E., and Wetzel, R.: *Die Krankheiten des Schweines*, 5th edn, M. and H. Schaper, Hannover (1950).

Goldfischer, S., Essner, E., and Novikoff, A. B.: The localization of phosphatase activities at the level of ultrastructure. J. Histochem. Cytochem., 12,(1964): 72-95.

Goldschmidt, R.: *Untersuchungen zur Ätiologie der Durchfallserkrankungen des Säuglings*. Jb. Kinderheilk. phys. Erzieh., 139,(1933): 318-358.

Goldstein, F., and Wirts, C. W.: Steatorrhea following gastrojejunotomy and vagotomy. Gastroenterology, 48,(1965): 819-819.

Gomori, G.: *Microscopic Histochemistry; Principles and Practice*. The University of Chicago Press, Chicago (1952).

Gomori, G.: Histochemical specificity of phosphatases. Proc. Soc. exp. Biol. Med., 70,(1949): 7-11.

Goodwin, M. H., Mackel, D. C., Ganelin, R. S., Weaver, R. E., and Payne, F. J.: Observations on etiology of diarrheal diseases in Arizona. Am. J. trop. Med. Hyg., 9,(1960): 336-342.

Gordon, J. E.: *Control of Communicable Diseases in Man.* 9th edn, American Public Health Association, New York (1960).

Gordon, J. E., Chitkara, I. D., and Wyon, J. B.: Weanling diarrhea. Am. J. med. Sci., 245,(1963): 129/345-161/377.

Gordon, J. E., Pierce, V., Ascoli, W., and Scrimshaw, N. S.: Studies of diarrheal disease in Central America. II. Community prevalence of *Shigella* and *Salmonella* infection in childhood. Am. J. trop. Med. Hyg., 11,(1962): 389-394.

Gordon, R. S., Feeley, J. C., Greenough, W. B., Sprinz, H., and Oseasohn, R.: Cholera. Ann. intern. Med., 64,(1966): 1328-1351.

Gordon, W. A. M., and Luke, D.: Gastroenteritis in young piglets associated with *Escherichia coli*. Vet. Rec., 70,(1958): 542-543.

Gossling, J., and Rhoades, H. E.: Serologic types of *Escherichia coli* isolated from certain pigs with enteric disorders. Cornell Vet., 56,(1956): 344-353.

Govan, C. D., and Darrow, D. C.: The use of potassium chloride in the treatment of the dehydration of diarrhea in infants. J. Pediat., 28,(1946): 541-549.

Graney, D. O.: Uptake of ferritin by intestinal lining cells of suckling rats. Report of the Ross Conferences on Pediatric Research 1963-1965, 50,(1965): 18-27.

Granger, B., and Baker, R. F.: Electron microscope investigation of the striated border of intestinal epithelium. Anat. Rec., 103,(1949): 459-459.

Graumann, W.: *Handbuch der Histochemie. Band II. Polysaccharide.* Gustav Fischer Verlag, Stuttgart (1964): pp. 13, 45, 329-332.

Gray, G. M.: Malabsorption of carbohydrate. Fedn Proc. Fedn Am. Socs exp. Biol., 26,(1967): 1415-1419.

Gray, G. M., and Santiago, N. A.: Disaccharide absorption in normal and diseased human intestine. Gastroenterology, 51,(1966): 489-498.

Gyles, C., and Barnum, D. A.: (1965) Unpublished data cited by Barnum, Glantz, and Moon (1967).

Haelterman, E. O., and Hooper, B. E.: Transmissible gastroenteritis of swine as a model for the study of enteric disease. Gastroenterology, 53,(1967): 109-113.

Halsted, J. A., Lewis, P. M., and Gasster, M.: Absorption of radioactive vitamin B12 in the syndrome of megaloblastic anemia associated with intestinal stricture or anastomosis. Am. J. Med., 20,(1956): 42-52.

Ham, A. W.: *Histology*, 5th edn, J. B. Lippincott Company, Philadelphia (1965).

Handforth, C. P., and Sorger, K.: Failure of regeneration of small bowel mucosa following epidemic infantile gastroenteritis. Can. med. Ass. J., 84,(1961): 425-427.

Hardy, A. V., and Watt, J.: Studies of the acute diarrheal diseases. XII. Etiology. Pub. Health Rep., 60,(1945): 57-66.

Hartman, R. S., Conrad, M. E., Hartman, R. E., Joy, R. J. T., and Crosby, W. H.: Ferritin-containing bodies in human small intestinal epithelium. Blood, 22,(1963): 397-405.

Heatley, N. G., and Florey, H. W.: An antibiotic from *Bacterium coli*. Brit. J. exp. Path., 27,(1946): 378-390.

Hess, R., Scarpelli, D. G., and Pearse, A. G. E.: The cytochemical localization of oxidative enzymes. II. Pyridine nucleotide-linked dehydrogenases. J. biophys. biochem. Cytol., 4,(1958): 753-760.

Hodes, H. L.: Etiology of infantile diarrhea. In *Advances in Pediatrics*, S. Z. Levine ed., Year Book Publishers, Chicago (1956).

Hofman, A. F., and Borgstrom, B.: Physico-chemical state of lipids in intestinal content during their digestion and absorption. Fedn Proc. Fedn Am. Socs exp. Biol., 21,(1962): 43-50.

Holt, J. H., and Miller, D.: The intestinal brush border as a digestive surface: Localization of aminopeptidase and phosphatase in the mucosal epithelial cell. J. Lab. clin. Med., 58,(1961): 827-827.

Hooper, B. E., and Haelterman, E. O.: Growth of transmissible gastroenteritis virus in young pigs. Am. J. vet. Res., 27,(1966): 286-291.

Hopps, C. H.: Bacterial diseases. In *Pathology*, 5th edn, Vol 1, W. A. D. Anderson, ed. The C. V. Mosby Company, St. Louis, Mo. (1966).

Hunt, R. D.: Microscopic histochemical methods for the demonstration of enzymes. In *Selected Histochemical and Histopathological Methods*, S. W. Thompson and R. D. Hunt. Charles C. Thomas, Springfield, Illinois (1966).

Isselbacher, K. J.: Biochemical aspects of lipid malabsorption. Fedn Proc. Fedn Am. Socs exp. Biol., 26,(1967): 1420-1425.

Ito, S.: The enteric surface coat on cat intestinal microvilli. J. Cell Biol., 27,(1965): 475-491.

Ito, S.: Structure and function of the glycocalyx. Fedn Proc. Fedn Am. Socs exp. Biol., 28,(1969): 12-25.

Jackson, S. H.: The effect of food ingestion on intestinal and serum alkaline phosphatase in rats. J. biol. Chem., 198, (1952): 553-559.

Jardetsky, O.: Simple allosteric model for membrane pumps. Nature, Lond., 211,(1966): 969-970.

Jelliffe, D. B., and Dean, R. F. A.: Protein-calorie malnutrition in early childhood. J. trop. Pediat., 5,(1959): 96-106.

Jennings, R. A.: Gastroenteritis in the pig. Vet. Rec., 71,(1959): 766-771.

Jensen, C. O.: (1889) C. O. Jensen's Selected Papers 1886-1908. Ejnar Munskgaard, Copenhagen, 1,(1948): 290, cited by W. J. Sojka, (1965).

Jensen, C. O.: *Ueber die Kälberruhr und deren Aetiologie.* Monatschr. prakt. Tierh., 4,(1892): 97-124.

Joe, L. K., Sahab, K., Yauw, G. S., and Makaliwy, C.: Diarrhea among infants and children in Djakarta, Indonesia, with special reference to pathogenic *Escherichia coli.* Am. J. trop. Med. Hyg., 9,(1960): 626-632.

Johnson, C. F.: Localization of hamster intestinal disaccharidase activity to a repeating unit associated with the microvillous plasma membrane. J. Lab. clin. Med., 68,(1966): 883-884.

Johnson, C. F.: Hamster intestinal brush-border surface particles and their function. Fedn Proc. Fedn Am. Socs exp. Biol., 28,(1969): 26-29.

Jubb, K. V. F., and Kennedy, P. C.: *Pathology of Domestic Animals, Volume 2.* Academic Press, New York (1963).

June, R. C., Ferguson, W. W., and Worfel, M. T.: Experiments in feeding adult volunteers with *Escherichia coli* 55, B5, a coliform organism associated with infant diarrhea. Am. J. Hyg., 57,(1953): 222-236.

Kaneko, J. J., Moulton, J. E., Brodey, R. S., and Perryman, V. D.: Malabsorption syndrome resembling nontropical sprue in dogs. J. Am. Vet. med. Ass., 146,(1965): 463-473.

Kassirer, J., Berkman, P. M., Lawrenz, D. R., and Schwartz, W. B.: The critical role of chloride in the correction of hypokalemic alkalosis in man. Am. J. Med., 38,(1965): 172-189.

Kauffman, F.: *Zur Serologie der Coli-Gruppe.* Acta path. microbiol. scand., 21,(1944): 20-45.

Kauffman, F.: The serology of the coli group. J. Immun., 57,(1947): 71-100.

Kauffman, F., and Dupont, A.: *Escherichia* strains from infantile epidemic gastroenteritis. Acta path. microbiol. scand., 27, (1950): 552-564.

Kauffman, F., and Perch, B.: *Über die Coliflora des gesunden Menschen.* Acta path. microbiol. scand., 20,(1943): 201-220.

Kenworthy, R.: Influence of bacteria on absorption from the small intestine. Proc. Nutr. Soc., 26,(1967): 18-22.

Kenworthy, R., and Allen, D.: Influence of diet and bacteria on small intestinal morphology with special reference to early weaning and *Escherichia coli*. Studies with germfree and gnotobiotic pigs. J. Comp. Path., 76,(1966): 291-296.

Kenworthy, R., Stubbs, J. M., and Syme, G.: Ultrastructure of small-intestinal epithelium in weaned and unweaned pigs and pigs with post-weaning diarrhoea. J. Path. Bact., 93,(1967): 493-498.

Kessner, D. M., Shaughnessy, H. J., Googins, J., Rasmussen, C. M., Rose, N. J., Marshall, A. L., Jr., Andelman, S. L., Hall, J. B., and Rosenbloom, P. J.: An extensive community outbreak of diarrhea due to enteropathogenic *Escherichia coli* 0111:B4 I. Epidemiological studies. Am. J. Hyg., 76, (1962): 27-43.

Kim, Y. S., Spritz, N., Blum, M., Tery, J., and Sherlock, P.: The role of altered bile acid metabolism in the steatorrhea of experimental blind loop. J. clin. Invest., 45,(1966): 956-962.

Kinnaird, P. J.: Progress of HyPAR pigs under commercial conditions at Dookie College. Vict. vet. Proc., 22,(1964): 37-38.

Kohler, E. M.: Studies of *Escherichia coli* in gnotobiotic pigs. V. Evaluation of the effects of oral and parenteral administration of immune serum. Can. J. comp. Med., 31,(1967): 283-289.

Kohler, E. M.: Personal communication, University of Illinois, October 3, 1968.

Kohler, E. M., and Bohl, E. H.: Studies of *Escherichia coli* in gnotobiotic pigs. I. Experimental reproduction of colibacillosis. Can. J. comp. Med., 30,(1966): 199-203.

Korn, E. D.: Current concepts of membrane structure and function. Fedn Proc. Fedn Am. Socs exp. Biol., 28,(1969): 6-11.

Kowlesser, O. D., Haeffner, L. J., Riley, E. M., and Sleisenger, M. H.: Comparative study of serum leucine amino-peptidase, 5-nucleotidase and non-specific alkaline phosphatase in diseases affecting the pancreas, hepatobiliary tree and bone. Am. J. Med., 31,(1961): 231-237.

Kraehenbuhl, J. P., Gloor, E., and Blanc, B.: *Resorption intestinale de la ferritine chez deux especes animales aux possibilites d'absorption proteique neonatale differentes.* Z. Zellforsch., 76,(1967): 170-186.

Leblond, C. P., and Carriere, R.: The effect of growth hormone and thyroxine on the mitotic rate of the intestinal mucosa of the rat. Endocrinology, 56,(1955): 261-266.

Leblond, C. P., and Messier, B.: Renewal of chief cells and goblet cells in the small intestine as shown by radioautography after injection of thymidine-H^3 into mice. Anat. Rec., 132,(1958): 247-259.

Leblond, C. P., and Stevens, C. E.: The constant renewal of the intestinal epithelium in the albino rat. Anat. Rec., 100, (1948): 357-377.

Lecce, J. G.: Absorption of macromolecules by neonatal intestine. Biologia Neonat., 9,(1966): 50-61.

Lecce, J. G., and Reep, B. R.: *Escherichia coli* associated with colostrum-free neonatal pigs raised in isolation. J. exp. Med., 115,(1962): 491-501.

Lehninger, A. L.: *The Mitochondrion; Molecular Basis of Structure and Function.* W. A. Benjamin, Incorporated, New York (1964).

Lesher, S., Walburg, H. E., Jr., and Sacher, G. A., Jr.: Generation cycle in the duodenal crypt cells of germ-free and conventional mice. Nature, Lond., 202,(1964): 884-886.

Levin, R. J.: Techniques, terminology, and parameters in intestinal absorption. Br. med. Bull., 23,(1967): 209-212.

Levitan, R., Fordtran, J. S., Burrows, B. A., and Ingelfinger, F. J.: Water and salt absorption in the human colon. J. clin. Invest., 41,(1962): 1754-1759.

Lifson, N., and Parsons, D. S.: Support of water absorption by rat jejunum *in vitro* by glucose in serosal fluid. Proc. Soc. exp. Biol. Med., 95,(1957): 532-534.

Lillie, R. D.: Histochemical comparison of the Casella, Bauer, and periodic acid oxidation-Schiff leucofuchsin technics. Stain Technol., 26,(1951): 123-136.

Lipkin, M.: Cell proliferation in the gastrointestinal tract of man. Fedn Proc. Fedn Am. Socs exp. Biol., 24,(1965): 10-15.

Losos, G. J.: Pathogenesis of enteric infection of swine. M.Sc. Thesis, University of Toronto (1964).

Lovell, R.: Classification of *Bacterium coli* from diseased calves. J. Path. Bact., 44,(1937): 125-139.

Lovell, R.: Infection and resistance in young animals. Lancet, 2, (1951): 1097-1100.

Luft, J.H.: Improvements in epoxy resin embedding methods. J. biophys. biochem. Cytol., 9,(1961): 409-414.

MacDonald, W. C., Trier, J. S., and Everett, N. B.: Cell proliferation and migration in the stomach, duodenum, and rectum of man. Radioautographic studies. Gastroenterology, 46,(1964): 405-417.

Marchesi, V. T.: Histochemical and biochemical studies of adenosine triphosphatase activity bound to membranes of pinocytotic vesicles. J. Histochem. Cytochem., 13,(1965): 713-714.

Maronpot, R. R., and Whitehair, C. K.: A sprue-like villous atrophy in pigs. Fedn Proc. Fedn Am. Socs exp. Biol., 25,(1966): 752-752.

Maronpot, R. R., and Whitehair, C. K.: Experimental sprue-like small intestinal lesions in pigs. Can. J. comp. Med., 31,(1967): 309-316.

Matthews, D. M.: Absorption of water-soluble vitamins. Br. med. Bull., 23,(1967): 258-262.

Mattisson, A. G. M., and Karlsson, B. W.: Sub-light microscopical changes in the epithelial cells of the intestine of the piglet reared with and without colostrum. Third European Regional Conference on Electron Microscopy, Prague, Czechoslovakia (1964).

Mattisson, A. G. M., and Karlsson, B. W.: Observations on structure of intestinal epithelial cells in newborn piglets. J. Ultrastruct. Res., 12,(1965): 243-243.

Mattisson, A. G. M., and Karlsson, B. W.: Electron microscopic and immunochemical studies on the small intestine of newborn piglets. Ark. Zool. Series 2, 18,(1966): 575-589.

Maunsbach, A. B.: Albumin absorption by renal proximal tubule cells. Nature, Lond., 212,(1966): 546-547.

McBryde, C. N.: Acute enteritis in young pigs due to infection with the colon group. J. Am. vet. med. Ass., 84,(1934): 36-50.

McHardy, G. J. R., and Parsons, D. S.: The absorption of water and salt from the small intestine of the rat. Q. J. exp. Physiol., 42,(1957): 33-48.

McKay, D. G., and Wahle, G. H.: Epidemic gastroenteritis due to *Escherichia coli* O111B4. II. Pathologic anatomy (with special reference to the presence of the local and generalized Schwartzman phenomenon). Archs Path., 60,(1955): 679-693.

McManus, J. F. A.: Histological and histochemical uses of periodic acid. Stain Technol., 23,(1948): 99-108.

McNabb, J. D., and Sandborn, E.: Filaments in the microvillous border of intestinal cells. J. Cell Biol., 22,(1964): 701-704.

Melnyk, C. S., Braucher, R. E., and Kirsner, J. B.: Colon mucosa response to injury. II. Histochemical study. Gastroenterology, 51,(1966): 50-58.

Michel, M. C.: *Activité metabolique de la flore totale isolée de l'intestin du porc.* Amino acides, Peptides, Proteines, 5, (1962): 157-173.

Miller, D., and Crane, R. K.: The digestive function of the epithelium of the small intestine. II. Localization of disaccharide hydrolysis in the isolated brush border portion of intestinal epithelial cells. Biochim. biophys. Acta, 52,(1961): 293-298.

Mitchell, P.: Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. Nature, Lond., 191,(1961): 144-148.

Moe, H., Rostgaard, J., and Behnke, O.: On the morphology and origin of virgin lysosomes in the intestinal epithelium of the rat. J. Ultrastruct. Res., 12,(1965): 396-403.

Moog, F.: Developmental adaptations of alkaline phosphatase in the small intestine. Fedn Proc. Fedn Am. Socs exp. Biol., 21, (1962): 51-56.

Moon, H. W.: The association of *Escherichia coli* with diarrheal disease of the newborn pig. Ph.D. Thesis, University of Minnesota, Minneapolis, Minnesota (1965), cited by Moon, Sorenson, and Sautter, 1966.

Moon, H. W., Sorenson, D. K., and Sautter, J. H.: *Escherichia coli* infection of the ligated intestinal loop of the newborn pig. Am. J. vet. Res., 27,(1966): 1317-1325.

Moon, H. W. Sorenson, D. K., Sautter, J. H., and Higbee, J. M.: Association of *Escherichia coli* with diarrheal disease of the newborn pig. Am. J. vet. Res., 27,(1966): 1007-1011.

Morton, R. K.: Acid and alkaline phosphatases. In *Biochemist's Handbook*, C. Long, ed., D. Van Norstrand Co., Inc., Princeton, N.J., (1961): 249.

Mowry, R. W.: Comparison of technical procedures for histochemical preservation of alkaline phosphatase. Bull. int. Ass. med. Mus., 30,(1949): 95-98.

Nachlas, M. M., Crawford, D. T., and Seligman, A. M.: The histochemical demonstration of leucine aminopeptidase. J. Histochem. Cytochem., 5,(1957): 264-278.

Nachlas, M. M., Monis, B., Rosenblatt, D., and Seligman, A. M.: Improvement in the histochemical localization of leucine aminopeptidase with a new substrate, l-leucyl 4-methoxy-2-naphthylamide. J. biophys. biochem. Cytol., 7,(1960): 261-264.

Nachlas, M. M. Tsou, K. C., Souza, E. D., Cheng, C. S., and Seligman, A. M.: Cytochemical demonstration of succinic dehydrogenase by the use of a new p-nitrophenyl substituted ditetrazole. J. Histochem. Cytochem., 5,(1957): 420-436.

Nachlas, M. M., Walker, D. G., and Seligman, A. M.: A histochemical method for the demonstration of diphosphopyridine nucleotide diaphorase. J. biophys. biochem. Cytol., 4,(1958): 29-36.

Neter, E., and Shumway, C. N.: *Escherichia coli* serotype D443: Occurrence in intestinal and respiratory tracts, cultural characteristics, pathogenicity, sensitivity to antibiotics. Proc. Soc. exp. Biol. Med., 75,(1950): 504-507.

Newey, H.: Absorption of carbohydrates. Br. med. Bull., 23,(1967): 236-240.

Nielsen, N. O.: Studies on edema disease of swine. Ph.D. Thesis, University of Minnesota, Minneapolis, Minnesota (1963), cited by Moon, Sorenson, and Sautter, 1966.

Nielsen, N. O., Moon, H. W., and Roe, W. E.: Enteric colibacillosis in swine. J. Am. vet. med. Ass., 153,(1968): 1590-1606.

Novikoff, A. B., and Masek, B.: Survival of lactic dehydrogenase and DPNH-diaphorase activities after formol-calcium fixation. J. Histochem. Cytochem., 6,(1958): 217-217.

Ordway, N. K.: Diarrhoeal disease and its control. Bull. World Health Organ., 23,(1960): 73-101.

Osborne, J. C.: *Escherichia coli* serotypes orally. I. Induced gastroenteritis and pathological changes in neonatal calves. Cornell Vet., 57,(1967): 204-217.

Osborne, J. C.: *Escherichia coli* serotypes intravenously. II. Pharmacological effects and tolerance. Cornell Vet., 57,(1967): 217-227.

Osborne, J. C.: *Escherichia coli* serotypes intravenously. III. Pathological changes and pathogenesis. Cornell Vet., 57, (1967): 227-238.

Padykula, H. A., and Herman, E.: Factors affecting the activity of adenosine triphosphatase and other phosphatases as measured by histochemical techniques. J. Histochem. Cytochem., 3, (1955a): 161-169.

Padykula, H. A., and Herman, E.: The specificity of the histochemical method for adenosine triphosphatase. J. Histochem. Cytochem., 3(1955b): 170-195.

Padykula, H. A., Strauss, E. W., Ladman, A. J., and Gardner, F. H.: A morphologic and histochemical analysis of the human jejunal epithelium in nontropical sprue. Gastroenterology, 40,(1961): 735-765.

Palade, G. E.: A study of fixation for electron microscopy. J. exp. Med., 95,(1952): 285-298.

Palay, S. L., and Karlin, L. J.: An electron microscopic study of the intestinal villus. II. The pathway of fat absorption. J. biophys. biochem. Cytol., 5,(1959): 373-383.

Panish, J. F.: Experimental blind loop steatorrhea. Gastroenterology, 45,(1963): 394-399.

Parsons, D. S.: Salt and water absorption by the intestinal tract. Br. med. Bull., 23,(1967): 252-257.

Payne, A. M. M., and Cook, G. T.: A specific serological type of *Bacterium coli* found in infants' home in absence of epidemic diarrhoea. Br. med. J., 2,(1950): 192-195.

Payne, L. C., and Marsh, C. L.: Absorption of gamma globulin by the small intestine. Fedn Proc. Fedn Am. Socs exp. Biol., 21, (1962): 909-912.

Pearse, A. G. E.: Extension of the limits of cellular pathology: The role of enzyme histochemistry. J. clin. Path., 11,(1958): 520-534.

Pearse, A. G. E.: *Histochemistry Theoretical and Applied*, 2nd edn, Little, Brown and Company, Boston, Mass. (1961a): 882.

Pearse, A. G. E.: *Histochemistry Theoretical and Applied*, 2nd edn, Little, Brown and Company, Boston, Mass. (1961b): 910.

Pearse, A. G. E.: Personal communication, Michigan State University, March 1, 1968.

Pearse, A. G. E., and Riecken, E. O.: Histology and cytochemistry of the cells of the small intestine, in relation to absorption. Br. med. Bull., 23,(1967): 217-230.

Pease, D. C.: *Histological Techniques for Electron Microscopy.* Academic Press, New York (1964).

Perch, B.: *Weitere Untersuchungen über die Coliflora des gesunden Menschen.* Acta path. microbiol. scand., 21,(1944): 239-247.

Peterson, M. R., and Leblond, C. P.: Uptake by the Golgi region of glucose labeled with tritium in the 1 or 6 position, as an indicator of synthesis of complex carbohydrates. Expl. Cell Res., 34,(1964): 420-423.

Phelps, P. C., Rubin, C. E., and Luft, J. H.: Electron microscope techniques for studying absorption of fat in man with some observations on pinocytosis. Gastroenterology, 46,(1964): 134-156.

Phillips, R. A.: Water and electrolyte losses in cholera. Fedn Proc. Fedn Am. Socs exp. Biol., 23,(1964): 705-712.

Phillips, R. A.: Cholera in the perspective of 1966. Ann. intern. Med., 65,(1966): 922-930.

Pierce, A. E., and Smith, M. W.: The *in vitro* transfer of bovine immune lactoglobulins across the intestine of new-born pigs. J. Physiol., 190,(1967): 19-34.

Pink, I. J., Curran, R. C., and Creamer, B.: The ultrastructure of the small intestine under normal and pathological conditions. In *Methods and Achievements in Experimental Pathology. Volume III, Ultrastructural, Histopathologic and Chemical Approaches*. E. Bajusz and G. Jasmin, ed., Year Book Medical Publishers, Inc., Chicago, U.S.A. (1967).

Pitman, F. E., and Pitman, J. C.: Electron microscopy of intestinal mucosa. Archs Path., 81,(1966): 398-401.

Pitts, R. F.: *Physiology of the Kidney and Body Fluids*. Year Book Medical Publishers, Inc., Chicago, U.S.A. (1963).

Platt, B. S., Heard, C. R. C., and Stewart, R. J. C.: The effects of protein-calorie deficiency on the gastrointestinal tract. In *The Role of the Gastrointestinal Tract in Protein Metabolism: A Symposium*. H. N. Munro, ed., F. A. Davis Company, Philadelphia, Pa. (1964): 227-238.

Porter, K. R.: Independence of fat absorption and pinocytosis. Fedn Proc. Fedn Am. Socs exp. Biol., 28,(1969): 35-40.

Public Health Department, London County Council: Gastro-enteritis in infancy. Brit. J. prev. soc. Med., 7,(1953): 194-204.

Raaflaub, J.: *Die schwellung isolieter Lebezellmitochondrien und ihre Physikalische-chemische beeinflusbarkeit*. Helv. Physiol. Acta, 11,(1953): 142-156.

Reynolds, E. S.: The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol., 17,(1963): 208-212.

Rhodin, J. A. G.: *An Atlas of Ultrastructure*. The W. B. Saunders Company, Philadelphia (1963).

Ribi, E., Anacker, R., Fukushi, K., Haskins, W. T., Landy, M., and Milner, K. C.: Relationship of chemical composition to biological activity. In *Bacterial Endotoxins*. M. Landy and W. Brown, ed., Institute of Microbiology, Rutgers, The State University, New Brunswick, N.J. (1964).

Riecken, E. O., Stewart, J. S., Booth, C. C., and Pearse, A. G. E.: A histochemical study on the role of lysosomal enzymes in idiopathic steatorrhoea before and during a gluten-free diet. Gut, 7,(1966): 317-332.

Robbins, S. L.: *Textbook of Pathology with Clinical Applications*, 2nd edn, W. B. Saunders Co., Philadelphia, Pa. (1962).

Robertson, J. D.: The Unit Membrane. In *Electron Microscopy in Anatomy*. J. D. Boyd, F. R. Johnson, and J. D. Lever, ed., Edward Arnold, London (1961): 74.

Robinson, J. R.: Metabolism of intracellular water. Physiol. Rev., 40,(1960): 112-149.

Rodriguez-Leiva, M.: *Escherichia coli* studies. VII. Incidence of enteropathogenic *Escherichia coli* in the acute diarrhea of infants in Santiago, Chile.. Am. J. Hyg., 72,(1960): 162-168.

Roe, W. E., Quamme, G., and Nielsen, N. O.: University of Saskatchewan, Saskatoon, Sask., unpublished data, 1968, cited by Nielsen, Moon, and Roe (1968).

Rosalki, S. B., and Wilkinson, J. H.: *Diagnostic Enzymology*. Dade Reagents, Miami, Florida (1966).

Ross, C. A. C., and Dawes, E. A.: Resistance of the breast-fed infant to gastro-enteritis. Lancet, 1,(1954): 994-998.

Rubin, C. E., and Dobbins, W. O.: Peroral biopsy of the small intestine. A review of its diagnostic usefulness. Gastroenterology, 49,(1965): 676-697.

Rutenberg, A. M., Monis, B., and Banks, B. M.: Histochemical distribution of hydrolytic enzymes in the alimentary tract of man in health and disease. J. Histochem. Cytochem., 6,(1958): 102-102.

Rutenburg, A. M., and Seligman, A. M.: The histochemical demonstration of acid phosphatase by a post-incubation coupling technique. J. Histochem. Cytochem., 3,(1955): 455-469.

Samloff, I. M., Davis, J. S., and Schenk, E. A.: A clinical and histochemical study of celiac disease before and during a gluten-free diet. Gastroenterology, 48,(1965): 155-172.

Sanford, P. A.: The inhibition and mechanism of intestinal absorption. Proc. Nutr. Soc., 26,(1967a): 12-17.

Sanford, P. A.: Inhibition of intestinal absorption. Br. med. Bull., 23,(1967b): 270-274.

Sandritter, W., and Lasch, H. G.: Pathologic aspects of shock. In *Methods and Achievements in Experimental Pathology. Vol. III. Ultrastructural, Histopathologic, and Chemical Approaches.* E. Bajusz and G. Jasmin, ed., Year Book Medical Publishers, Inc., Chicago, U.S.A. (1967).

Saunders, C. N., Stevens, A. J., Spence, J. B., and Betts, A. O.: *Escherichia coli* infection: Reproduction of the disease in "pathogen-free" piglets. Res. vet. Sci., 4,(1963): 347-357.

Saunders, C. N., Stevens, J. A., Spence, J. B., and Sojka, W. J.: *Escherichia coli* infection in piglets. Res. vet. Sci., 1, (1960): 28-35.

Saunders, C. N., Stevens, A. J., Spence, J. B., and Sojka, W. J.: *Escherichia coli* infection: Reproduction of the disease in naturally reared piglets. Res. vet. Sci., 4,(1964): 333-346.

Scarpelli, D. G., and Pearse, A. G. E.: Physical and chemical protection of cell constituents and the precise localization of enzymes. J. Histochem. Cytochem., 6,(1958): 369-375.

Sears, H. J., Brownlee, I., and Uchiyama, J. K.: Persistence of individual strains of *Escherichia coli* in the intestinal tract of man. J. Bact., 59,(1950): 293-301.

Senecal, J.: *Les diarrhées infantiles en Afrique-Occidentale francaise.* Bull. World Health Organ., 21,(1959): 321-336.

Senior, J. R.: Intestinal absorption of fats. J. Lipid Res., 5,(1964): 495-521.

Seyderhelmen, R., Lehmann, W., and Wichels, P.: *Experimentelle intestinale perniziöze anämie beim Hund.* Klin. Wochschr., 3,(1924): 1439-1440.

Shanks, R. A., and Studzinski, L. P.: *Bacterium coli* in infantile diarrhoea. Brit. med. J., 2,(1952): 119-123.

Sheehy, T. W., and Floch, M. H.: *The Small Intestine its Functions and Diseases*. Hoeber Medical Division, Harper and Row, New York (1964).

Sherratt, H. S. A., and HÜbscher, G.: Properties of mitochondrial preparations from small-intestinal mucosa of the guinea-pig. Biochem. biophys. Acta, 69,(1963): 403-405.

Shiner, M.: *Feinstrukturelle Untersuchungen am Zottenepithel des menslichen Dünndarms a) Die normale Feinstruktur, b) Feinstrukturelle Veränderungen bei der Einheimischen Sprue (Cöliakie, idiopathische steatorrhae).* Internist, 7,(1966): 217-223.

Shiner, M.: Ultrastructure of jejunal surface epithelium in untreated idiopathic steatorrhea. Br. med. Bull., 23,(1967a): 223-225.

Shiner, M.: Personal communication (1967b), cited by Sanford (1967b).

Shiner, M.: The structure of the small intestine and some interesting relations to its function. Proc. Nutr. Soc., 26,(1967c): 1-4.

Shnitka, T. K.: Enzymatic histochemistry of gastrointestinal mucous membrane. Fedn Proc. Fedn Am. Socs exp. Biol., 19,(1960): 897-904.

Silhar, A. S., and Maru, A. M.: Morbidity in children. Indian J. Child Hlth., 9,(1960): 507-515.

Skou, J. C.: Enzymatic basis for active transport of Na^+ and K^+ across cell membrane. Physiol. Rev., 45,(1965): 596-617.

Smith, D. T., Conant, N. F., and Overman, J. R.: *Microbiology*. 13th edn, Appleton-Century-Crofts, New York (1964).

Smith, H. L., and Etteldorf, J. N.: Parenteral fluid regimens in the treatment of severe diarrhea in infants. J. Pediat., 58, (1961): 1-16.

Smith, H. W.: The ecology of the intestinal bacterial flora of the calf with particular reference to *Escherichia coli*. Vet. Rec., 72,(1960): 1178-1183.

Smith, H. W.: Observations on the flora of the alimentary tract of animals and factors affecting its composition. J. Path. Bact., 89,(1965): 95-122.

Smith, H. W., and Crabb, W. E.: The typing of *Escherichia coli* by bacteriophage, its application to the study of the *E. coli* population of the intestinal tract of healthy calves and of calves suffering from white scours. J. gen. Microbiol., 15,(1956): 556-574.

Smith, H. W., and Halls, S.: Observations by the ligated intestinal segment and oral inoculation methods on *Escherichia coli* infection in pigs, calves, lambs, and rabbits. J. Path. Bact., 93,(1967): 499-529.

Smith, H. W., and Halls, S.: Studies on *Escherichia coli* enterotoxin. J. Path. Bact., 93,(1967): 531-543.

Smith, H. W., and Jones, J. E. T.: Observations on the alimentary tract and its bacterial flora in healthy and diseased pigs. J. Path. Bact., 86,(1963): 387-412.

Smith, T.: Studies on pathogenic *B. coli* from bovine sources. III. Normal and serologically induced resistance to *B. coli* and its mutant. J. exp. Med., 46,(1927): 141-154.

Smith, T.: The relation of the capsular substance of *B. coli* to antibody production. J. exp. Med., 48,(1927): 351-361.

Smith, T., and Little, R. B.: Studies on pathogenic *B. coli* from bovine sources. I. The pathogenic action of culture filtrates. J. exp. Med., 46,(19 7): 123-131.

Smith, T., and Orcutt, M. L.: The bacteriology of the intestinal tract of young calves with special reference to the early diarrhea ("scours"). J. exp. Med., 41,(1925): 89-106.

Smyth, D. H.: Intestinal absorption. Br. med. Bull., 23,(1967): 205-208.

Smyth, D. H., and Whittam, R.: Membrane transport in relation to intestinal absorption. Br. med. Bull., 23,(1967): 231-235.

Sojka, W. J.: Escherichia coli *in Domestic Animals and Poultry*. Commonwealth Agriculture Bureaux, Farnham Royal, Bucks, England (1965).

Sprinz, H., Kundel, D. W., Dammin, G. J., Horowitz, R. E., Schneider, H., and Formal, S. B.: The response of the germfree guinea pig to oral bacterial challenge with *Escherichia coli* and *Shigella flexneri*. Am. J. Path., 39,(1961): 681-695.

Staley, T. E.: Clinical implications of normal and abnormal intestinal epithelial maturation in the neonatal pig. Journal of the Oklahoma Veterinary Medical Association, 21,(1969): 10-14.

Staley, T. E., Jones, E. W., and Marshall, A. E.: The jejunal absorptive cell of the newborn pig. An electron microscopic study. Anat. Rec. (In Press), (1968a).

Staley, T. E., Jones, E. W., and Corley, L. D.: The fine structure of the duodenal absorptive cell in the newborn pig before and after feeding of colostrum. Am. J. vet. Res., (In Press), (1968b).

Staley, T. E., Jones, L. D., and Corley, L. D.: Intestinal monocontamination in the neonatal pig -- microbiological and microscopic studies. Paper B-Z. *Abstracts. Symposium on Gnotobiology: Experimental and Clinical Aspects.* Statler Hilton Hotel, Buffalo, New York (1968c).

Staley, T. E., Jones, E. W., and Corley, L. D.: Intestinal moncontamination in the neonatal pig: Microbiological and microscopic studies. Gnotobiotic Symposium, October 1968 (In Press), (1968d).

Steedman, H. F.: Alcian blue 8GS: A new stain for mucin. Q. Jl Microsc. Sci., 91,(1950): 477-479.

Stevens, A. J.: *A Laboratory Handbook of Veterinary Diagnostic Procedures.* Animal Health Branch Monograph No. 3, F.A.O., Rome (1961).

Stevens, A. J.: Coliform infections in the young pig and a practical approach to the control of enteritis. Vet. Rec., 75,(1963): 1241-1246.

Stowens, D.: *Pediatric Pathology* 2nd edn, The Williams and Wilkins Co., Baltimore (1966): 218.

Strauss, E. W.: Electron microscopic study of intestinal fat absorption *in vitro* from mixed micelles containing linolenic acid, monoolein, and bile salt. J. Lipid Res., 7,(1966): 307-323.

Takeuchi, A.: Electron microscope studies of experimental *Salmonella* infection. I. Penetration into the intestinal epithelium by *Salmonella typhimurium*. Am. J. Path., 50,(1966): 109-136.

Takeuchi, A., and Sprinz, H.: Electron microscope studies of experimental *Salmonella* infection in the preconditioned guinea pig. II. Response of the intestinal mucosa to the invasion by *Salmonella typhimurium*. Am. J. Path., 51,(1967): 137-161.

Takeuchi, A., Sprinz, H., LaBrec, E. H., and Formal, S. B.: Experimental bacillary dysentery. An electron microscopic study of the response of the intestinal mucosa to bacterial invasion. Am. J. Path., 47,(1965): 1011-1044.

Taylor, J., and Charter, R. E.: The isolation and serological types of *Bacterium coli* in two residential nurseries and their relation to infantile gastro-enteritis. J. Path. Bact., 64,(1952): 715-728.

Taylor, J., Powell, B. W., and Wright, J.: Infantile diarrhoea and vomiting. Brit. med. J., 2,(1949): 117-125.

Teree, T. M., Mirabal-Font, E., Ortiz, A., and Wallace, W. M.: Stool losses and acidosis in diarrheal disease of infancy. Pediatrics, 36,(1965): 704-713.

Thake, D. C.: Jejunal epithelium in transmissible gastroenteritis of swine. Am. J. Path., 53,(1968): 149-168.

Thompson, S. W., and Hunt, R. D.: *Selected Histochemical and Histopathological Methods*. Charles C. Thomas, Springfield, Illinois, U.S.A. (1966).

Ticktin, H. E., and Trujillo, N. P.: *Serum Enzymes in Diagnosis*. Year Book Medical Publishers, Inc., Chicago (1966).

Torres-Pinedo, R., Rivera, C. L., Fernandes, S., and Maldonado, M.: Studies on infant diarrhea. II. Absorption of glucose and net fluxes of water and sodium chloride in a segment of the jejunum. J. clin. Invest., 45,(1966): 1916-1922.

Trapp, A. L., Sanger, V. L., and Stalnaker, E.: Lesions of the small intestinal mucosa in transmissible gastroenteritis-infected germfree pigs. Am. J. vet. Res., 27,(1966): 1695-1702.

Triantaphyllopoulos, E., and Tuba, J.: Changes in intestinal and serum alkaline phosphatase levels during absorption of certain amino acids. Can. J. Biochem. Physiol., 37,(1959): 711-719.

Trier, J. S., Phelps, P. C., and Rubin, C. E.: Electron microscopy of mucosa of small intestine. J. Am. med. Ass., 183,(1963): 768-774.

Trier, J. S., and Rubin, C. E.: Electron microscopy of the small intestine: A review. Gastroenterology, 49,(1965): 574-603.

Tuba, J., and Dickie, N.: The role of alkaline phosphatase in intestinal absorption. IV. The effects of various proteins on levels of the enzyme in intestinal mucosa. Can. J. Biochem. Physiol., 33,(1955): 89-92.

Tuba, J., and Robinson, M. I.: The response of intestinal alkaline phosphatase of fasted rats to forced feeding of fat. J. biol. Chem., 203,(1953): 947-951.

Ugolev, A. M.: Membrane (contact) digestion. Physiol. Rev., 45, (1965): 555-595.

Venable, J. H., and Coggeshall, R.: A simplified lead citrate stain for use in electron microscopy. J. Cell Biol., 25,(1965): 407-408.

Vernon, D. F.: Idiopathic sprue in a dog. J. Am. vet. med. Ass., 140,(1962): 1062-1067.

Verzar, F., and McDougall, E. J.: *Absorption from the Intestine.* Longmans, Green, and Co., London (1936).

Visscher, M. B., Varco, R. H., Carr, C. W., Dean, R. B., and Erickson, D.: Sodium ion movement between the intestinal lumen and the blood. Am. J. Physiol., 141,(1944): 488-505.

Vodovar, N.: *Intestine grele du porc. II. Structure histologique des parois et plus particulierement de la tunique muqueuse en fonction de l'age de l'animal.* Annls Biol. anim. Biochem. Biophys., 4,(1964): 113-139.

Voigt, A., and Abadjieff, W.: *Untersuchungen zur Pathogenese der Kolienterotoxämie des Schweines unter besonderer Berücksichtigung der Bedeutung der Resistenz des Makroorganismus und der Herdenprophylaxe.* Archs exp. vet. Med., 21,(1967): 155-164.

Wallick, H., and Stuart, C. A.: Antigenic relationships of *Escherichia coli* isolated from one individual. J. Bact., 45,(1943): 121-126.

Wasti, S. M.: Diarrhoea, the worst killer of child life. Pakistan J. Hlth., 9,(1959): 70-72.

Watson, M. L., and Adridge, W. G.: Methods for the use of indican as an electron stain for nucleic acids. J. biophys biochem. Cytol., 11,(1961): 257-261.

Watten, R. H., Morgan, F. M., Songkhla, Y. N., Vanikiata, B., and Phillips, R. A.: Water and electrolyte studies in cholera. J. clin. Invest., 38,(1959): 1879-1889.

Waxler, G. L., Schmidt, D. A., and Whitehair, C. K.: Technique for rearing gnotobiotic pigs. Am. J. vet. Res., 27,(1966): 300-307.

Weijers, H. A., Van de Kamer, J. H., Dicke, W. K., and Ijsseling, J.: Diarrhoea caused by deficiency of sugar splitting enzymes. Acta Paediat., 50,(1961): 55-71.

Wheeler, W. E.: *Fluorescent Antibody Detection of Enteropathogenic* Escherichia coli. Script of film No. M-1456. U. S. Department of Health, Education and Welfare, Public Health Service, Chamblee, Georgia (1967).

Whitehair, C. K., Grummer, R. H., Phillips, P. H., Bohstedt, G., and McNutt, S. H.: Gastroenteritis in pigs. Cornell Vet., 38,(1948): 23-39.

Whittam, R., and Ager, M. E.: The connexion between active cation transport and metabolism in erythrocytes. Biochem. J., 97, (1965): 214-227.

Wilson, T. H.: *Intestinal Absorption.* W. B. Saunders Co., Philadelphia, Pa. (1962).

Wirts, C. W., and Goldstein, F.: Studies of the mechanism of post-gastrectomy steatorrhea. Ann. intern. Med., 58,(1963): 25-36.

Wiseman, G.: Absorption of amino-acids using an *in vitro* technique. J. Physiol., 120,(1953): 63-72.

Wiseman, G.: *Absorption from the Intestine.* Academic Press, London (1964).

World Health Organization Report: *Manual of the International Statistical Classification of Diseases, Injuries, and Causes of Death; 6th Revision of the International Lists of Diseases and Causes of Death based on the Recommendations of the 7th Revision Conference 1955: and adopted by the 9th World Health Assembly under W.H.O. nomenclature regulations.* World Health Organization, Geneva (1957).

Wright, J., and Boden, A. T.: *Escherichia coli* 055B5 infection in a gastro-enteritis ward. Epidemiological applications of H antigen type determinations. Am. J. Hyg., 58,(1953): 133-147.

Yekutiel, P.: Epidemiological methods used in the study of infantile diarrhoea in Israel. Bull. World Health Organ., 21,(1959): 374-381.

Zamcheck, N.: Dynamic interaction among body nutrition, gut mucosal metabolism and morphology and transport across the mucosa. Fedn Proc. Fedn Am. Socs exp. Biol., 19,(1960): 855-864.

Zilversmit, D. B.: The composition and structure of lymph chylomicrons in dog, rat, and man. J. clin. Invest., 44,(1965): 1610-1622.

Zweifach, B. W.: Microcirculatory derangements as a basis for the lethal manifestations of experimental shock. Br. J. Anaesth., 30,(1958): 466-484.

Zweifach, B. W.: Vascular effects of bacterial endotoxin. In *Bacterial Endotoxins*. M. Landy and W. Brown, ed., Institute of Microbiology, Rutgers, The State University, New Brunswick, N.J. (1964).

Zweifach, B. W., Nagler, A. L., and Thomas, L.: The role of epinephrine in the reactions produced by the endotoxins of gram negative bacteria. J. exp. Med., 104,(1956): 881-896.

VITA

Bruce Robert CHRISTIE

Born: 22nd. September 1932 at Colac, Victoria, Australia.

Father: Richard William Christie

Mother: Christina Christie (nee Lemon)

Married: Greta Constance Straton

19th. May 1956 at Hobart, Tasmania.

Children:

Richard James Iain Christie

Bruce Angus Straton Christie

Fiona Christine Christie

Catriona Clark Christie

Education:

1936-40: Meeniyan State School, Victoria

1941-42: Hamilton State School, Victoria

1943-47: Hamilton High School, Victoria

1948-50: Ballarat College, Victoria

1951: Melbourne University, Victoria

1952-56: University of Sydney, New South Wales

1964-69: Michigan State University, Michigan, U.S.A.

Academic Degrees:

1957: Bachelor of Veterinary Science
Faculty of Veterinary Science
University of Sydney
Sydney, New South Wales, Australia

1967: Master of Science
Department of Pathology
Michigan State University
East Lansing, Michigan, U.S.A.

1969: Doctor of Philosophy
Department of Pathology
Michigan State University
East Lansing, Michigan, U.S.A.

Professional Certification:

1957: Registered Veterinary Surgeon, State of Victoria, Australia

Professional Posts:

1957: Veterinary Officer
Livestock Division, Department of Agriculture
Melbourne, Victoria

1957-61: District Veterinary Officer
Livestock Division, Department of Agriculture
Horsham, Victoria

1957-61: Lecturer in Veterinary Science
Longerenong Agricultural College
Dooen, Victoria

1961-64: Veterinary Research Officer (Cattle Infertility)
Cameron Laboratory
State Research Farm
Werribee, Victoria

1962: Representative
Technical Committee on Cattle Infertility
Launceston, Tasmania

1964-66: Assistant Instructor
Department of Pathology
College of Veterinary Medicine
Michigan State University
East Lansing, Michigan

1965-69: Post Graduate Research Fellow
Australian Wool Board
Michigan State University
East Lansing, Michigan

1966-69: Senior Graduate Assistant
Department of Pathology
College of Veterinary Medicine
Michigan State University
East Lansing, Michigan

Professional Societies:

Australian Veterinary Association - Member
Honorary Secretary Victorian Division 1963-64
Member Federal Council and State Representative 1963
Australian Institute of Agricultural Science - Member
Australian and New Zealand Association for the Advancement of Science - Member
Australian Society of Animal Production - Member
Register of Scientists and Engineers for Commonwealth of Australia - Member
Phi Zeta
Sigma Xi

Research and Publications:

Christie, B. R.: Actinobacillosis of Sheep. Vict. vet. Proc., 17, (1958-1959): A22.

Christie, B. R.: Trace Elements in Plant and Animal Nutrition - The Animal. Proceedings, Conference of the Grassland Society of Victoria, Melbourne, May, 1961.

Christie, B. R.: A Muscular Dystrophy of Merino Lambs in Western Victoria. Vict. vet. Proc., 20, (1961-62): 30-32.

Christie, B. R.: Nutrition and Management of Beef Cattle as Applied to Reproductive Failure under Victorian Conditions. Vict. vet. Proc., 20, (1961-62): 16-16.

Christie, B. R.: Ovine Muscular Dystrophy in Victoria. Aust. vet. J., 38, (1962): 392-394.

Christie, B. R.: Hysterectomy Produced Artificially Reared Pigs and Their Possible Use in the Control of Disease. Aust. vet. J., 39, (1963): 148-152.

Christie, B. R.: Experimental Colibacillosis in Gnotobiotic Pigs. M. S. Thesis, Michigan State University, East Lansing, Mich., 1967.

Christie, B. R.: The Pathogenesis of Colibacillary Diarrhea of the Newborn. An Electron Microscopic and Histochemical Study of the Disease in Neonatal Gnotobiotic Pigs. Ph.D. Thesis, Michigan State University, East Lansing, Mich., 1969.