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A Morphological and Histological and Histochemical Study of the Intracellular Inclusion Bodies in the Intestingal Epithemium of the Prenatal and Postnatal Rat

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A MORPHOLOGICAL AND HISTOCHEMICAL STUDY OF THE INTRACELLULAR INCLUSION BODIES IN THE INTESTINAL EPITHELIUM OF THE PRENATAL AND POSTNATAL RAT

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF PLATES AND FIGURES

66. Photomicrograph of ileum of 6 day old animal demonstrating amino groups within inclusion bodies (Alloxan-Schiff reaction).

PLATE XXXIV

Figure:

67. Photomicrograph of ileum of 10 day old animal treated with the Feulgen reaction.

68. Photomicrograph of ileum of 9 day old animal showing distribution of succinic dehydrogenase in epithelium.

ABSTRACT

A morphological and histochemical study of the epithelium of the small intestine of the developing rat was performed using the following techniques: (1) hematoxylin and eosin; (2) periodic acid-Schiff; (3) Alcian blue; (4) alkaline phosphates; (5) acid phosphates; (6) non-specif esterace; and (7) leucine aminopeptidase. In addition to those listed above, other reactions were performed including: (1) dihydroxy-dinaphthyl-disulphide; (2) alloxan-Schiff; (3) nile blue sulfate; (4) Ziehl-Neelsen method for acid fastness; (5) Feulgen; and (6) succinic dehydrogenace. To provide a means of demonstrating whether swallowed amniotic fluid is absorbed by the fetal intestine, a colloidal iron preparation was injected into the amniotic cavity of fetal animals. Following this procedure, sections of the intestinal tissues of these animals were treated with the Prussian blue reaction to demonstrate the course taken by the iron containing material.

During the fetal developmental period, the intestinal epithelium consisted of a stratified layer of cells at 16 and 17 days gestation. In the 18 day old fetuses, the epithelium in the cephalic portion of the small intestine had transformed to the simple columnar type and by 20 days gestation, transition was complete along the entire small intestine. Beginning with the 19

day old fetuses, small, PAS-positive droplets accumulated in the supranuclear cytoplasm of the columnar cells. Similarly, granules or droplets containing iron were demonstrated in the same portion of these cells in animals which had received iron by the intra-amniotic route.

In the postnatal animals, large supranuclear inclusion bodies were present in the columnar absorptive cells of the ileum and jejunum from 1 day to approximately 20 days of age at which time they disappeared. All of these instructures were PAS positive and Alcian blue negative and contained no succinic dehydrogenase. The largest of these bodies gave positive reactions for lipofuscins, sulfhydryl groups, and amino groups producing characteristics similar to "meconium corpuscles" described in the human fetus.

In regard to the enzyme technique, strong reactions with the acid and alkaline phosphatase and the non-specific esterase procedures were obtained in the intracellular inclusion bodies of the suckling animals. In the developmental process, non-specific esterase, acid and alkaline phosphatase and aminopeptidase enzyme activities appeared in the intestinal epithelial cells at 17, 18, and 19 days gestation, respectively. At the onset, the esterase enzyme was localized in the form of scattered deposits in the basal portions of the epithelium which was later replaced by the zone of activity in the supranuclear cytoplasm of the villus epithelium. The phosphatases and amino-peptidase were localized in the cuticular border of the columnar cells in all segments of of the small intestine by birth.

In the postnatal animals, alkaline phosphatase showed little change in activity in the striate border following birth, whereas

definite decreases in the activity of acid phosphatase and amino-peptidase occurred in that portion of the cells. At weaning a transition occurred in which the pattern of distribution of all enzyme studied changed to the adult configuration.

On the basis of the results of the present study and information obtained from other studies in this area, the following conclusions were made: (1) the intestine of the fetal rat absorbs material from swallowed amniotic fluid; (2) the enzyme of the intestinal epithelium became active before birth; (3) the intracellular inclusion bodies appeared to be related to lysosomes; (4) the morphological and histochemical characteristics of the inclusion bodies of the intestinal epithelium of the suckling rat were very similar to "meconium corpuscles" previously described in the human fetus; and (5) the inclusion bodies represented a developmental adaptation occurring during a certain period of ontogeny.

CHAPTER I

INTRODUCTION

Since the observations of Heidenhain (1) were published on the newborn dog in 1888, several investigators have demonstrated that the intestinal epithelial cells of the newborn of several species of animals contain a system of large inclusion bodies. Later, it became known that similar structures are contained in the intestinal epithelium of the developing human fetus (2).

In the original study, Heidenhain (1) observed that the inclusion bodies ranged in size from small droplets to large globules. In the latter situation, the cytoplasm of the luminal portions of the columnar absorptive cells was displaced by these structures leaving a thin cytoplasmic "rim" at the periphery of these cells. These bodies contained a structureless material which stained with a variety of dyes including methyl green, acid fuchsin and methyl orange. Heidenhain (1) also pointed out that, in contrast to the picture obtained in the young pup, bodies of this type were absent from the intestinal epithelium of the newborn rabbit and guinea pig.

In the dog, the inclusion bodies were seen to appear shortly after birth and remained until the 12th (1) or the 14th (2) day when they disappeared from the epithelial cells. Schmidt (2) who repeated studies on the epithelium of the dog in 1905, also reported that small droplets of intracellular material accumulated in these cells during

the late stages of fetal development.

In addition to his observations on the newborn dog, Schmidt (2) described intracellular inclusion bodies in the intestinal epithelium of the human fetus which were very similar in appearance to those seen in the intestine of the young animals. The inclusion bodies of the human fetus, which were also described by several authors quoted by Patzelt (3), were shown to contain globules of homogeneous material giving a yellow-brown color in unstained sections.

In the fetus, the inclusion bodies generally were most prominent during the middle trimester of intrauterine life. During the remaining third of the fetal development period, these bodies gradually receded, usually disappearing completely by the time of birth (2,3). At times however, a small number of globules may remain in some of the cells at the tips of the villi in the newborn infant (4).

During all phases of development, the inclusion bodies were absent from the cephalic portion of the small intestine down to the middle jejunum where small globules were present. Beyond this point, these bodies were larger in size reaching the greatest dimensions in the distal ileum. In regard to the distribution of the inclusion bodies on each villus, the cells located on the apices contained the largest of these structures. On the lateral sides of the villi, these bodies were smaller and disappeared from the cells at the neck of the crypts (3).

At the height of their development, which occurred during the 6th and 7th months of gestation, the inclusion bodies were very large in the distal ileum. At this point, the supranuclear portion of the columnar cells was crowded and filled by these structures (3).

In unstained sections, the color of these inclusion bodies was very similar to that exhibited by meconium (3). Meconium which consists of vernix caseosa mixed with extruded epithelial cells and secretions of the alimentary tract, is generally located in the lumen of the distal portion of the intestinal tract. According to Patten (5) it is understood that the vernix caseosa, which is composed of lanugo hairs and desquamated cells and secretions from the skin, is suspended in the amniotic fluid. These materials are introduced into the intestinal lumen when this liquid is swallowed by the fetus (5).

Since the inclusion bodies were very similar in appearance to meconium, many of the earlier investigators concluded that the material contained within these structures was bile-stained material that had been absorbed from the intestinal lumen (2,3). In fact these structures so closely resembled corpuscular globules of meconium (termed "meconium corpuscles") seen in smears of meconium that Schmidt (2) assumed that the inclusion bodies were "meconium corpuscles" that had been absorbed <u>in toto</u> by the columnar cells. This fact has never been demonstrated but the term "meconium corpuscles" is used at the present time to refer to the large inclusion bodies located in the columnar epithelial cells of the human fetus (6).

In 1925, Tobeck (7) presented evidence that the color exhibited by the intracellular bodies may have been due to a substance other than bile-stained meconium. In his study, intracellular bodies demonstrating the usual color and appearance for "meconium corpuscles" were identified in the epithelium distal to a locus of congenital intestinal atresia. In this condition, neither bile nor materials contained in swallowed amniotic fluid could gain entrance to this portion of the intestine and,

on this basis, Tobeck (7) concluded that the materials seen in the inclusion bodies was thickened mucous.

In 1924, the list of animals known to contain inclusion bodies was expanded by Parat (8) to include the newborn mouse, cat, sheep and guinea pig. The latter presents an interesting contrast with the work of Heidenhain (1) who observed no structures in the intestine epithelium of the newborn guinea pig.

As it became apparent that intracellular inclusion bodies were located in the intestinal epithelium of the newborn of several species of animals, speculations concerning their functional significance developed. Since Ehrlich in 1892 (9) showed a transfer of maternal antibodies to suckling mice through mammary secretions, it was postulated that unaltered proteins were absorbed in the intestine of the suckling young. In 1924, von Mollendorff (10) produced evidence to support this theory by demonstrating that the intestinal epithelium of newborn animals possessed powers of unselective absorption. In these experiments, von Mollendorff (10) obtained vital staining of both the epithelial tissue and the intracellular globules when various acid dyes were introduced into the intestinal lumen of suckling mice ranging in age from newborn to 20 days.

Additional work with the intestinal tissue of young animals has indicated that intracellular inclusion bodies are also found in the intestinal epithelium of several other species of animals including the newborn of some of the ruminants. In the newborn calf, for example, Comline et al. (11) have shown that the columnar absorptive cells of the distal segments of the small intestine contained 1 or 2 large supranuclear globules ranging in size from 10 to 24 microns in diameter.

In addition to these, clusters of smaller droplets (2-6 microns in diameter) could be seen in this position, and simultaneously, small bodies 2 microns in diameter were found in the apical cytoplasm immediately within the cuticular border. The material contained within the globules appeared to be similar to that seen in the intestinal lumen, and occasionally, droplets with similar material were seen within the lacteals. These findings led Comline et al. (11) to conclude that colostrum proteins were absorbed and transported by these structures.

In 1925, Smith (12) showed that some of this material began to accumulate in droplets within the columnar epithelial cells of the calf intestine during the late stages of fetal life. Comline et al. (11) felt that colostrum absorption probably took place during the first 36 to 48 hours of postnatal life, corresponding to the period when the inclusion bodies were most highly developed. After this point of development, the intracellular structures underwent regression and in animals studied at 63 and 65 hours of age, only a few small bodies remained within the columnar cells.

After piglets and kittens had ingested colostrum a similar picture showing inclusion bodies contained within the columnar cells was seen (13). The intracellular material, which stained black with iron hemotoxylin and red with azo carmine, was found in globules of 0.5 to 10 microns in diameter. In the pig, these structures were seen in the supranuclear cytoplasm of the columnar cells 6 to 8 hours after colostrum was first ingested. In 15 and 18 hour old individuals, however, these structures had migrated downward into the cell and were seen in an infranuclear position. In contrast to the above, only pale

unstained vacuoles were seen in the intestinal cells of 48 hour old animals that had not been permitted to suckle. Similarly, unstained vacuoles were also identified in the epithelium of these animals during the last 2 to 3 weeks of intrauterine development (13).

In another study, Hill and Hardy (14) observed that the columnar epithelial cells of lambs and kids which received colostrum proteins contained many large eosinophilic globules 2.5 to 15 microns in diameter. These bodies were most prominent during the first few hours following the ingestion of colostrum and began to recede after 36 hours of age. The picture concerning these bodies was different from those described in the calf since the majority of these structures were located in an infranuclear position. In animals sacrificed 3 days after birth, the columnar absorptive cells were completely devoid of this material. Similar to the calf, a number of small droplets with contents which were thought to be similar to material contained in the intestinal lumen and in the large supranuclear bodies, were seen in the lacteals. In addition, small droplets immediately below the cuticular border of the columnar cells were seen in the lamb and kid (14,15).

Inclusion bodies were identified in the intestinal epithelial cells of newborn guinea pigs by Schmidt (2) and Parat (8). More recently, they were studied more thoroughly by Leissring and Anderson (16) who observed these structures in the epithelial cells and saw droplets in the lacteals of the ileum of the guinea pig during the first postnatal week.

In a similar manner, Anderson and Leissring (17) observed small basophilic granules in the cells lining the small intestine of the

fetal guinea pig beginning with 55 days of gestation. At 65 days these bodies became more numerous.

In another study, Leissring and Anderson (18) observed an uptake of labelled materials in guinea pig fetuses by the intestine from the amniotic cavity after 50 days of gestation. They observed that this function was performed largely by the yolk sac epithelium prior to the 50 day stage but following this time, it was gradually taken over by the fetal gut which ultimately became the predominant transfer organ. In a like manner, Wright and Nixon (19) have observed absorption of amniotic fluid in the gut of fetal sheep. These studies substantiated observations made by Wislocki (20), who in 1920, obtained vital staining of intestinal tissue of fetal guinea pigs and cats following intra-amniotic injections of trypan blue.

Inclusion bodies appearing to be similar to those described in the other animals have been observed in the epithelium of the distal portion of the small intestine in newborn rats (21,22) and mice (10,21). In the rat, these structures, which are definitely eosinophilic and give a positive PAS reaction, are negative to the Alcian blue reaction (22). Clark (21), who performed electron microscopy on the jejunal epithelium of the suckling rats and mice, reported numerous vacuoles and inclusions in the apical portions of the columnar cells enclosed by an encircling membrane. These structures were identified up to 20 days of age when they disappeared. The large apical bodies contained granular material, dense bodies, and frequently, complex arrangements of parallel membranes. Numerous pinocytotic vesicles, postulated by Clark (21) to be associated with processes of absorption, were identified in the apical cytoplasm beneath the luminal border of the columnar cells.

Similar to the observations of von Mollendorff (10), Clark (21) demonstrated an uptake of various colloidal materials by the columnar cells of the jejunum when administered to suckling rats and mice less than 18 days of age. Substances which were taken up by these cells included India ink, trypan blue, Evans blue, saccharated iron oxide, colloidal gold, whole blood, plasma and bovine globulins and albumin. With the use of the electron microscope, he was able to trace the uptake of electron dense materials through a system of pinocytotic vesicles and globules which ultimately coalesced with the large supranuclear body.

With fluorescence microscopy, Clark (21) observed that absorbed proteins were also transported into the supranuclear globules. Following the feeding of proteins, this material was so extensively absorbed that it caused marked enlargement of the inclusion bodies literally filling the entire apical portion of the columnar cell. As an example, Clark (21) observed that in the cells of the jejunum, where the inclusion bodies are relatively small, these bodies became very large after protein feeding, approaching the size of the bodies commonly seen in the ileum.

During the course of these experiments, Clark (21) made another interesting observation. He observed that lipids, presumably absorbed by processes of micropinocytosis were absorbed primarily in the proximal segments of the intestine. This phenomenon was in contrast to the absorption of proteins and other colloids which were absorbed in the distal portions of the small intestine.

Similar to the situation observed in some of the other animals, small PAS-positive droplets having an affinity to a wide variety of stains have been identified by Owman (23) in the intestinal epithelium of fetal rats. These structures were shown to be clustered in the

supranuclear Golgi zone of the columnar absorptive cells of the small intestine in 20 to 22 day old fetal animals. Occasionally, small droplets were also demonstrable in the lacteal ducts during this period.

The droplets observed in the epithelial cells of the fetal rats, which had a diameter of about 2 microns, became much larger and more numerous in the epithelium when the rats were pinealectomized in utero (23). The structures which developed as a result appeared to be very similar in size and form to those typically seen in the columnar cells of the suckling individuals. Owman (23) postulated that these structures consisted of a mucoprotein since these bodies were negative to the Sudan black B method for lipids. In addition, they resisted extraction in pyridine and acetone and were digested with proteases.

In another study, Owman (24) demonstrated that the large inclusion bodies seen in the fetal animals following pinealectomy contain materials absorbed from the intestinal lumen. This was demonstrated when fluorescence labelled macroproteins, which were injected into the amniotic cavities of these animals, were absorbed by the columnar cells and incorporated into the intracellular corpuscles.

In 1962, Moe and Behnke (25) observed small cytoplasmic bodies appearing to be very similar to "dense bodies" of liver cells in the cells of the crypt anlage of the duodenum of the newborn rat. With the aid of the electron microscope, it was shown that these bodies contained fragments of degenerating mitochondria, ribosomes, rough surfaced endoplasmic reticulum and other membranes.

One year later, Behnke (26) demonstrated cytoplasmic bodies which appeared to be similar to those he described with Moe (25) in the

epithelium of the duodenum of fetal rats. In his study, he observed that the epithelium of the rat fetus at 14 days gestation consists of high, tapering columnar cells. On the 16th day, small granules, which stained with toluidine blue and gave a positive PAS-reaction, occurred in the apical cytoplasm. During the following days of development, intensive mitotic activity took place in these cells resulting in a stratified epithelium consisting of 4-6 layers of cells by the 18th day of gestation.

During this period of time, certain changes developed in the mesenchyme of the intestinal wall and in the cells of the epithelium which were involved in processes of villus development. The first noticeable changes were the formation of clefts in the epithelium and the evagination of buds of mesenchyme from the intestinal wall into the epithelial layer. The mesenchyme ultimately formed the connective tissue cores of the villi and at the same time, the epithelial cells covering these structures had transformed to the single columnar type (26).

In this period of cellular rearrangement, a variety of cytoplasmic bodies appeared in these cells. These structures were first seen on the 16th day, increased in number and reached a maximum during the 18th day. These inclusions presented a variation in size ranging from less than 1 micron to several microns in diameter. Some of these bodies contained PAS-positive material and others were stained with pyronine. Most of these structures were strongly basophilic. Incubation of frozen sections for acid phosphatase showed that many of these bodies contained this enzyme (26).

Electron microscopy of these tissues indicated that the intracellular bodies were enclosed by unit membranes and contained a series of granules, mitochondria, ribosomes, smooth and rough surfaced endoplasmic reticulum and lamellae (myelin figures). These structures appeared to arise from a fusion of various vesicles and lamellae found in the cytoplasm of these cells. They appeared to be able to gain in mass by apposition of additional layers of cytoplasmic components (26).

Because of the nature of their contents, Behnke (26) postulated that these cytoplasmic bodies represented autophagocytic bodies or cytolysomes presumably involved in the remodeling processes of the intestinal epithelium. It seemed natural to assume that during the transition from a stratified to a simple epithelium some of the epithelial cells died and the cellular waste products were eliminated by digestion. If the intracellular bodies described by Behnke (26) were sites of autophagocytosis, their presence presents a mechanism by which the cellular waste products can be hydrolyzed and removed. This fact would correspond favorably with the concept of Glucksmann (27) that the death of certain cells during the developmental process plays an important part in organogenesis. The fact that similar "autophagocytic bodies" have been seen in the cells of the regressing tail of the tadpole undergoing metamorphosis (28) and in chick blastoderm (29) provides additional illustrations of this phenomenon.

In regard to processes of intestinal absorption, information obtained from studies published by Clark (21), Comline et al. (11,13), Hill and Hardy (14) and von Mollendorff (10) provides substantial support that the epithelial cells of the distal part of the small intestine of the young of several species of animals are capable of

absorption of macromolecular materials from the intestinal lumen. It was also seen that this phenomenon usually corresponded to the time when the large inclusion bodies were seen in the columnar cells. With this in mind, these structures were interpreted by Owman (23) as representing a stage in the absorption of large molecular materials, particularly intact antibody proteins, which are transferred from the mother to the young animals through suckled milk.

The fact that antibodies are absorbed at this age of development had been demonstrated morphologically by Clark (21). In addition to this, other investigators have demonstrated the intestinal absorption of antibodies in newborn animals with immunological and electrophoretic techniques in situations involving absorption of normal colostrum and immune sera. These studies include those of Halliday (30) on the rat, Deutsch and Smith (31) in the goat and calf, and Payne and Marsh (32) in the pig.

Very recently, electron microscopy of the intestinal epithelium of the human fetus has provided substantial evidence that the large inclusion bodies seen in this tissue also serve as focal points for the collection of absorbed materials (33). At this level of magnification, Bierring et al. (33) described numerous pinocytotic vesicles and other small globules in the apical cytoplasm beneath the luminal border of the columnar cells. These authors postulated that these small droplets were associated with an uptake of materials from the intestinal lumen after which they migrated down into the depths of the cell. Here, they coalesced with other bodies, forming larger vesicles which ultimately united with the large "meconium corpuscle." During the

process of transport from the smaller to the larger bodies an increase in the density of the intravesicular material was seen.

On the ultrastructural level, the "meconium corpuscles" were shown to consist of globules of electron dense, homogeneous material containing dense bodies, membranes and "myelin figures." These structures in turn, were enclosed by an encircling unit membrane. Because of their apparent phagocytic origin, Bierring et al. (33) interpreted these bodies as a type of lysosome.

Lysosomes, which are vesicular subcellular structures of a digestive nature (34), were first discovered in the mitochondrial fraction of homogenized liver cells by de Duve (35) in 1955. More recently, lysosomes or "dense bodies" have been identified with electron microscopy in a wide variety of cells including those of the liver (36,37), nerve tissue (38), phagocytes (28), protozoans (39) and others. No common structural characteristics have been determined for these bodies seen in various tissues since they have been shown to contain a variety of structurally different materials (34). In regard to function, these bodies seem to be involved in processes of far reaching significance. For example, they have been implicated in the process of cell death during holocrine secretion (39) and in embryonic development (27,28). In addition, they have been associated with digestive processes of phagocytic cells (28) and many other processes.

At the present time at least 12 different enzymes exhibiting an optimum activity at an acid pH have been demonstrated in lysosomes (41). Of these, acid phosphatase is the most easily demonstrated by histochemistry, and according to Novikoff (42), is the most reliable cytochemical characteristic for the identification of lysosomes.

To provide further verification that the structures demonstrated in the human fetal intestine with the electron microscope were lysosomes, a companion histochemical study on this tissue was performed by Andersen et al. (43). In this study, several enzymes were investigated as well as several staining reactions for various other tissue components. In their results, Andersen et al. (43) localized activities for the non-specific esterase, aminopeptidase and acid phosphatase enzymes in the inclusion bodies of the columnar cells of the ileum of the human fetus. Activity for lactic dehydrogenase, which gave a general cytoplasmic reaction, was also apparent in these structures. On the other hand, no intravesicular reactions were obtained for alkaline phosphatase nor succinic dehydrogenase. These findings led Andersen et al. (43) to postulate that these structures were associated with digestive processes in addition to their absorptive capabilities.

Andersen et al. (43) found that the inclusion bodies gave a positive reaction with the periodic acid-Schiff procedure and were uncolored by Alcian blue similar to the picture obtained in the suckling rat. They also demonstrated that the inclusion bodies were negative to tests for bile pigments and iron complexes indicating that these globules may not contain absorbed bile. On the other hand, positive demonstration for lipofuscins was obtained supporting a hypothesis that the characteristic yellow-brown color of these structures may be derived from lipofuscin pigments.

Other tests performed by Andersen et al. (42) included positive identification of phospholipids in the small bodies and amino groups in the largest of the inclusion bodies. At the same time, tests for sulfhydryl groups and tyrosine were positive and reactions for

ribonucleic acids were negative. On this basis, they concluded that these structures seen in the fetus probably were composed primarily of muco- or glycoproteins.

After reviewing the existing literature on the subject, it became apparent that a histochemical investigation concerning the possible presence of enzymes within the absorptive granules in the columnar cells of the intestinal epithelium of young animals should be performed. The fact that high levels of non-specific esterase activity were demonstrated in the ileum of newborn and 10 day old rats by Koldovsky (44) increases the possibility that enzymes are contained in the inclusion bodies when in the cells of the intestinal epithelium of these animals.

With this in mind, a histochemical study emphasizing the demonstration of enzymes contained within the inclusion bodies of the epithelium of the suckling rat seemed justified and was performed. The enzyme techniques used in this study include some of those previously studied in the human fetus. These are: acid phosphatase, alkaline phosphatase, non-specific esterase, aminopeptidase and succinic dehydrogenase. It is hoped that information obtained from these procedures will give more insight into the nature of these bodies and at the same time, provide a comparison with information previously obtained in the human fetus.

CHAPTER II

MATERIALS AND METHODS

White rats of Holtzman stock, bred and raised in this laboratory, were utilized in this study. The animals studied ranged in age from 16 days gestation to 26 days after birth. A chart indicating the actual number of animals studied on each day of development is included in Table 1. In addition to these, 3 adult animals were used for comparison with the young animals.

TABLE 1. LIST OF ANIMALS USED WITH REFERENCE TO AGE, WEIGHT OR CROWN-RUMP LENGTH

Age*	Number of Animals Studied**	Ave. Crown-Rump Length or Wt. in Grams
16 days gest.	(5) 3	16 mm.
17 " "	2	19 mm.
	(9) 3	20 mm.
18 " "	(13) 2	25 mm.
	(10) 3	27 mm.
19 " "	(3) 2	29 mm.
	(13) 3	30 mm.
	-- 2	30 mm.
20 " "	(10) 3	---
	2	---
	(11) 3	40 mm.
21 " "	- 6	---
	(7) 4	40 mm.
22 " "	(12) 4	43-45 mm.
	6	45 mm.
Newborn	5	
2 hrs.	3	
5 hrs.	8	
6-7 hrs.	1	

TABLE 1--Continued

Age*	Number of Animals Studied**	Ave. Crown-Rump Length or Wt. in Grams
12 hrs.	2	
1 day	4	
2 days	4	
3 days	4	8 gm.
4 days	3	12 gm.
5 days	4	14 gm.
6 days	3	15 gm.
7 days	3	19 gm.
8 days	4	16 gm.
9 days	2	----
10 days	4	----
11 days	3	20 gm.
12 days	2	22 gm.
13 days	2	25 gm.
14 days	6	30 gm.
15 days	5	28 gm.
16 days	2	30 gm.
17 days	5	32 gm.
18 days	2	40 gm.
19 days	2	38 gm.
20 days	2	40 gm.
21 days	4	47 gm.
22 days	2	40 gm.
23 days	1	50 gm.
24 days	2	60 gm.
26 days	1	78 gm.

* When reference is made to the age of the fetal animals the figure given is always the number of days following the onset of gestation.

**Number in parenthesis indicates number of animals in litter.

The pregnant animals and those supporting suckling litters were maintained on a diet consisting of Purina Laboratory Chow and water supplied ad libitum. Normal breeding stock was used to supply the fetal and postnatal animals used in this study.

The fetal animals were collected on appropriate days of gestation from female rats whose approximate time of fertilization was known. This point was determined by a procedure involving examination of vaginal washings of breeding animals for the presence of sperm as described below.

During the period of breeding, male animals were placed with the females only during the night-time hours to permit closer approximation of the time of fertilization. Vaginal smears were taken from the breeding females by flushing with a small volume of distilled water from a glass pipette during each morning of the breeding period. These washings, which were examined for sperm under a microscope, were taken every day until sperm were found in the smear. When sperm were seen, the animal was assumed to have been fertilized and the period of gestation was counted in daily increments from this time. More sophisticated means for closer determination of the exact time of breeding seemed unnecessary since it has been shown that the actual fertilization of the ova occurs anywhere from 8-20 hours after copulation (45).

The procedure of collecting the fetal animals involved inducing deep anesthesia in the mother animals with lethal doses of Diabutol (veterinary Nembutal; 35mg/kg) followed by opening the maternal abdominal cavity by means of a midline incision. The fetal specimens were quickly removed from the uterine cavity and tissues from these animals were treated immediately with the fixation or the quenching procedures indicated below. Because of their small size, the 16 and 17 day old fetuses were fixed <u>in toto</u>. To permit proper penetration

of fixative into the intestinal tissue, the abdominal cavities of these animals were opened prior to fixation. In the older fetal specimens, the loops of the small intestine were removed and fixed as these animals were collected.

The postnatal animals used in this study were selected from normal litters which had been allowed to suckle and feed normally up to the time of sacrifice. These animals were sacrificed by intraperitoneal injections of lethal doses of Diabutol and segments of the duodenum, jejunum and ileum were removed as soon as these animals were insensitive to pain. These tissues were then divided and fixed in the procedures described below. When possible, the intestinal segments collected for study from the older suckling animals were cut open prior to fixation to permit better penetration of the fixative.

FIXATION PROCEDURES

1. Gluteraldehyde fixation: representative sections of the segments of the intestine from all animals were fixed in 3% gluteraldehyde (46) in 0.1 M phosphate buffer (pH 7.0) at 4°C for two hours. After fixation, these tissues were then transferred to a 0.1 M phosphate (pH 7.0) "wash buffer" containing 5% sucrose and maintained at 4°C for at least 12 hours before further treatment. Further treatment of these tissues, which were primarily used in enzyme studies, is described below.
2. Formalin fixation: for morphological studies, segments of the various portions of the intestine of the animals were

fixed in 10% formalin buffered with phosphate buffer (pH 7.0) (47) for at least 24 hours.

3. Quenching: representative segments of the intestinal tissue were quenched in isopentane cooled to - 165°C in liquid nitrogen. These tissues were processed immediately or stored at -18° to -20°C for periods of time not longer than 2-3 days before further treatment. These tissues were used in the aminopeptidase and succinic dehydrogenase techniques.

EMBEDDING AND SECTIONING

Fixed tissues were dehydrated through graded series of ethyl alcohols up to 95%, cleared in butyl acetate for at least 24 hours and vacuum embedded in 56° Tissuemat (paraffin). Sections were cut at 5 microns on an AO Spencer 820 rotary microtome and mounted on albumin coated slides.

STAINING PROCEDURES

After drying, the mounted paraffin embedded sections were deparaffinized in xylene and brought to water through a series of graded alcohols before being stained. Upon completion of the respective staining techniques, the tissues were again dehydrated through a series of alcohols up through absolute ethanol, cleared in xylene and mounted in Permount.

The following staining techniques were performed on these tissues:

1. A routine hematoxylin and eosin technique was performed to demonstrate the general morphology of these tissues.
2. Mowry's (48) modification of the Alcian blue technique and the periodic acid-Schiff (49) technique were usually performed

in combination for the morphological demonstration of the intracellular inclusion bodies. The techniques used in these procedures are listed below in sequence.

A. Alcian blue.

1. Treat sections for 2 hours in 1% Alcian blue 8 G X in 3% acetic acid.
2. Treat with 3% acetic acid for 5 minutes.
3. Rinse in tap water for 5 minutes.
4. Treat for 30 minutes in 0.3% sodium carbonate.
5. Rinse in distilled water.

B. Periodic acid-Schiff (49).

1. Treat hydrated sections with 0.5% periodic acid for 5 minutes.
2. Wash in glass distilled water.
3. Treat with Schiff's reagent for 15 minutes.
4. Rinse with 3 changes of fresh sulfurous acid (6 ml 10% $Na_2S_2O_5$ (sodium metabisulfite) and 5 ml 1 N hydrochloric acid made up to 100 ml with distilled water)-2 minutes per change.
5. Wash in running tap water for 10-15 minutes.
6. Briefly counterstain in Harris hemotoxylin (optional).
7. Wash in running tap water for 10-15 minutes.
8. Dehydrate and mount sections.

   For controls, (1) hydrolysis in periodic acid was eliminated, and (2) sections were incubated in salivary amylase.

ENZYME STUDIES

Gluteraldehyde fixed tissues were frozen by quenching in isopentane cooled to -165°C by liquid nitrogen and mounted in ice. These tissues were sectioned at 5 microns in an International cryostat regulated at -18° to -20°C. The sections were mounted on coverslips and allowed to dry at room temperature. To prevent loss of tissue sections during the subsequent incubation, the coverslips were previously coated with a film of gelatin which was produced by drying 1 or 2 drops of a 1.0% gelatin solution on the glass. Unfixed frozen sections were sectioned in a similar manner, but were mounted without the aid of an adhesive film.

After air drying, the mounted sections were incubated on the coverslips according to the techniques described below. When the incubation procedures were completed, the sections were washed in distilled water and mounted in glycerol gel.

A. Gluteraldehyde fixed frozen tissues sections were incubated for alkaline phosphatase (after Burstone) according to the following procedure (50).

   1. 10 ml stock substrate solution (0.1 mg naphthol AS-TR phosphate*/1 ml 0.05 M Tris buffer adjusted to pH 8.4).
   2. 10 mg Fast blue RR salt.
   3. 2 drops 0.5 M magnesium chloride (activator).

   *in the control medium the substrate was omitted.

B. Wash and mount sections.

2. Sections incubated for acid phosphatase were treated according to the procedure outlined below (51):

   A. Incubate at 37°C for 15 minutes in the following incubating medium.

      1. 4 to 5 mg naphthol AS-BI phosphate* dissolved in 0.25 ml N,N-dimethylformamide.
      2. 10 ml 0.2 M acetate buffer (pH 5.2).
      3. 10-15 mg Garnett GBC salt.
      4. Add 2 drops 10% manganese chloride (activator).

      *the substrate was omitted in the control medium.

   B. Wash and mount sections.

3. Gluteraldehyde fixed frozen sections were incubated for the presence of non-specific esterase according to Pearse's (52) modification of the Gomori technique.

   A. The sections were incubated for 15 minutes at room temperature in the following incubation medium:

      1. 9.9 ml 0.2 M phosphate buffer at pH 6.8.
      2. 0.1 ml propylene glycol.
      3. 15 mg Garnett GBC salt.
      4. 0.1 ml acetone containing the dissolved substrate, naphthol AS-D acetate* (concentration: 4 mg/ml).

      *in the control sections the substrate was omitted.

   B. Wash and mount sections.

4. Both unfixed and gluteraldehyde fixed frozen sections were incubated for leucine aminopeptidase according to the method of Nachlas, Crawford and Seligman (53).

A. These sections were incubated at 37°C for 25-30 minutes in an incubating medium containing the following materials:

1. 0.5 ml stock substrate solution (L-leucyl-4-methoxy-2-naphthylamide* at a concentration of 4 mg/ml.
2. 5 ml 0.1 M acetate buffer (pH 6.5).
3. 4 ml sodium chloride (0.85%).
4. 0.5 ml 0.02 M potassium cyanide.
5. 5 mg Fast blue B salt.

*in the control medium the substrate was omitted.

B. Rinse sections in 0.85% saline for 2 minutes.

C. Treat with 0.1 M cupric sulfate for 2-5 minutes.

D. Rinse in saline.

E. Fix unfixed sections in 10% formalin or 3% gluteraldehyde fixative.

F. Wash and mount sections.

OTHER STUDIES

In addition to the procedures described above, the following procedures were performed on a limited basis on a small number of animals to provide data for comparison with information previously obtained from the human fetus.

1. The dihydroxy-dinaphthyl-disulphide (DDD) reaction for sulfhydryl (SH) groups was performed on formalin fixed paraffin-embedded tissues according to the procedure of Barnett and Seligmann (54) listed below.

A. Deparaffinize and dehydrate sections.

B. Incubate sections for 1 hour at 50°C in a solution containing 35 ml 0.1 M veronal acetate buffer (pH 8.5)

and 15 ml absolute ethanol in which has been dissolved 25 mg of the DDD reagent.

C. Cool to room temperature.

D. Rinse briefly in distilled water.

E. Wash for 10 minutes in two changes of distilled water acidified to pH 4-4.5 with acetic acid.

F. Extract the free naphthol by passage through a graded series of alcohols and wash twice in absolute ether (5 minutes each).

G. Rinse in distilled water.

H. Stain for 2 minutes at room temperature in a freshly prepared solution of 50 mg Fast blue B salt in 50 ml 0.1 M phosphate buffer at pH 7.4.

I. Wash in running tap water.

J. Dehydrate and mount in Permount.

2. The alloxan-Schiff method for protein bound amino groups was performed on paraffin embedded formalin fixed tissues according to the method of Yasuma and Ichakawa (55) as listed in the following:

A. Deparaffinize and hydrate sections.

B. Treat sections in 1.0% alloxan in absolute ethanol for 16-20 hours at 37°C*.

C. Wash gently in running water for 2-5 minutes.

D. Treat section with Schiff's reagent for 15 minutes.

E. Wash in running water for 10 minutes.

F. Dehydrate, clear and mount in Permount.

*Control sections - eliminate step in alloxan.

3. The Nile blue sulphate method for lipofuscins was performed on formalin fixed paraffin embedded sections according to the procedure of Lillie (56).

   A. Bring sections to water.

   B. Stain sections for 20 minutes in 0.05% Nile blue A in 1.0% sulfuric acid.

   C. Wash in running water for 10-20 minutes.

   D. Mount in glycerol gel.

4. The Ziehl-Neelsen method for acid fast lipofuscins was performed on formalin fixed paraffin-embedded sections (57).

   A. Bring sections to water.

   B. Stain in the carbol fuchsin solution for 3 hours at 60°C. This solution was made up as following:

Basic fuchsin	1 gm.
Phenol	5 gm.
Alcohol	10 ml.
Distilled water	100 ml.

   C. Wash in running water.

   D. Differentiate in 1.0% acid alcohol until cells are just faint pink.

   E. Counterstain lightly with 0.5% toluidine blue.

   F. Wash in running water.

   G. Dehydrate, clear, and mount in the routine manner.

5. Unfixed frozen cryostat sections mounted on coverslips were incubated for succinic dehydrogenase using nitro blue tetrazolium according to the procedure of Nachlas et al. (57).

   A. Incubate sections for 20-25 minutes in an incubating medium consisting of 0.1 M sodium succinate* and 0.1%

nitro blue tetrazolium buffered to pH 7.6 with 0.1 M phosphate buffer.

B. Wash sections in distilled water.

C. Fix sections in buffered 10% formalin.

D. Wash sections.

E. Mount in glycerol gel.

*in control media the substrate was omitted.

6. The Feulgen reaction was utilized to determine whether nucleic acids were contained in the inclusion bodies (47) according to the following procedure.

A. Deparaffinize formalin fixed paraffin embedded tissues and bring to water.

B. Rinse sections in cold 1 N hydrochloric acid for 1 minute.

C. Hydrolyze in 1 N hydrochloric acid at 60°C for 8 minutes*.

D. Rinse in cold 1 N hydrochloric acid and follow with rinse in distilled water.

E. Treat with Schiff's reagent for 15-30 minutes.

F. Rinse in freshly prepared bisulphite solution (6 ml 10% sodium metabisulfite 5 ml 1 N hydrochloric acid and add water to 100 ml).

G. Wash in running water for 15 minutes.

H. Dehydrate, clear and mount in Permount.

*controls: eliminate hydrolysis in heated acid.

FETAL ABSORPTION

In an attempt to demonstrate absorption of materials from the amniotic fluid by the fetal intestinal epithelium, a colloidal iron

preparation ("Co-Ferrin"), was injected into the amniotic cavity of fetal rats. In order to provide a morphological demonstration of any possible absorption of this material by the intestinal cell, sections of intestinal tissue taken from these animals were later treated with the Prussian blue reaction for the demonstration of iron. It was assumed that this material could gain entrance into the intestine by swallowing amniotic fluid.

In this procedure, female animals carrying litters timed to be of 19 and 20 days gestation were anesthetized with ethyl ether and, using semi-sterile techniques, the abdomen was opened by a midline incision. One of the uterine horns was brought into view and the amniotic cavities of selected fetuses were injected with approximately 0.3 to 0.5 cc of a 50:50 mixture of colloidal iron and 0.9% saline. In an attempt to reduce a build-up of pressure when this fluid was introduced into the amniotic sac, quantities of amniotic fluid were withdrawn prior to the injection of the iron-containing fluid.

At the conclusion of this procedure, the abdomen was closed and the development of the fetuses was allowed to proceed up to the 23rd day of gestation. At this stage of development, the fetuses were collected and the intestinal tissues were fixed in formalin. This was followed by routine paraffin embedding as described above and, after sectioning, these tissues were treated with Lison and Bunting's modification of Perl's Prussian blue reaction (57) for the demonstration of iron according to the following procedure:

1. Deparaffinize and bring mounted sections to water.
2. Treat sections with a fresh mixture of equal parts of 2.0% potassium ferrocyanide and 2.0% hydrochloric acid. Instead

of using the recommended 30 minutes or longer period of incubation, treatment for 2-5 minutes proved to be more satisfactory.

3. Wash in distilled water.

4. Dehydrate and mount in Permount.

During the course of this study, all sections were studied by light microscopy. Photographs were taken on High Contrast Copy film using a Series 10 AO Microstar compound microscope with 35 mm camera attachment. The photographic film was developed in Microdol X and prints made at various magnifications on paper which gave the best contrast.

CHAPTER III

OBSERVATIONS

To facilitate the description of results this section is divided into 4 parts: (A) Fetal, (B) Postnatal, (C) Weanling and Adult, and (D) Other Procedures performed on selected animals.

During the course of this study, most emphasis was placed on the investigation of the inclusion bodies located within the columnar absorptive cells of the intestinal epithelium. However, in order to provide an aid to the understanding of the level of tissue development attained at the particular stage under investigation, a description of the morphology of the intestinal tissue seen at these respective stages is given in the first 3 sections.

In regard to the description of the results obtained with the enzyme techniques, the levels of enzyme activity demonstrated by these procedures were grouped into 3 broad divisions; strong, moderate, and weak. Activity was classified as strong when the dye deposits were very heavy and dense or when bright color was demonstrated. When the density of the dye deposits was lower or if the coloration obtained was dull, the enzyme activity was classified as moderate. In situations where the dye deposits were sparse or if the color of the final reaction product was pale, this activity was placed in the weak classification.

A. FETAL ANIMALS

1. MORPHOLOGICAL OBSERVATIONS

On gross examination, the diameter of the proximal small intestine was greater than that of the distal portions of the small intestine which was smaller and more constricted in appearance. In the earliest fetal stages studied, the intestinal loops were herniated into the unbilical cord. As growth progressed in the subsequent days of development, the body cavity became much larger providing room for the intestinal tract which returned into the body by 18 days gestation. At the same time, numerous coils developed in the intestinal tract.

Microscopic examination of this tissue showed that the intestine of the developing fetus consisted of 2 elements, (a) a hollow epithelial tube surrounded by (b) a wall of mesenchyme or primitive connective tissue (Figures 1 and 2). In the fetus, the most prominent structures to develop in the intestinal mucosa were the villi. These structures, along with epithelial differentiation and the establishment of intraepithelial enzyme activity, were first formed in the cephalic portion of the intestine at 18 and 19 days gestation. During this period, processes of differentiation were delayed in the distal portions of the small intestine by 1 or 2 days.

The intestinal wall of the 16 and 17 days old fetuses consisted of a relatively thick layer of mesenchyme supporting the epithelial tube. On the histological level, 2 distinct morphological zones were seen which consisted of (a) a subepithelial zone in which the majority of the spindle-shaped mesenchymal cells were arranged around the epithelial tube in a circular manner; and (b) an outer zone consisting of scattered elements of mesenchyme (Figure 1). At the periphery of

the outer zone and near the presumptive serosal border of the intestinal wall, a distinct circular band of smooth muscle 3 to 4 cells in thickness was observed in transverse sections (Figures 1 and 2).

The intestinal epithelium of the 16 and 17 day old fetuses consisted of a smooth, thick layer of stratified cells surrounding a narrow lumen which varied in shape from circular to a narrow slit. Cells containing mitotic figures were numerous throughout the epithelium during these stages of development. At 16 days gestation, the epithelium was relatively thin, composed of 4 to 6 layers of stratified cells containing oval nuclei arranged at right angles to the basement membrane, similar to stratified or pseudostratified epithelium (Figure 1). In the 17 day old fetus, the epithelium increased in thickness to 6 and 8 or more layers of cells in certain sections. In the latter, thickened plates of cells, clefts, folds and upheavals of the epithelial layer were observed in the cephalic portion of the intestinal tube. In Figure 2 thickened epithelial plates and clefts in the epithelium are illustrated.

In the 18 day old fetus, the diameter of the cephalic intestine increased considerably and for the first time, the intestinal wall was relatively thin in relation to the enlarged lumen (Figure 3). In the cephalic segments, thick, short villi were formed which were covered by epithelium which was reduced in thickness consisting of a layer of cells varying from a single layer of short columnar cells (Figure 4) to 2 to 4 layers of transitional cells. At the same time, mitotic cells were confined to the presumptive crypt areas. Furthermore, a number of small mucinogen containing goblet cells were observed in the process of development in the epithelium of the proximal intestine (Figure 5).

The distal segments of the small intestine were small in diameter with a stratified epithelium showing folds and furrows (Figure 6). In other sections, other formations including intraepithelial (extracellular) "blisters" or sinuses and small finger-like processes of epithelial cells protruding into the intestinal lumen were seen (Figure 7).

At 19 days gestation, the villi were more numerous and increased in length assuming a characteristic finger-like shape. At the same time, the villus epithelium consisted of a single layer of short columnar cells with a centrally placed nucleus. In the basal regions between the villi, patches of stratified epithelium containing mitotic cells remained. At this stage, small PAS-positive droplets appeared in the cytoplasm above and below the nuclei of some of the columnar cells and occasionally, in the connective tissue core of the villi (Figure 8). A folded, stratified epithelium still remained in the distal portions of the small intestine at 19 days.

In the 20 day fetus, transition to the simple columnar epithelium was completed along the entire length of the small intestine with the exception of a small number of "stacked" or stratified cells in the crypt areas. The villi continued in their growth and development during the remainder of the intrauterine period increasing both in size and number. By 22 days gestation, the villi had developed into long delicate structures projecting into an enlarged lumen. The intestinal wall, on the other hand, was relatively thin on the final day of fetal development containing very shallow crypts lined by stratified cells many of which were undergoing mitosis.

The number of PAS-positive droplets observed within the cells became greater during the last 3 days of fetal life. By 22 days

gestation, they were represented by clusters of small droplets in the Golgi zones of every cell of the villus epithelium (Figure 9). In addition, clear vacuoles of various sizes were observed in the apical cytoplasm of the columnar cells in close association with the PAS-positive droplets (Figure 9). These vacuoles were also well demonstrated with the hemotoxylin and eosin stain as illustrated in Figure 10.

Dense material positive to both the PAS and Alcian blue reactions was observed concentrated in the lumen of the distal portion of the intestine (Figure 11).

2. ENZYME STUDY

(a) Alkaline Phosphatase

Alkaline phosphatase activity, localized by blue-black precipitates of the Fast blue RR salt, was clearly demonstrated in the luminal border of the villus epithelium of the 18 day old fetus (Figure 12). Zones of this activity were extended along the villus epithelium and reached nearly into the bottom of the presumptive crypt areas. At the same time, scattered deposits of the localizing dye were found in the cytoplasm of the columnar cells which surrounds the nucleus (Figure 12).

In the 19 and 20 day old fetuses, alkaline phosphatase activity in the cuticular border of the villus epithelium increased to moderate levels which were maintained during the remainder of the gestation period.

An increase in the alkaline phosphatase activity was observed in the apical cytoplasm of the columnar villus epithelial cells in the 22 day old fetus (Figure 13). This activity was demonstrated by scattered deposits of localizing dye crystals concentrated in the

cytoplasmic areas between the cuticular border and supranuclear Golgi apparatus. In sections where the previously described "clear vacuoles" were present, the activities of the enzyme appeared with precipitates of the dye concentrated in the same general vicinity of these structures (Figure 14).

(b) Acid Phosphatase

At 18 days gestation, activity for acid phosphatase, localized by red-brown precipitates of the Garnett GBC dye, was weakly concentrated in the luminal border of the villus epithelium of the proximal intestine where cells had differentiated to the columnar type (Figure 15). At the same time, weakly stained crystals were widely scattered throughout the remainder of the cytoplasm.

In the 19 and 20 day old fetuses, the level of activity of acid phosphatase in the cuticular border was greater, becoming moderately strong along the entire length of the small intestine. Moderate activity in the cuticular border was maintained up through the remainder of the intrauterine period of development (Figure 16). Beginning with 20 days gestation, a weak zone of activity appeared in the supranuclear Golgi zone of the columnar cells and remained up through 22 days gestation (Figure 16).

Similar to the picture obtained in the distribution of alkaline phosphatase, acid phosphatase activity increased in the supranuclear cytoplasmic zone of the columnar cells in sections where clear vacuoles were in evidence (Figure 17).

(c) Non-specific Esterase

Non-specific esterase activity was first observed in the intestinal epithelium at 17 days gestation. This activity, which was

demonstrated by scattered granules or deposits of the localizing dye (Garnett GBC) in the cytoplasm of the stratified epithelium, was most prevalent in the basal portions of the cells bordering upon the basement membrane (Figure 18). Scattered sites of enzyme activity became more pronounced at 18 days gestation and remained concentrated in the basal cytoplasm bordering upon the basement membrane of the intestinal epithelium up to 20 days gestation. At this time, these sites disappeared from the cells of the villus epithelium and another zone of activity, more intense than the former, appeared in the supranuclear Golgi zone remaining there up to the time of birth (Figure 19). In the cells of the crypt epithelium, activity as seen by scattered precipitate, remained in the basal cytoplasm during the remainder of the intrauterine period of development.

At 22 days of gestation, non-specific esterase activity increased in the apical cytoplasm of the columnar cells in areas comparable to the increases in phosphatase activity noted previously (Figure 20).

(d) Aminopeptidase Activity

Throughout the developmental period, sections of fixed and unfixed tissue were compared for levels of aminopeptidase activity. In fixed tissue, the enzyme activity was usually low in intensity but was relatively well localized whereas in unfixed tissue, activity was relatively high with poorer localization.

In sections of unfixed tissue, aminopeptidase activity was first demonstrated in the intestinal epithelium at 18 days gestation. At the onset, this activity was very weak and diffuse in which no clear pattern of enzyme localization was seen in the epithelial cells. In the 19 day old fetus, however, aminopeptidase activity increased

becoming relatively strong in unfixed tissue. In unfixed tissue, localization was indistinct with the greatest amount of activity in the apical cytoplasm of the columnar cells of the villus epithelium. Furthermore, sites of enzyme activity appearin the cytoplasm beneath the nucleus and on occasion, in the nucleus itself (Figure 21). In fixed tissue, where the intensity of the enzyme activity was reduced, the localization of this activity was more clearly localized in the cuticular border with some diffusion into the subjacent cytoplasm of the columnar cells (Figure 22). In the fetus, aminopeptidase was relatively evenly distributed in the villus epithelium from apex to base (Figure 22).

3. FETAL ABSORPTIVE STUDY

In the fetal absorption study, the absorption of iron was successfully demonstrated in fetuses of 2 litters in which this material had been deposited within the amniotic cavity.

With the Prussian blue reaction, the absorbed iron was demonstrated in small droplets or granules in the supranuclear area of the columnar cells (Figure 23). In addition, diffuse staining of the cytoplasm within this reaction was common in many of the cells which had absorbed iron. In control animals which did not receive the injections of iron, no iron reaction was demonstrated.

The animals which demonstrated the absorption of iron from the amniotic fluid received intra-amniotic injections at 19 and 20 days gestation. Those injected at 19 days were sacrificed shortly after delivery while the animals receiving the iron at 20 days were collected at 22 days gestation.

4. DISCUSSION

On the basis of previous studies performed in this area, it seems likely that the formation of epithelial clefts, folds, and thickened plates of cells seen in the intestinal tract of the rat fetus were involved in the early stages of the formation of villi. The exact mode of villus development was not clearly elucidated during the course of the present study but Behnke (26) showed that in the duodenum of the fetal rat, villi were preceded by the growth of buds of mesenchyme which originated from the intestinal wall. He observed that an increase in the length of these buds beneath the epithelium produced upheavals which protruded into the intestinal lumen (26). When the buds reached a certain height, Behnke (26) further observed that clefts developed between the presumptive villi breaking the epithelial surface up into "blocks." It is conceivable therefore, that the epithelial clefts seen in the 17 day old fetuses of the present study were associated with processes similar to those observed by Behnke.

In the light of observations made on the development of the intestinal villi in the human fetus published by Johnson (59) in 1910, it is probable that some of the other epithelial formations seen during the onset of the period of villus development were also involved in processes of villus formation. For example, Johnson (59) observed that the formation of large sinuses or cavities in the epithelium preceded development of the villi in the duodenum. In the present study, similar formations were seen in sections of the intestine of the 18 day old fetus.

Similarly, Johnson (59) observed that villus formation in the jejunum of the human was preceded by separated thickenings of the

epithelium which increased in length forming long buds of cellular processes. This compares favorably with finger-like processes composed of epithelial cells seen in the middle segments of the intestine of the rat at 18 days gestation.

In the ileum, on the other hand, villus formation was preceded by the development of longitudinal epithelial folds, similar to observations made in the distal intestine of the 18 and 19 day old fetuses. According to Johnson (59), these folds later broke up into villus-like processes.

Similar to the situation seen concerning the mode of villus formation, different interpretations have arisen concerning the origin of the intestinal folds. In contrast to Behnke's (26) interpretation, Johnson (59) suggested that in the human, the folds in the epithelium were produced by rapid growth and expansion of the epithelial layer causing the epithelium to buckle and produce upheavals which protruded into the intestinal lumen. In addition, Coulombre and Coulombre (60), who studied villus formation in the developing chick embryo, asserted that contraction of the intestinal musculature played an important role in throwing the intestinal epithelium into a series of longitudinal folds which in turn, developed into villi. Whether these observations represented a manifestation of species differences is difficult to determine but in the developing rat, small mesenchymal buds were seen in the earlier stages similar to Behnke's observations (26) whereas in the 18 and 19 day old fetuses, folds similar to the observations of Johnson (59) were observed in the distal segments of the intestine.

While this information may be inconclusive, the structural formations seen during the course of the present study do seem to

indicate that other processes of villus formation in addition to the process described by Behnke are active in the intestine of the fetal rat. Behnke (26) limited his studies to the duodenum of the earlier fetal stages and this fact may provide an explanation for his failure to observe other forms of villus development.

Similar to observations made during the course of the present study, Johnson (59) described simple columnar epithelium on the villi as these structures were formed. As pointed out in the introduction, Behnke (26) postulated that transition of the epithelium from the stratified to the columnar type during the process of intestinal remodeling was facilitated by functions of cell death and degeneration. The sites of "cell death," which has been shown to be an important process which occurs during organ formation in the fetal organism (27), were presumably in cytolysomes or "autophagocytic" bodies which in light microscope sections were manifested by small basophilic, PAS-positive, acid-phosphatase containing inclusion bodies within the cells (26).

While it is assumed that similar processes occurred in the course of epithelial remodeling in the tissue observed in the present study, no bodies similar to those Behnke (26) described were seen in the intestinal epithelium of the 17 day old fetus. In his studies, Behnke (26) used Vestopal W embedded sections for light microscopy which conceivably may have allowed better resolution of the tissue components than the routine paraffin embedded and frozen sections used during the course of the present study. In the latter, only widely scattered crystals of the localizing dye were seen in the intestinal tissue following incubation for acid phosphatase in the 16 and 17 day old

fetuses. Because no pattern of localization was established with this technique until 18 days gestation, it was assumed that in the earlier stages the dye precipitates were the result of background staining artifact.

By circumstance, it seems that the PAS-positive droplets seen in the intestinal epithelium of the last 3 days of fetal development were associated with processes of absorption of materials from the intestinal lumen. These structures, which were similar to those described by Owman (23) in the rat and by others in the prenatal guinea pig (17), dog (2), and calf (12) were observed to accumulate with the progress of development, increasing both in number and in size. In addition, it has been shown that the fetuses of several animals including the guinea pig (18), rat (24,61), and sheep (19) are capable of absorbing amniotic fluid through the intestine as demonstrated by the uptake of antibodies when introduced into the amniotic cavity.

In the present study, the fact that iron was demonstrated in the supranuclear cytoplasm of the columnar absorptive cells of the rat fetus following absorption from the amniotic cavity provides morphological evidence for these absorptive processes. In order to obtain more conclusive evidence that the PAS-positive droplets contained absorbed materials, it would have been wise to subject Prussian blue treated sections to the PAS-reaction to provide simultaneous demonstration of both the iron-containing droplets or granules and the PAS-positive droplets. The fact that the granules of iron were concentrated in the supranuclear Golgi zones of the columnar cells similar to the distribution of the PAS-positive droplets produces

circumstantial evidence, at least, that the PAS-positive droplets were associated with processes of absorption from the intestinal lumen.

During the course of the fetal absorption studies, death occurred in a high percentage of the fetuses which had received the intra-amniotic injections. In doing preliminary work in order to determine a suitable medium to demonstrate absorption, the use of colloidal iron at full strength and the use of thorotrast at any dilution ranging from 1/4 to full strength resulted in the death of all the treated fetuses. Up to the present time, the most satisfactory results were obtained with the use of colloidal iron diluted to 1/2 strength with 0.85% saline. Even at this dilution, at least 1 of the fetuses died in every litter in which injections had been made.

Since fatalities were common among the fetuses which had received intra-amniotic injections, it becomes apparent that these injected materials or the procedure of injection had a harmful effect. What these effects were, were not determined but they could have involved a variety of factors which may have included: (a) an upset in the osmotic pressure of the amniotic fluid; (b) too great an increase in the fluid pressure of the amniotic fluid; (c) separation of the placenta from the uterine wall due to mechanical trauma of handling the uterine horns; and (d) a possible toxic effect of the injected iron material.

It was interesting to observe that when the surgical procedures were performed on pregnant animals carrying litters of 20 days gestation, a tendancy to deliver the fetuses followed within a few hours. This did not occur at 19 days gestation. Again, this may have been produced by a variety of causes. These may include: (a) death of the fetuses; (b) increases in the intrauterine pressure; (c) by

stimulation of the uterine musculature due to handling and manipulation of the uterus; or (d) other causes.

Some difficulties arose in an attempt to interpret the significances of some of the enzyme reactions obtained in the fetal animals. For example, when evaluating the scattered esterase active "granules" seen in the 17 day fetus, one problem to consider was the possibility of staining artifact. However, artifact did not seem to be indicated since the sites of activity remained consistently in the basal portions of the epithelium until they were replaced by activity in the supranuclear cytoplasm of the columnar absorptive cells. Therefore, an interpretation that the localization of the non-specific esterase reaction represented an actual concentration of enzyme in this area is supported. It is not known whether these reaction sites were the result of weak and diffuse enzymatic activity in the cytoplasm or if they were a representation of inclusion bodies or granules of activity located in this portion of the cells. At any rate, Moe and Behnke (25) identified lysosome-like particles in the crypt epithelium of newborn rats which may lead to the suggestion that the esterases, which were localized in the crypt epithelium during the last portion of the fetal period, may be located in small lysosomes. However, this hypothesis is not entirely acceptable since no histochemical reaction for acid phosphatase was obtained failing to meet the requirements of lysosomes proposed by Novikoff (42).

The increases in the activities of the various enzymes localized in the cuticular border of the intestinal epithelium during the last portion of the fetal developmental period is similar to patterns which have been established previously for alkaline phosphatase and non-specific

esterase. The activity of alkaline phosphatase, which has been most extensively studied, was observed to increase rapidly in the intestinal epithelium during the last few days in the fetal mouse (62) and guinea pig (17) and in the chick embryo before hatching (63). In the chick embryo, a similar increase in non-specific esterase activity was observed in the intestinal epithelium following 17 days incubation (64).

According to Moog (63), the increase in the activity of alkaline phosphatase is correlated with the development of the striated border of the columnar cells, a finding which appears to be supported by the present study. For example, none of the enzymes normally localized in the cuticular border (i.e. acid and alkaline phosphatase and amino-peptidase) demonstrated activity in the epithelium until 18 and 19 days gestation when differentiation of the epithelium into columnar cells was completed in certain segments of the gut. It is assumed that the primitive microvilli were formed or developed as columnar cells were formed since a "bilaminar" cuticular border was seen in the luminal border of these cells by 19 and 20 days gestation.

Since increases in enzyme activities were demonstrated shortly before birth, it appears that the intestine was preparing itself for the functional processes of digestion and absorption required of it in the postnatal period of existance. As a matter of fact, the functional ability of the alimentary tract has been shown to develop in the rat relatively early in its developmental history. Hartmann and Wells (65) have shown that in implanting various food materials in the stomach of 20-22 day old fetal rats, the fetuses had the ability to digest such foods as milk and starch. The ability to digest meat, on the other

hand, was poorly developed or absent. This would be most logical since the first materials required to be digested by the intestine are easily digestible materials contained in the milk while solid materials are not normally ingested until near the age of weaning.

It is not known whether the concentrated material seen in the lumen of the distal portion of the small intestine consisted of intestinal secretions or if it represented a mixture of materials derived from the amniotic fluid and intestinal secretions. Speculation would lead to the assumption that the latter was more likely. It is apparent that this material contained both acid and neutral mucopolysaccharides due to its coloration with both the Alcian blue and the PAS techniques indicating a contribution by intestinal mucin. The presence of this material is interesting since Patzelt (3) indicated that intestinal secretions and other material do not collect in the intestinal lumen of the fetal animals as in the human.

B. NEWBORN AND SUCKLING ANIMALS

1. MORPHOLOGICAL OBSERVATIONS

In the newborn, the intestinal morphology was very similar to that presented by the fetal animals at 22 days gestation. Following birth, a rapid growth of the intestinal tissue occurred as shown by an increase in the length of the small intestine from approximately 12 cm. (formalin fixed) in the newborn up to about 46 cm. at 15 days. During the first half of the suckling period, the diameter of the duodenum was larger than the remainder of the small intestine on gross inspection. On the histological level, this portion of the intestine contained a thicker wall and a larger lumen than the remainder of the small intestine.

In these animals, the columnar cells of the villus epithelium were observed to increase in length following birth assuming the characteristic long, columnar shape with basally placed nuclei by 2 to 3 days of age. In the proximal intestine, the thickness of the intestinal wall increased gradually. This was followed by an initiation of growth of the crypts by 3 to 4 days of age. In the distal segments, deeper crypts were observed beginning with 6 to 8 days. When compared with the columnar cells of the villus, the crypt epithelium consisted of shorter basophilic cells.

When studying tissues of animals from this period, particular attention was paid to the supranuclear inclusion bodies contained within the columnar cells of the villus epithelium. These structures, which were PAS-positive and negative to the Alcian blue reaction, were observed during the period ranging from 1 to approximately 20 days of age (range: 19-21 days). The results of the other histochemical tests performed during the course of this study are shown in Table 2 and are described in the following sections.

The first day of the neonatal period appeared to be associated with processes involved in the formation of inclusion bodies. In the unsuckled newborn animal, the pattern presented by these bodies remained very similar to the animals at 22 days gestation (Figure 24). During the first few hours following birth, the number of PAS-positive droplets gradually increased in the apical cytoplasm of the columnar absorptive cells in the distal segments of the intestine. The accumulation of these droplets was most pronounced in the ileum where coalescence of these structures was seen forming small circular inclusion bodies by 12 hours (Figure 25). The clear vacuoles observed

TABLE 2. TABLE OF RESULTS

Histochemical Test	Reaction in Inclusion Bodies	Other Positive Cytoplasmic Structures
1. Acid phosphatase	positive	cuticular border, apical cyto.
2. Alkaline phosphatase	"	" " " "
3. Non-specific esterase	"	apical cytoplasm
4. Aminopeptidase	negative	cuticular border
5. Succinic dehydrogenase	"	cytoplasmic mitochondria
6. PAS	positive	----------
7. Alcian blue	negative	goblet cell mucinogen
8. DDD reaction (SH groups)	positive (large bodies)	
9. Alloxan-Schiff (NH_2)	slightly positive	
10. Nile blue	slight (large bodies)	
11. Acid Fast lipofuscins	positive (large bodies)	
12. Feulgen	negative	cell nuclei

in the supranuclear cytoplasm became less prominent after birth as the apical portion of these cells were gradually filled with the PAS-positive droplets. These vacuoles did not disappear completely, however, since clear spaces were seen in the columnar cell cytoplasm which surrounds the inclusion bodies in tissue obtained from animals sacrificed throughout the entire suckling period. This was most evident in the portion of the cell immediately superior to these structures.

In the 1 day old animal, the amount of PAS-positive material had increased considerably, filling the apical cytoplasm of the villus epithelium of the ileum. In the jejunum the accumulation of this material was on a lower scale in which it was concentrated in the form of smaller droplets restricted to the supranuclear Golgi zone. In the duodenum, where no change was observed following birth, only occasional small PAS-positive droplets were observed in the apical cytoplasm of the villus epithelium.

During the 2nd and 3rd days after birth, the amount of PAS-positive material located in the supranuclear area increased in density producing the characteristic inclusion bodies (Figure 26). These structures were present during the remainder of the postnatal period up to the age of weaning when regression occurred.

In the intestinal tissues of all animals studied from the suckling stage, the following picture concerning the distribution of the inclusion bodies was observed: none or very few were seen in the duodenum; small bodies (2-6 microns in diameter) were found in the jejunum; and the largest (10-30 microns in diameter) were contained in the ileum. In an attempt to obtain a more precise picture concerning the distribution of these bodies along the length of the small

intestine, the small intestines from a series of newborn, 3, 8, and 15 day old animals (2 per age group) were divided into 10 equal lengths. Representative sections were removed from each of these segments and examined for the presence of inclusion bodies within the villus epithelium. In taking sections in descending order from the duodenum to ileum, occasional small PAS-positive droplets were seen in the apical cytoplasm of the villus epithelial cells of the duodenum and proximal jejunum (Figure 27). In the middle sections of the small intestine, small droplets 2-4 microns in diameter became more numerous and were consistently encountered in the supranuclear Golgi zone of every columnar absorptive cell (Figure 28). Distal to this point, the inclusion bodies gradually became larger reaching the largest dimensions in the distal half of the ileum (Figure 26). In the most terminal end of the ileum, however, these bodies were again reduced in size. In regards to the distribution of the inclusion bodies on each villus, the largest were found in the cells located on the luminal end and were reduced in size becoming progressively smaller in the cells located on the lateral sides of the villi (Figure 29). No inclusion bodies were observed in the crypt epithelium.

No change in the relative distribution of the inclusion bodies was observed when tissues taken from the 3, 8, and 15 day old animals were compared.

In microscopic sections, the inclusion bodies were seen as large globules with a smooth oval outline which, in the terminal ileum, assumed enormous proportions which served to displace the apical cytoplasm to the periphery of the cells. As a result, the nuclei of the columnar epithelium, which were often flattened by this pressure, were

displaced deep into the basal pole of these cells (Figure 30). During the period of time ranging from approximately 3 to 10 days of age, these globules were the largest in size when structures with dimensions measuring up to 15 by 30 microns were seen. During the remainder of the suckling period, the inclusion bodies were slightly smaller in size with dimensions of 12 by 15 microns. The presence of the large inclusion bodies within the cytoplasm of the columnar cells of the villus epithelium of the ileum caused these cells to increase considerably in size creating a swollen or "ballooned" appearance (Figure 30).

Some differences in the morphological detail of the inclusion bodies were obtained when sections of tissue fixed in formalin and in gluteraldehyde were compared. With formalin fixation, shrinkage or fixation artifact in many of the large globules occurred. This was manifested by vacuolization, shrinkage, and disintegration of the intravesicular material, particularly in the larger bodies. In many instances the intravesicular material seemed to be reduced in volume by 1/3 or 1/2 leaving many "empty" and clear spaces (Figure 30). In the smaller structures located in more proximal portions of the ileum, shrinkage was not as extreme (Figure 26). Instead, the apical cytoplasm and intracellular material of the columnar cells contained numerous small vacuoles giving a "foamy" appearance. In formalin fixed tissues, these bodies reacted strongly to the PAS technique and were uncolored with Alcian blue. The goblet cells, on the other hand, stained with both of these reactions and when the tissue was treated with PAS and followed with Alcian blue as a counterstain, the mucinogen was colored deep blue-violet. With hematoxylin and eosin, the globules were moderately eosinophilic with the exception of the largest structures

in which a strong affinity for eosin was observed (Figure 30).

On the microscopic level, sections of tissue fixed with gluteraldehyde appeared to be more dense than formalin fixed tissue. This served to reduce the optical contrast of many of the cytoplasmic structures and tissue components during observation. Vacuoles seen in the apical cytoplasm were restricted to the area immediately superior to the inclusion body (Figure 31). Shrinkage of the material contained within the inclusion bodies was less than the picture obtained in formalin fixation but large clear spaces were still seen occasionally in the apical and basal ends of the larger globules of the ileum (Figure 32).

In regard to the morphological detail of the inclusion bodies seen in tissues preserved in gluteraldehyde, the intravesicular contents of these globules consisted of structureless, artificial-appearing material which completely filled the inclusion bodies with the exception of the "clear spaces" described above.

In reference to the tissue staining obtained in gluteraldehyde fixed tissue, the inclusion bodies, columnar cell cytoplasm, and the remainder of the tissue components were heavily stained with eosin which served to interfere with the differentiation of these structures with the hemotoxylin and eosin stain. With the PAS reaction, the inclusion bodies were colored very intensely. After tissue sections were treated with this procedure, the cytoplasm of the cells and the rest of the tissue were also stained with a Schiff-positive background stain. The background reaction in these tissues was not affected by either prior digestion of the tissue sections with salivary amylase or the elimination of the oxidation step with periodic acid in the

performance of this reaction. With the Alcian blue reaction, the material located within the globules was uncolored but, at the same time, the membranes enclosing these structures were weakly positive, creating a weakly positive Alcian blue colored rim around the globules (Figure 33). As with formalin fixed tissues, goblet cell mucinogen was strongly positive to both PAS and Alcian blue.

In addition to the apical inclusion bodies, small PAS-positive droplets were often seen in the basal portions of the columnar cells in the distal small intestine, predominantly in those located at the apices of the villi (Figure 32). Similarly, PAS-positive droplets 2 to 4 microns in diameter were seen in the connective tissue cores of the villi and occasionally, within what appeared to be the lacteals (Figure 31).

2. ENZYME STUDY

With the enzyme techniques, strong reactions for alkaline and acid phosphatase and non-specific esterase were obtained within the inclusion bodies of the intestinal epithelium of these animals.

In figures 34-37, the typical pattern of localization and the activity of the enzyme reactions studied in the columnar absorptive cells during the various postnatal stages of development, i.e. newborn, suckling, and weanling, are shown in diagramatic form to present some of the differences in these patterns obtained during the various stages. In these figures, the 10 day and 24 day blocks serve to represent the typical reactions obtained in the suckling and weanling stages respectively.

The classification of the intensity of the enzyme reactions, based upon the intensity of the precipitate of the localizing dye, is described at the beginning of Chapter III.

More complete descriptions of the results obtained from these reactions are included in the subsequent paragraphs. To facilitate the description of these findings, observations obtained with each technique are described separately below.

(a) Alkaline Phosphatase

In the newborn, the pattern of alkaline phosphatase activity in the intestinal epithelium was very similar to that obtained in the 22 day old fetus. After this point of time, very little change in activity developed in the microvillus border of the columnar cells until the age of weaning when certain changes occurred.

As illustrated in the diagram in Figure 34, alkaline phosphatase activity was very prominent in the inclusion bodies of the jejunum and ileum. In those segments of the intestine, enzyme activity developed in the supranuclear cytoplasm of the columnar cells shortly after birth as described in the following paragraphs. However, in the epithelial cells of the duodenum, in which no inclusion bodies developed, very little change in the alkaline phosphatase activity occurred in the post partum animal until the age of weaning was reached. In animals sacrificed from the suckling period, cytoplasmic alkaline phosphatase activity of the duodenal epithelium consisted of widely scattered deposits in all portions of the cytoplasm. Occasionally, acitivity was slightly stronger in the supranuclear Golgi zone of these cells (Figure 38).

In regard to the development of enzyme activity in the jejunum and ileum, gradual increases in the level of this activity occurred in the apical cytoplasm of the villus epithelium during the first few

hours after birth. In the jejunum, the sites of greatest cytoplasmic enzyme activity were concentrated in the Golgi zone of the columnar cells, whereas in the ileum, the sites of enzyme activity were more scattered. By 12 hours, this activity became moderately heavy (Figure 39).

Once the pattern of enzyme activity was established, the picture presented by the distribution of the enzyme in the columnar cells of the jejunum was similar to the picture obtained with the PAS reaction, that is to say, the sites containing the enzyme were confined to a small oval area in the supranuclear Golgi zone of these cells (Figure 40) which corresponds with the small inclusion bodies normally encountered in the jejunum (Figure 28). In the ileum the picture was somewhat different as large inclusion bodies were formed in the apical cytoplasm of the columnar absorptive cells by the end of the first day. In the 1 day old animal, no alkaline phosphatase activity was demonstrated within these bodies but sites of cytoplasmic enzyme activity were seen in the surrounding apical cytoplasm. This served to create a picture resembling a ring of cytoplasmic activity surrounding an inactive central area (Figure 41).

With the alkaline phosphatase technique, activity appeared and increased in the inclusion bodies of the ileum following 2 days. A gradual increase followed reaching strong levels of activity at 5 days (Figure 42) and remained with little change during the rest of the suckling period. Activity in the inclusion bodies was accompanied by scattered sites of enzyme activity in the apical cytoplasm which surrounded these structures.

The control sections were completely free of dye precipitates.

(b) Acid Phosphatase

In the newborn, the level of acid phosphatase activity was similar to the picture seen in the 22 day old fetus. The developmental changes which occurred after birth are given below. In regard to the cuticular or striated border of the columnar cells of the villus epithelium, acid phosphatase activity was maintained at moderate levels until 2 or 3 days of age when this activity decreased and reached low levels, particularly in the duodenum and jejunum (Figures 35 and 43). This activity remained at low levels until the age of weaning when higher activities again appeared.

In the duodenum, no appreciable change in the acid phosphatase activity was observed in the cytoplasm of the columnar absorptive cells during the preweaning period. During this time, cytoplasmic activity was very weak in these cells with only scattered deposits and a faint concentration in the Golgi zone.

Similar to the picture obtained with alkaline phosphatase, acid phosphatase activity increased in the apical cytoplasm of the villus epithelial cells of the jejunum and ileum. As previously described, this involved gradual increases of activity shortly after birth which became relatively strong by 12 hours (Figure 44). In the jejunum, the localization of acid phosphatase was more irregular in the Golgi zone than with the alkaline phosphatase with more scattering of the dye deposits in the surrounding cytoplasm. Similar to alkaline phosphatase, activity remained relatively strong in the supranuclear Golgi zone of the columnar cells of the jejunum where small enzyme positive inclusion bodies were usually seen (Figure 43).

Similar to the situation seen with alkaline phosphatase, no acid phosphatase activity appeared in the inclusion bodies seen in the intestinal epithelium of the 1 day old animal (Figure 45). At 2 days, moderate acid phosphatase activity appeared and during the subsequent days of development, this activity gradually increased in intensity reaching strong levels by 4-5 days of age (Figure 46). In this instance, strong activity was indicated by bright red coloration of the globules by the localizing dye. At high magnification, scattered dye deposits were prevalent in the apical cytoplasm immediately surrounding the inclusion bodies (Figures 35 and 46). This picture remained essentially unchanged during the rest of the suckling period up to the age of weaning.

In the control sections, no enzyme localization was obtained.

(c) Non-specific Esterase

The level of non-specific esterase activity obtained at birth was very similar to that seen in the 22 day old fetuses (Figure 47). In addition, activity remained in the basal cytoplasm of the cells of the crypts during the first 2-3 days following birth (Figure 47) after which this activity disappeared.

Other than the above noted changes observed in the crypts, no change was seen in the esterase activity of the duodenal epithelium during the suckling period up to the age of weaning. In these cells, weak enzyme activity was demonstrated by scattered dye deposits and, occasionally, esterase-positive droplets in the Golgi zone. In contrast to the phosphatases, no esterase activity was observed in the cuticular border of the villus epithelial cells.

Non-specific esterase activity increased rapidly in the apical cytoplasm of the villus epithelium of the jejunum and ileum during the first few hours after birth reaching relatively strong levels by 12 hours (Figure 48). In the jejunum at 1 day of age, further increases were noted as demonstrated by strong esterase activity which was concentrated in the Golgi zone of the columnar absorptive cells. In the adjacent cytoplasm, scattered sites of activity surrounded this locus. No further change in this pattern occurred during the rest of the suckling period.

Similar to the situation seen in the demonstration of the phosphatases, only weak non-specific esterase activity was apparent in the supranuclear inclusion bodies of the ileum at 1 day (Figure 49). At 2 days, activity was greater followed by further increases reaching strong levels by 5 days (Figure 50). Under high magnification, granular sites of enzyme activity were also observed in the apical cytoplasm surrounding the inclusion bodies (Figure 50).

Control sections were completely free of dye precipitates.

(d) Aminopeptidase

During the postnatal period, aminopeptidase activity was confined to the cuticular border and subjacent cytoplasm of the columnar cells. When the reactions obtained in fixed and unfixed tissues were compared, the activity in the unfixed tissue was the strongest but the distribution in both instances was usually similar. In some instances, activity was demonstrated in the nuclei of the columnar cells and more often, in cytoplasmic areas basal to the nuclei in unfixed tissue. These locations were not observed in fixed tissue. In fixed tissues,

coloration of the dye deposits was red or some shade thereof (monocoupling of the dye and substrate) and in unfixed tissues, both reds and blues (dicoupling) were observed.

In the newborn, aminopeptidase activity, which was moderate in fixed tissues and at strong levels in unfixed tissues, was evenly distributed in the cuticular border of the cells along the entire length of the villi. At the same time the intensity of the activity of this enzyme appeared to be even in the various sections of the small intestine.

At 2-3 days of age, leucine aminopeptidase activity decreased in the apithelium of the distal portions of the villi becoming very weak or disappearing completely (Figures 51 and 52). This condition, which was observed in both fixed and unfixed tissue, remained during the remainder of the suckling period up to the time of weaning when a return of activity in the villus epithelium was observed.

Throughout the suckling period, no aminopeptidase activity could be demonstrated in the supranuclear inclusion bodies of the columnar absorptive cells as demonstrated in Figure 53.

In the control sections, no staining of the tissues was obtained.

3. DISCUSSION

Tissues fixed in buffered formalin proved to be the most useful in the morphological studies of the intestinal tract. This was because the tissue stains and reactions obtained with hematoxylin and eosin and with the PAS and Alcian blue reactions were of the normally encountered appearance and intensity. Preservation of the tissue elements was better following gluteraldehyde fixation but because of certain differences obtained in the tissue stains and reactions, interpretation

of paraffin embedded tissue sections became difficult. This was most marked with the PAS reaction which produced background staining serving to mask out cell membranes and connective tissue fibers. Since a similar reaction (unchanged by salivary amylase digestion) was obtained in unoxidized control sections, it is likely that this coloration was produced by a reaction with an aldehyde residue derived from the fixative. However, this was not of any great consequence during the course of the present study since the inclusion bodies were preserved very well and reacted very intensely to the PAS reaction permitting easy visualization of these bodies in this tissue. Serious difficulties probably would arise in studies where closer scrutiny of membranes and fibers is required.

The shrinkage exhibited by some of the large globules, as evidenced by clear spaces in and around the globules, was likely to have been due to either extraction of some of the components of the inclusion body or shrinkage (removal of water) during the procedures involved in the processing of the tissue. Gluteraldehyde fixed tissue, in which tissue morphology has been shown to be preserved very well by electron microscopy (46), also exhibited a certain amount of shrinkage as evidenced by clear spaces within the globules following paraffin embedding. On the other hand, the inclusion bodies seen in the columnar cells in gluteraldehyde fixed tissue that had been quenched showed very little evidence of shrinkage during most of the suckling stage. On this basis, it is likely that some of this shrinkage was produced by subjecting the tissues to dehydration and heat during the process of tissue embedding.

The significance of the clear vacuoles observed in the apical cytoplasm of the columnar cells of animals sacrificed during the suckling stage is not clear. With electron microscopy, it has been shown that numerous pinocytotic vesicles and vacuoles are located in the apical portions of these cells in the suckling rat (21). Therefore, the vacuoles seen with light microscopy may have represented coalesced absorptive droplets which have had their contents extracted. If this was true, it is conceivable that such an artificial enlargement of these vacuoles, which could have made them large enough to be seen with the light microscope, could have resulted from the rupture of the vesicular membranes leading to the subsequent coalescence of adjacent vesicles during the procedures of tissue dehydration and embedding.

The interpretation of the histochemical staining reactions obtained with the periodic acid-Schiff and Alcian blue procedures presents an interesting problem. For example, a large number of materials which give a positive PAS reaction are normally found in various body tissues. Some of these materials include: glycogen, neutral mucopolysaccharides, mucoproteins, glyocproteins, glycolipids, proteins (very weakly positive) (50) and lipofuscins (57). On the basis of their histochemical characteristics some of these materials can be eliminated. For example, glycogen is eliminated as a major contributor to the PAS reaction in this particular case because incubation of these tissue sections with salivary amylase (which would theoretically digest any glycogen that might be present) resulted in no change in the PAS reaction in the inclusion bodies. This finding is supported by previous work of Taylor and Flaa (22) who observed no change in this reaction in the inclusion bodies following diastase digestion.

Dehydration with organic solvents probably removes most of the lipid materials (57) leaving neutral mucopolysaccharides, glyo- and mucoproteins, and lipofuscins as possible materials responsible for the positive PAS reaction. Lipofuscins, which are discussed in another section, were found in only a small number of inclusion bodies and must be considered as insignificant. Similarly, neutral mucopolysaccharides are not reputed to be prevalent in mammalian tissue where they make up only a small portion of the gastric mucus (57). Therefore, the most likely suspects appear to be muco- and glycoproteins although certain carbohydrates could also be involved. For example, lactose is present in the milk of cattle (66) and it is conceivable that this and possibly other carbohydrates may be present in the milk of rats in sufficient quantities to produce a positive PAS reaction following ingestion into the cells.

According to Pearse (57), mucoproteins are hexosamine-containing polysaccharides which have a hexosamine content greater than 4%. Glycoproteins, on the other hand, are materials containing less than 4% hexosamine. However, the exact source of the intravesicular material, if indeed it is muco- or glycoprotein, is not clear. One possible source is milk casein, which in bovine milk consists of 0.31% galactose qualifying it as a glycoprotein. Whether rat milk casein is composed of similar material has not been disclosed but if it is, it is doubtful that it contributed significantly to the PAS reaction unless significant concentration of this material followed absorption into the epithelial cells since Pearse (57) stated that materials containing less than 0.5% polysaccharide do not give any recognizable color with the PAS reaction. Mucoproteins (57,66) and possibly glycoproteins are contained within

some of the gastrointestinal mucins which could conceivably be absorbed by the intestinal epithelial cells. It is unlikely that intestinal mucin is involved, at least not in its unaltered form since goblet cell mucin contains, among other materials, acid mucopolysaccharides which give a positive Alcian blue reaction. Therefore, because several inconsistencies exist it is difficult to make any further conclusion concerning the source of the PAS positive material without the aid of additional information.

In regard to the enzyme studies, frozen sections of gluteraldehyde fixed tissues were successfully incubated for the acid and alkaline phosphatase, non-specific esterase, and aminopeptidase enzymes. In sections of quenched gluteraldehyde fixed tissues, tissue morphology was very good and for the most part, the enzyme activity was good and clearly localized with only slight amounts of discernable diffusion. Following incubation, gluteraldehyde fixed tissues exhibited slightly more background staining than that seen following formalin fixation in comparable tissues. It was assumed that this was the result of absorption of the azo dye contained in the incubation media. However, this did not interfere with evaluation of the histochemical enzyme reactions.

One important practical advantage made possible by the use of gluteraldehyde was that these tissues could be stored for relatively long periods of time amounting to at least several weeks in which no great change in enzyme activity was observed. On the other hand, when formalin is used as a fixative for enzymes, enzyme activity is greatly reduced after fixation for periods of time longer than 24 hours.

In the evaluation of results obtained with the enzyme technique, there may have been some question as to whether the demonstration of enzyme activity within the inclusion bodies was derived from diffusion of the dye products from the sites of enzyme activity in the surrounding cytoplasm. In order to clarify this, sections were incubated for shorter periods of time than normal, (beginning with a length of 1 minute) and it was observed that activity for this enzyme was weakly demonstrated within the inclusion body before sites were stained in the surrounding cytoplasm. The possibility that this was the result of diffusion and absorption of the dye product is almost eliminated by this information. Furthermore, these results were recently confirmed by Cornell and Padykula (67) who demonstrated acid and alkaline phosphatase, esterase, pyro-phosphatase and ATPase within the inclusion bodies of the suckling rat. Conversely, whether the sites of enzyme activity demonstrated in the cytoplasm which surrounds the inclusion bodies were derived from diffusion out of these structures or they were actual sites of cytoplasmic enzyme activity remains unsolved.

The pattern of enzyme localization presented by the aminopeptidase reaction in the intestinal epithelium of the suckling animals created some problems in regard to interpretation. For example, there may be some question as to whether the loss of activity on the villi of the suckling animals was due to an artifact resulting from diffusion or inactivation of the enzyme. However, similar patterns of activity were usually obtained in fresh frozen tissues and, at the same time, this phenomenon was consistent only in the suckling animals which makes it more likely that this was the actual pattern of enzyme distribution during this stage of development. The question of nuclear staining

observed in some of the unfixed tissues may be associated with diffusion artifact, however.

Leucine aminopeptidase, which has previously been described in the intestinal mucosa by biochemical studies (41) and identified in the microvillus border by Holt and Miller (68), is associated with hydrolysis of peptide bonds. Therefore, it seems that the presence of this enzyme in the rat intestinal mucosa indicated some degree of proteolytic activity. However, the absence of this enzyme in the villus epithelium does not necessarily indicate that no proteolytic activity took place in the intestine of the suckling animals since other proteolytic enzymes with a specificity toward substrates other than the leucyl naphthylamide used in this study could have been present.

The presence of acid phosphatase and non-specific esterase in the inclusion bodies provided some evidence that these structures were associated with processes of digestion. Acid phosphatase is an enzyme mediating hydrolysis of phosphate esters at acid pH's (50). Similarly, non-specific esterase is commonly considered a hydrolytic enzyme with which hydrolysis of esters of alcohols, phenols, and naphthols is facilitated (50).

The presence of alkaline phosphatase within the inclusion bodies is more troublesome to explain. Traditionally, this enzyme has been considered with processes of absorption (active transport mechanisms in particular) of glucose, fats, and other materials (69). This is subject to debate, however, since Koldovsky (44) demonstrated no correlation between glucose absorption and the development of alkaline phosphatase activity in the suckling rat. It has been suggested that the alkaline phosphatase located within the Golgi regions of the

columnar cells have hydrolytic functions (69). Whether the alkaline phosphatase demonstrated within the inclusion bodies was associated with processes of digestion of absorption is unclear but the fact that they were found within the inclusion bodies may support a hypothesis that they were partially at least, associated with processes of digestion.

When the criteria of Novikoff (42) are applied, it seems that the enzyme-containing inclusion bodies must be classified as lysosomes. However, it is probable that these bodies also contained materials which were ingested by the cells which indicates these structures probably were "digestive vacuoles." An explanation of this classification and definitions of these terms are given below.

As pointed out in the introduction, lysosomes are membrane-enclosed vesicles containing hydrolytic enzymes, indicating that these bodies are digestive in nature (34). According to deDuve (34,70), 4 functional forms of inclusion bodies are covered by the lysosome concept. He has classified these into the following: (1) digestive vacuoles; (2) storage granules; (3) autophagocytic vacuoles; and (4) residual bodies. The inter-relationships of the different varieties of these bodies have been equated with an intracellular digestive tract, which in unicellular organisms such as the protozoans (34) are the most complete. In deDuve's scheme (34,70), "storage granules" are bodies transporting active hydrolytic enzymes to sites of hydrolysis. He proposed that these enzymes were produced in the ribosomes and were "packaged" in the Golgi apparatus similar to the formation of zymogen granules in the production of other cellular secretory products composed of proteins. Recently, additional

evidence has shown more definitely that enzyme containing vesicles (i.e. lysosomes) are derived from the tubules and saccules of the Golgi apparatus (39,71,72).

In the intracellular digestion of material ingested by the cells, the following scheme has been proposed by deDuve (34,70). First of all, the pinocytosed material is collected into a membrane bound vesicle termed "phagosome." deDuve (34,70) suggested that the "storage granules" liberate their enzymes into the phagosome producing a "digestive vacuole" where the digestion of the ingested food materials take place. "Autophagocytic" vacuoles are similar zones of digestion in which the enzymes have been released into the cellular cytoplasm creating sites of cellular lysis. "Residual bodies" represent vacuoles containing an accumulation of indigestable residues and enzymes remaining after digestion. In the ideal situation, residual bodies are eliminated from the cells but deDuve (70) pointed out that this factor varies among the various cells of the body. Some of these cells do not have the ability to eliminate these materials which gives rise to an accumulation of these bodies within the cell. In the liver, the enzymes are thought to be "reused" since "old" lysosomes fuse with new lytic bodies (70).

When making a comparison with this background information, the inclusion bodies contained within the intestinal epithelial cells of the rat seem to fit into the scheme proposed by deDuve (34,70). Electron microscopy has shown that the materials which are ingested by processes of pinocytosis are ultimately incorporated into the large inclusion bodies (21) which possibly are similar to the "phagosomes" described above. With the results of the present study,

the inclusion bodies have been shown to contain enzymes indicating the processes of digestion may take place there.

As mentioned previously, it is not known whether the activity demonstrated in the cytoplasm surrounding the inclusion bodies represented a collection of enzyme containing bodies or "storage granules" or if it was merely a manifestation of diffusion of enzyme or reaction product into the surrounding cytoplasm. If it can be shown that these sites of activity represented storage granules, the source of enzymes within the inclusion bodies could be explained.

C. TRANSITION AT WEANING

At the age of weaning, certain changes which involved the regression and loss of the inclusion bodies and a transition in the pattern of enzyme activity were observed in the intestinal epithelium. This change began at approximately 15 days of age and ended at about 21 or 22 days when the morphological transition to the type of epithelium seen in the weanling animals was completed.

1. MORPHOLOGICAL OBSERVATIONS

Beginning with 15 days of age, shrinkage and disintegration of the intravesicular material became more marked than that seen during the preceding days of the suckling stage. These features were most noticeable in formalin fixed tissue but similar changes were seen in sections of tissue fixed with gluteraldehyde. Tissue fixed in the latter fixative showed a break-up of the intravesicular material into smaller globules (Figure 54). This was in contrast to the appearance previously seen in gluteraldehyde fixed tissues during the suckling period when the intravesicular material was represented by a single

relatively homogeneous mass (Figure 34).

Degeneration of the intravesicular material became very extensive by 20 and 21 days of age. At times, only sparse amounts of degenerate material remained within the inclusion bodies. On other occasions, the contents of the globules disappeared completely leaving large empty vacuoles in the apical cytoplasm of the villus epithelial cells. This picture, which was most evident at the apical ends of the villi, was observed in both gluteraldehyde and formalin fixed tissue (Figures 55 and 56).

In the animals studied, the inclusion bodies disappeared from the distal small intestine as early as 19 days of age in 1 animal. Usually, however, the inclusion bodies remained until 21 days of age after which (22 days and beyond) these structures were consistently absent from the intestinal epithelium.

In the process of regression, the inclusion bodies of the jejunum disappeared before those in the ileum. At the same time, globules contained in the cells of the basal portions of the villi disappeared before those located at the apical end of these processes (Figure 55). Therefore, at the termination of this period, inclusion bodies remained in the cells located on the apices of the villi for the longest time.

Concurrent with the regression or disappearance of the inclusion bodies, changes in the intensity of the activity of the enzymes located within the inclusion bodies were demonstrated. At the same time, changes in the intensity of activity and pattern of localization of the enzymes contained in the cytoplasm of the columnar cells occurred.

Considering the inclusion bodies first, gradual decreases in the activities of all 3 enzymes developed beginning at approximately 15 days of age. This activity decreased to the point where little or no coloration of the vesicles was obtained at 20 and 21 days of age. Figure 57, which is a demonstration of acid phosphatase activity and was similar to the picture obtained with the other techniques, provides an illustration of this.

Changes which occurred in the activity of the enzymes of the cytoplasm of the cells are described separately below.

(a) Acid and Alkaline Phosphatase

Beginning with approximately 18 days, increases in the activity of the acid and alkaline phosphatase enzymes developed in the cuticular border of the villus epithelium of the duodenum and jejunum. By 22 and 23 days of age, these enzymes reached strong levels of activity very clearly localized in the cuticular border of the villus epithelium (Figures 58 and 59). In the ileum on the other hand, acid and alkaline phosphatase activity decreased in the cytoplasm and striate border of the columnar cells as the globules disappeared.

(b) Non-specific Esterase

Similar to the above, a gradual increase in the non-specific esterase activity occurred in the cytoplasm of the apical portions of the columnar cells of the duodenum and jejunum in which the picture seen in the weanling animal was attained by 22 days of age (Figure 60). Similar to the observations made with the phosphatase reactions, cytoplasmic enzyme activity in the villus epithelium of the ileum decreased.

(c) Aminopeptidase

Aminopeptidase activity in the cuticular border of all portions of the small intestine gradually increased at the end of the suckling period, becoming re-established in the epithelium along the entire length of the villi. In 1 animal this pattern was established as early as 19 days of age (Figure 61) whereas in the other animals sacrificed during this period, the transition was not completed until 23 days of age. It was interesting to observe that the same 19 day old specimen demonstrated early maturation with all enzyme techniques and, likewise, a loss of inclusion bodies.

3. DISCUSSION

The decrease in the intravesicular enzyme activity and the break-up of the included material observed beginning with 15 days of age seemed to be associated with processes involving degeneration of the inclusion bodies. What happens to the intracellular material during this process is unknown. However, it can be postulated that: (a) the material may remain within the cells before the cells themselves are extruded from the epithelium; or (b) the material is eliminated from the cells before they are removed from the epithelial surface. The fact that empty or nearly empty vacuoles were seen during the final days of the suckling period before complete transition of the intestinal epithelium occurs may have indicated that this material was absorbed, digested, or otherwise eliminated before extrusion of the columnar absorptive cells.

To be sure, the complete absence of the intravesicular enzymes seen in the 20 and 21 day old animals was probably a manifestation of a complete loss of the material contained within the inclusion bodies.

However, since a gradual trend in which the intensity of enzyme reactions were gradually reduced prior to this point, it is assumed that these changes in activity were involved in a gradual reduction of the metabolic function of the inclusion bodies before they were eliminated from the epithelium.

It is assumed that the change from a liquid to a solid diet at the age of weaning had no effect on the change in intestinal morphology seen at this stage since Halliday (73) has shown that alterations in the diet of suckling rats had no effect on their ability to absorb antibodies and produced no change in the developmental timetable. Furthermore, metabolic control of the process of intestinal maturation is in evidence since changes similar to that seen at weaning were observed following the administration of adrenal steroids. Clark (21) for example, administered cortisone acetate to 8 and 10 day old rats and mice which resulted in alterations of the columnar absorptive cells within 72 hours. At this time, these cells attained a morphology similar to those seen in the epithelium of the adult animals and the ability to ingest proteins was lost. Similarly, Moog and Thomas (74) observed an increase in the activity of alkaline phosphatase of the intestinal epithelium of suckling mice and Halliday (75) observed similar increases in the same enzyme in suckling rats and mice following the administration of steroid hormones. These authors (74,75) correlated the increase in alkaline phosphatase activity with a loss in the ability to absorb proteins.

The influence exerted by the adrenal cortical hormones on the intestinal epithelium is not clearly understood but according to Moog (76) these hormones have a maturing effect causing them to differentiate

prematurely. However, she also demonstrated that this influence was not effective until a certain age was attained (i.e. 8 days in the mouse) indicating that these cells must pass through a certain stage of development before the hormone has an effect (76).

During the course of normal development, the increases observed in the activity of alkaline phosphatase during the process of weaning in the present study were similar to previous observations made by Moog (62) in the mouse. In the guinea pig, however, no change in enzyme activity was observed at weaning (17).

D. WEANLING AND ADULT

1. MORPHOLOGICAL OBSERVATIONS

The morphological pattern presented by the cells of the intestinal tissue of the weanling animals was very similar to that of the adult. To be sure, the gross dimensions of the intestine of the weanling animal were less than in the adult, but on microscopic examination, the structural proportions of the epithelium and villi were very similar in the adult and weanling animal.

In contrast to the newborn animals, the villi in the weanling animals had developed into thicker structures supported by a well established connective tissue core. The characteristic shapes of the villi were more definitive at this time; those in the duodenum were long and finger-like with blunted ends, in the jejunum, the villi were more pointed, whereas in the ileum, shorter thick villi were seen.

At the same time, crypts were better developed as demonstrated by prominent tubules at the basal ends of the villi.

The villus epithelium was represented by tall columnar cells with basally placed oval nuclei. No inclusion bodies were observed in the cytoplasm of these cells with the exception of a discrete zone of very fine PAS-positive droplets in the supranuclear Golgi zone.

2. ENZYME REACTIONS

The patterns of enzyme distribution presented in the columnar absorptive cells of the postweanling and adult animals were very similar. On critical evaluation, the level of enzyme activity demonstrated in the adult epithelium was usually slightly stronger than in the weanling animal, but for practical purposes of reporting they will be considered essentially the same since no precise values were given to the levels of enzyme activity. A comparison of the levels of enzyme activity attained in the weanling animals with the suckling and newborn is demonstrated in Figures 34-37.

(a) Acid and Alkaline Phosphatase

Activity for both the phosphatase enzymes became strong in the cuticular border of the villus epithelium of the duodenum by 22 days of age (Figures 58 and 59). In the jejunum similar increases were observed but the final level of activity attained was slightly lower. In the cytoplasm of the villus epithelium of the ileum, alkaline phosphatase was absent (Figure 62) and similarly, acid phosphatase activity was very weak.

In addition to sites of activity in the cuticular border of these cells, cytoplasmic phosphatase activity was seen in the subcuticular cytoplasm of the villus epithelium of the jejunum and duodenum. In the same sections, weak enzyme activity was observed in the basal cytoplasm and in a discrete zone of concentrated

activity in the Golgi zone (Figures 58 and 59). No activity was demonstrated in the cells of the crypt epithelium but, in the case of acid phosphatase, Brunner's gland epithelium was highly active.

(b) Non-specific Esterase

In the weanling animals, non-specific esterase activity was localized in the form of fine brown granules of the localizing dye in the apical cytoplasm of the columnar cells of the intestinal epithelium. This activity, which was moderately strong in the epithelium of the duodenum and jejunum and weak in the ileum (see Figure 36), was evenly distributed throughout the supranuclear cytoplasm between the nucleus and the striate border (Figure 60). The microvillus border, which contained no enzyme, was clearly set off from the remainder of the cell. On occasion, particularly in the adult animals, a concentrated zone of the enzyme activity was observed in the supranuclear Golgi zone of the columnar cells. Where reactions were positive in the weanling and adult animals, the deposit of the localizing dye was relatively fine and evenly distributed in contrast to the suckling animals in which coarse and uneven precipitates were deposited in areas of activity.

Non-specific esterase was evenly distributed among the cells covering the length of the entire villus in the weanling and adult animals although some "coarseness" and "clumping" of the dye product was evident in the cells at the apices, particularly in the weanling animals (Figure 60). No esterase activity was observed in the crypt epithelium, but similar to the acid phosphatase, non-specific esterase activity was strong in the Brunner's gland epithelium.

(c) Aminopeptidase

In the weanling animals, leucine aminopeptidase of the mucosa returned to the villus epithelium producing a pattern of distribution similar to that seen in the newborn animal (see Figure 37). The activity of this enzyme was strongest in the cuticular border of the cells of the villus epithelium with diffuse activity evident in the subjacent cytoplasm of these cells as well. Typically, this enzyme was relatively evenly distributed in the cells over the length of the villi but was absent from the crypts (Figure 61). In the adult, leucine aminopeptidase was observed in all portions of the small intestine but slight differences in activity was apparent in the various intestinal segments. In regard to the latter, this activity was the strongest in the duodenum, at slightly weaker levels in the jejunum, and seemed to be the weakest in the ileum.

3. DISCUSSION

It was interesting to observe that levels of high enzyme activity shifted from the caudal to the cephalic end of the intestine at weaning. At first glance, this process may appear to be contrary to the rule of cephalo-caudal differentiation which normally occurs during the course of ontological development. However, these enzymes appear to be associated with a process of intracellular digestion of ingested materials involved in a so-called primitive mechanism (21) of obtaining food materials. In this process, large macromolecular materials are taken in by the cells where they are subsequently digested providing the organism with a source of nutrition. This is similar to the means by which various invertebrates acquire nutritive materials (3). At the same time, cytoplasmic bodies are common in

endodermal epithelium of the embryonic stages of certain vertebrates (3). Corresponding with this hypothesis, cytoplasmic granules and droplets of fluid material have been observed in yolk sac epithelium of guinea pig fetuses with electron microscopy (77). Therefore, the enzyme containing inclusion bodies seen in the suckling rat probably represented a developmental adaptation which may have provided a source of nutrition at this particular stage of development.

Absorption of food materials by processes of pinocytosis per se does not seem to be unique to the intestinal epithelium of young animals since the absorption of lipid materials in the adult organism are also associated with pinocytosis (78). Therefore, it appears that this means of absorption is not lost entirely at weaning but rather becomes more selective in which the materials absorbed by this process are restricted. How this occurs is not clearly understood but Bennett (79) has suggested that in the mechanism of pinocytosis (perhaps more correctly endocytosis) the selection of materials to be ingested depends upon the formation of a specific bond between the plasma membrane of the cell and the particles to be absorbed from the environment. In this manner, the material which has been attached to the plasma membrane is brought into the interior of the cell as this membrane is invaginated. In a situation where all materials in the environment evoke pinocytosis, that is to say are attached to the plasma membrane and stimulate invagination of the plasma membrane, no selectivity in this process would be expressed. On the other hand, if only certain materials are attracted and attached to the membranes a certain degree of selectivity in this process would occur. Therefore, if pinocytosis is evoked only by certain substances in the environment of

the epithelial cells of the weanling and adult animals, it becomes likely in the light of the information given above that selectivity in this process which developed at weaning could have resulted from a change or a reorientation of the molecules of the plasma membranes of the epithelial cells during the process of maturation. This in turn, could have a selective effect on the materials that would be absorbed or ingested by the cells due to changes in the ionic changes or free chemical groups in the plasma membranes.

Along with a change in the distribution of the epithelial enzymes, there is some indication that the enzymes themselves may have changed at weaning. Moog (80) suggested that in the developing mouse, the intestinal alkaline phosphatase is replaced by a different alkaline phosphatase at weaning indicated by changes in specificity of this enzyme towards different substrates. Since the food materials ingested by the weanling and adult animals are different from the milk normally ingested by the suckling animal, it is logical that such alterations in enzymes should occur. However, it does not seem likely that the changes in the enzyme activity observed during the course of normal development were induced solely by changes in the substrate (i.e. diet). For example, similar changes in alkaline phosphatase activity and the absorptive abilities of the epithelial cells were induced by the administration of cortisome acetate (74,75), which tends to indicate that metabolic influences were primarily involved in these changes. In addition, the effect of diet on the intestinal epithelium of the suckling animals seems negligible since Halliday (73) observed no change in the ability or inability to absorb proteins when the diets of suckling and weanling rats were altered.

During the course of the present study, the distribution of the enzymes seen in the weanling and adult animals was similar to previous descriptions concerning alkaline phosphatase (69,81), acid phosphatase (69,41), non-specific esterase (69) and aminopeptidase (41,69).

E. OTHER PROCEDURES

Since some of these procedures were performed on a limited number of animals, only a brief sketch describing the reactions observed in each of these tests is included in this section.

1. RESULTS

Nile blue reaction

The Nile blue sulfate reaction was performed on 11 animals. The tissues treated with this method were colored with a background cast in which the cell nuclei and cytoplasm were stained pale blue. The inclusion bodies of the epithelial cells were similarly colored with the exception of a small number of large globules located in sections of the ileum which were slightly darker. This is indicative (Figure 63) of a positive reaction for lipofuscins.

Ziehl-Neelsen reaction for acid fast lipofuscins

Tissue sections treated with the Ziehl-Neelsen reaction, which was performed on 4 animals, were colored a faint pink. Following destaining in acid, the material contained within many of the large globules of sections of the ileum remained colored indicating acid fastness (Figure 64). This was limited primarily to the largest globules of the ileum and acid fastness was not indicated in the small inclusion bodies.

Dihydroxy-dinaphthyl-disulfide (DDD) reaction

The DDD reaction for sulfhydryl groups was performed on the intestinal tissue of 8 animals. The tissues treated with this procedure were colored with a weak background stain. Positive reactions, which were demonstrated by darker coloration, were obtained in the larger inclusion bodies of the ileum and in the erythrocytes located in the blood vessels of the tunica propria (Figure 65). At the same time, positive reactions were not obtained in the smaller sized inclusion bodies of the middle sections of the intestine.

In regard to the developmental process, no sulfhydryl groups were demonstrated in the intestinal epithelial cells of the newborn but were seen as early as 3 days of age which was the youngest stage studied with this reaction.

Alloxan-Schiff reaction

The alloxan-Schiff reaction for amino groups was performed on 8 animals. Tissues treated with this method were diffusely colored with the Schiff reagent in which no difference in the intensity of this reaction was observed in the small inclusion bodies. In the larger structures of the ileum, however, weakly positive reactions were observed which were indicated by a greater intensity of the color reaction (Figure 66).

With the control reaction, a stain of slightly lower intensity than in the experimental sections was also obtained in the large globules.

Feulgen reaction

The Feulgen reaction for nucleic acids, performed on the intestinal tissue of 32 animals, was negative in the inclusion bodies

(Figure 67). On the other hand, good nuclear reactions were obtained in both fetal and postnatal animals. In the early fetal animals, numerous mitotic figures were well demonstrated in the epithelium and mesenchymal wall of the intestinal tube.

Succinic Dehydrogenase

The succinic dehydrogenase reaction, which was performed on 4 animals, produced a dark blue precipitate of the localizing dye, nitro blue tetrazolium, in the cytoplasm of all the intestinal epithelial cells. No morphological demonstration of mitochondria was obtained since the precipitates of nitro blue tetrazolium were too coarse for precise localization.

The nuclei and inclusion bodies of the columnar cells were negative to this reaction, producing a negative morphological image in contrast to the activity in the cytoplasm which surrounded these structures (Figure 68).

2. DISCUSSION

Diffuse background coloration or "staining" of the tissues was evident in each of these reactions except with the Feulgen and succinic dehydrogenase procedures. However, this did not interfere significantly with the interpretation of the reactions obtained except in the Nile blue and Alloxan-Schiff procedures where the positive reactions were very weak. In regard to the former, positive reactions, which according to Lillie (56) are dark blue or blue green, were identified in only a small number of inclusion bodies in which the coloration was slightly stronger than that of the surrounding material of the tissue section. With the Alloxan-Schiff reaction, the situation was more acute since the positive reaction was weak. With unoxidized

Schiff controls, coloration of the inclusion bodies was nearly as great as in the experimental sections. This finding tends to indicate that a large portion of the coloration obtained with this reaction was due to a reaction with free aldehydes contained in this material. For a clearer and more definite demonstration of amino groups, oxidation with ninhydrin instead of alloxan is recommended (55).

In order to provide a double-check on the reactions obtained with the Nile blue sulfate method, the Ziehl-Neelsen acid fast reaction for lipofuscins was performed. According to Pearse (57) acid-fastness is a reliable criteria for the demonstration of lipofuscins in tissue sections. The results obtained with this method were very clearly positive in the largest sized inclusion bodies.

Lipofuscins are defined as auto-oxidation products of phospholipids and unsaturated lipids (57). In addition to the lipid content, they may also contain certain protein components (i.e. lipoproteins) (57). Pearse (57) presented a scheme which indicated that these materials are reactive to a variety of histochemical reactions during the various phases of oxidation. In this scheme, he suggested that as lipids are oxidized into the lipofuscin pigments, they develop an increased basophilia and lose reactivity to Sudan black B, a stain for lipids. In the early stages of oxidation, lipofuscins are PAS-positive and are acid fast. Both of these characteristics disappear with further oxidation.

When the results obtained during the course of the present study are correlated with this information, it is very probable that some of the material contained within some of the larger inclusion bodies were lipofuscins since PAS-positive, acidfast, and weakly positive

Nile blue reactions were obtained. The source of the lipid or bound lipid material which ultimately developed into lipofuscin could very likely have been derived from the ingested milk.

The presence of an oxidized material within the membrane-enclosed vesicles gives additional support for a hypothesis that digestion occurs within these structures. As a matter of fact, Barka and Anderson (50) have indicated that lipofuscins are usually located within lysosomes, lending support to this hypothesis. In addition, lipofuscins in nervous tissue have been found in association with acid phosphatase and non-specific esterase (57) and in other situations cathepsins (enzymes associated with protein hydrolysis) have been found in association with lipofuscins pigments (50). The former is not unlike the situation seen in the course of the present study.

The positive DDD reaction for SH groups, which was weak in the inclusion bodies, may have indicated the presence of methionine or other SH-containing materials within the inclusion bodies. The fact that erythrocytes, which contain methionine in the hemoglobin, were strongly positive lends support to the validity of this reaction.

Why only the larger bodies were shown to contain the lipofuscins and the amino and SH groups is somewhat puzzling. One reason could be that the ingested material contained within the larger inclusion bodies remained within these structures for a longer period of time than in the smaller inclusion bodies. This would, of course, result in a longer period of oxidation of the lipid materials producing more lipofuscin material than otherwise might be possible. In regard to the amino and SH groups, Anderson (43) postulated that in the human fetus, these chemical groups are freed from bonds with other tissue

groups by processes of digestion which occur within the inclusion bodies. In this manner, these groups are made available for the histochemical reaction. Therefore, if the materials are contained within the largest inclusion bodies for the longest periods of time, it would seem natural to assume that the process of digestion would be the most complete hydrolyzing more of the tissue bonds.

Another possibility could lie in the fact that the volume of the material contained within the small inclusion bodies might be insufficient to produce positive reactions in opposition to the larger bodies which contain greater volumes of material.

A third reason, which may be the most likely of the 3, is that the increased staining observed in the larger bodies might have been caused by a greater concentration of the material contained within. Electron micoscopic studies have demonstrated that as the material ingested by the cell is transported to the inclusion bodies, concentration of this material occurred (21). Therefore, a possible correlation in which the degree of condensation (concentration) of the intravesicular material is directly related to the size of the inclusion bodies may exist.

Turning to some of the other tissue reactions performed, the fact that no succinic dehydrogenase was demonstrated within the inclusion bodies seems to indicate that no mitochondria or mitochondrial remnants containing active enzymes were located within these structures. Similarly, the fact that the Feulgen reaction was negative in the inclusion bodies gave evidence that no unaltered deoxyribonucleic acids were contained within. These findings lend more support to the hypothesis that the inclusion bodies are digestive in nature.

During the course of performing preliminary studies in this area, pilocarpine, a parasympathomimetic drug, was administered to 4 suckling animals which were sacrificed at 15, 30, 45, and 60 minute intervals in an attempt to learn whether the inclusion bodies had any secretory functions. Following the injections, the animals exhibited the systemic changes produced by pilocarpine, i.e. shivering, lacrimation and increased salivation. The last 2 symptoms are indicative of a generalized glandular reaction to pilocarpine. After this procedure, however, no histological changes in the inclusion bodies could be detected. From this information, it can be concluded that the inclusion bodies probably do not have any secretory function, at least not under parasympathetic control.

GENERAL DISCUSSION

When the known information concerning the intracellular inclusion bodies is correlated, it appears that the significance of these structures may have been twofold. In the first place, it has been shown on several occasions that the inclusion bodies were associated with absorption of antibodies from the intestinal lumen (11,13,14,15,21,30,31,32). Secondly, information derived from the present study and that of others (15,67) infers that these structures were also associated with processes of intracellular digestion. It seems reasonable to assume that digestion of materials within the cell is a mechanism which serves to provide the organism with a source of nutritive material at an age when the glandular apparatus for producing digestion within the intestinal lumen was immature. If this is true, the materials absorbed by the cell could provide the

developing organism with 2 important functions which would involve protection from infection and contribution to its growth and development.

On account of high levels of enzyme activity in the intestinal mucosa and circumstances in which the effective intestinal digestion is greater than the enzymes of the succus entericus (intestinal juice) would indicate, it seems that processes of intracellular digestion may occur in the intestinal epithelium of adult animals (41,82,83,84). Recently, this hypothesis has been further substantiated by work of Hsu and Tappel (41) who demonstrated the presence of 6 lysosomal enzymes within the intestinal mucosa of the adult rat. However, it seems that a basic difference occurs between the process of intracellular digestion seen in the adult and that of the developing organism. In the latter, it seems that large macromolecular materials are taken up by the cell whereas in the former, this process appears to be associated with the final digestion of small molecules (dipeptides and so forth (41)).

As previously mentioned, the process of ingestion of materials by the columnar absorptive cells of the suckling animals appears to be indescriminate in which any compound contained in the environment of the cells involved is taken in (10,21). However, in studies on absorption in which uptake of antibodies from the intestine was measured, it has been demonstrated that some degree of functional selectivity is in operation since homologous antibodies (from the same species) were absorbed more rapidly than heterologous varieties (21, 85). Since the inclusion bodies and ingested materials were surrounded by membranes and were, therefore, essentially

extracytoplasmic, it can be suggested that the selective barrier was in the membranes surrounding these structures. Properties which could determine the ability of a substance (i.e. antibodies and other materials) to penetrate this membrane might include solubility, particle size and charge, affinities developed between the substance and the membranes, and its susceptibility to intracellular digestion (21).

In the process of ingestion of materials by the intestinal epithelial cells of the suckling animals, it is highly probable that digestible as well as indigestible materials are taken in by the cells. What happens to the indigestible residues is not clear but deDuve (70) has suggested that in certain cells, these residues are not eliminated and are allowed to accumulate within the cell. If this is true, the significance of the material contained within the inclusion bodies could be explained on a basis that it represented in part, at least, an accumulation of indigestible waste materials. Certainly, certain hydrolyzable materials were also present, but if all the material contained within these structures was absorbed by the cell, it seems that the size of the inclusion bodies would be more variable (i.e. they would probably decrease in size between feedings and enlarge again at feeding).

In regard to the intracellular route taken by the absorbed materials during the process of transport from the inclusion bodies, information is available only on the macromolecular antibodies. When techniques involving the recovery of fed antibodies are used, Comline et al. (86) learned that these materials were transported to the lymphatics and not into the blood stream following absorption from

the gut. In this manner it is conceivable that the small droplets seen in the basal portions of the columnar absorptive cells and in the connective tissue core of the villi during the course of the present study were associated with this process. It is not known what other materials aside from antibodies are transported in this manner, but in the case of the digestible materials it is likely that digestion preceded absorption. In this manner, absorption probably involved simple processes of diffusion.

Of incidental interest is the fact that no aminopeptidase was demonstrated within the inclusion bodies. This may have been of some significance since it would be detrimental to have digestion of absorbed antibodies before they are transported into the body fluids. Of course, it is not known how many other proteolytic enzymes may have been present within these bodies.

In regard to the acquisition of passive immunity in developing animals, a significant uptake of antibodies by intestinal absorption has also been implicated in the fetal stages of certain species. For example in the developing rat, which receives maternal antibodies both before and after birth, antibodies have been recovered from the stomach contents of these fetuses as well as from the amniotic and exocelomic fluids leading to the conclusion that transfer of antibodies through the process of intestinal absorption might be an important route of antibody uptake (87).

In the developing rabbits and guinea pigs, which receive maternal antibodies primarily during the prenatal period of development, it has also been demonstrated that transfer of antibodies occurs by means other than through direct transfer to the fetal circulation by

placental absorption (18,88). In the guinea pig, Leissring and Anderson (18) suggested that antibody transfer in the late fetal stages was through the absorption of these proteins from the amniotic fluid by the gut. In the fetal rabbit, antibodies have been recovered from the stomach contents which would suggest a similar mode of transfer (88,89,90). However, Brambell (88,89,90) maintained that absorption of antibodies from the uterine lumen through the yolk sac into the vitelline circulation is the most important pathway of transfer and that the antibodies contained within the fetal stomach represented a "reservoir of antibody" (89) which could be absorbed by the fetal gut if necessary. Whatever the significance of this material is, the fact that the fetal intestine is capable of absorbing antibodies seems to be supported in at least 3 species of animals.

Similar to the rabbit and guinea pig, the human fetus receives all of its maternal antibodies during the fetal stages (88). Since antibodies have also been identified in the stomach contents and the amniotic fluid of the human fetus, it has been postulated that maternal antibodies are transferred to the human fetus by similar processes of intestinal absorption (89,91). However, this theory has been repudiated by work of Roulet and von Muralt (92) who demonstrated similar accumulations of antibodies within the intestinal lumen of the human fetus in cases of congenital intestinal atresia. These investigators (92) then concluded that the antibodies demonstrated in the intestinal contents and amniotic fluids of the human fetus were derived from diffusion from the fetal circulation and were not associated with significant transfer of antibodies from the mother of the fetus. As a matter of fact, it seems that an efficient transfer

of antibodies through the fetal membranes and into the amniotic fluid seemingly would be hampered in the human fetus since its fetal membranes are more complete than in the rabbit or guinea pig (88). At the same time, the yolk sac in the human fetus is very rudimentary (5) and could not play a significant part in the role of absorption.

The inclusion bodies seen in the intestinal epithelium of the suckling rats during the course of the present study were remarkably similar in morphological appearance and distribution to the inclusion bodies described in the human fetus (2,3,33,43). Furthermore, certain histochemical similarities of the intracellular bodies of these 2 organisms were also demonstrated. For example, the inclusion bodies of the human fetus and suckling rats were PAS-positive and negative to the Alcian blue reaction and were shown to contain acid-fast lipofuscins and amino- and sulfhydryl groups. At the same time, hydrolytic enzymes were contained in both which lends support to a hypothesis that the inclusion bodies of the human fetus were digestive in nature (43) similar to observations made in the suckling animals. Some differences in the pattern of enzyme contents were seen however, as the inclusion bodies of the intestinal epithelium seen in the human fetus contained aminopeptidase and no alkaline phosphatase (43) whereas in the suckling rat, the reverse was true. Whether this was due to an actual difference in the enzyme content of these bodies or the result of differences in techniques is not certain. It is conceivable that in the fetus, which represents a different physiological entity than the postnatal animal, certain differences in the enzyme patterns could exist.

While this information indicates that the inclusion bodies of both the human fetus and the suckling animals probably were associated with processes of absorption and digestion of materials from the intestinal lumen, the physiological significance of these functions may have been different in each case. For example, in the suckling animals, these bodies probably were associated with the uptake of antibodies from the intestinal lumen and with the absorption and digestion of food materials. In the fetus, on the other hand, it is doubtful that any significant quantities of antibodies were absorbed from the amniotic fluids. The inclusions, which undoubtedly arose from processes of absorption on the basis of electron microscopic evidence, probably contained materials derived from meconium. The nutritive value of this material and absorbed amniotic fluid is questionable but certainly the digestion of some of this material could have served to salvage any proteins and other materials contained in the luminal contents. Perhaps the significance of the amino-peptidase in the inclusion bodies of the human fetus is explained on this basis.

The absorption and digestion of meconium from the intestinal lumen could have conceivably served another function to the fetus. For example, the volume of the amniotic fluids has been shown to be regulated in part by swallowing and absorption in the intestine (93). In the same manner, the absorption of meconium could have also served to regulate the volume of this material contained within the intestine.

CHAPTER IV

SUMMARY AND CONCLUSIONS

A morphological and histochemical study of the epithelium of the small intestine of the developing rat ranging in age from 16 days gestation to 26 days post partum, was performed using the following techniques: (1) hematoxylin and eosin; (2) periodic acid-Schiff; (3) Alcian blue; (4) alkaline phosphatase; (5) acid phosphatase; (6) non-specific esterase and; (7) leucine aminopeptidase. In addition to the above, other studies were performed including: (1) the DDD reaction for sulfhydryl groups; (2) the alloxan-Schiff reaction for amino groups; (3) the Nile blue sulfate method for lipofuscins; (4) the Ziehl-Neelsen method for acid fast lipofuscins; (5) the Feulgen reaction; and (6) succinic dehydrogenase. A colloidal iron preparation was injected into the amniotic cavity of fetal animals to demonstrate the absorption of amniotic fluid by the intestinal tract. To provide a means of demonstrating absorbed iron, these tissues were treated with the Prussian blue reaction.

During the fetal developmental period, the intestinal epithelium consisted of a stratified layer of cells at 16 and 17 days gestation. In the 18 day old fetuses, simple columnar epithelium developed in the proximal intestine and by 20 days gestation, transition was completed along the entire small intestine. Beginning with the 19 day stage of gestation, small PAS-positive droplets accumulated in the supranuclear

cytoplasm of the columnar cells. Granules or droplets containing iron were demonstrated in the supranuclear portion of the columnar cells following intra-amniotic administration of iron.

In the postnatal animals, large supranuclear inclusion bodies were seen in the columnar absorptive cells of the ileum and jejunum from 1 day to approximately 20 days when they disappeared. These bodies were extremely large in the ileum with dimensions ranging up to 15 by 30 microns whereas in the jejunum sizes of 2-6 microns were common. With the histochemical reactions, these bodies were PAS-positive and were uncolored by Alcian blue. The largest gave positive reactions for lipofuscins, sulfhydryl groups and amino groups and contained no nucleic acids nor succinic dehydrogenase comparing favorably with "meconium corpuscles" described in the human fetus.

In regard to the enzyme techniques, strong reactions with the acid and alkaline phosphatase and the non-specific esterase procedures were obtained in the intracellular inclusion bodies during the suckling period. Developmentally, non-specific esterase, acid and alkaline phosphatase, and aminopeptidase enzyme activities appeared in the intestinal epithelial cells at 17, 18, and 19 days gestation, respectively. At the onset, the non-specific esterase was localized in the form of scattered deposits in the basal portions of the epithelium. This was replaced by a zone of activity more extensive than the former, in the supranuclear cytoplasm of the villus epithelium at 20 days gestation. The phosphatases and aminopeptidase enzymes were localized in the cuticular border of the columnar cells reaching moderately strong levels in all segments of the small intestine by birth.

In the postnatal animals, alkaline phosphatase showed little change in activity in the striate border following birth whereas definite decreases in acid phosphatase and aminopeptidase activities occurred in that portion of the cells during the suckling period. In regards to the latter, enzyme activity was markedly reduced in intensity becoming very weak in or absent from the cells located on the distal portions of the villi. In the suckling animals, cytoplasmic non-specific esterase activity was localized in the form of scattered sites in the apical cytoplasm of the columnar cells. At weaning, a transition involving changes in the patterns of these enzymes to the adult configuration occurred.

On the basis of the results of the present study and information obtained from the literature, the following conclusions have been made:

1. Absorption of materials occur in the fetal rat intestine. This fact was suggested previously by the demonstration of antibodies in the fetal stomach contents and amniotic fluid. In the present study, this was demonstrated morphologically by the absorption of iron.

2. The intestinal enzymes become established in the fetal stages of development. This probably was a manifestation of the developmental process preparing the intestine for the tasks of digestion and absorption required in postnatal life.

3. The inclusion bodies, which contain the non-specific esterase and phosphatase enzymes seemed to be associated with processes of intracellular digestion. Because of the enzyme contents, these bodies fit the histochemical criteria for lysosomes. It is postulated that ingestion of macromolecular materials followed by intracellular digestion may be an important means of assimilating food materials

in the suckling animals.

4. Because of certain histochemical and morphological similarities, it is suggested that the inclusion bodies of both the human fetus and the suckling rats are involved in similar processes of ingestion of materials and intracellular digestion. However, it was postulated that the functional significances in each case may have been different.

5. The loss or regression of the inclusion bodies is a gradual, ordered process which occurs during the course of maturation of the intestinal tissue. This loss occurs at a certain point of maturation during the course of normal epithelial cell replacement. "Turn over" of the epithelium presumably occurred during the entire suckling period but did not result in a change of cells involving the loss of inclusion bodies until the level of maturation was reached. Therefore, it seems likely that these structures represented a developmental adaptation which occurred at a certain period of ontogeny.

In order to provide more information in this area, additional studies concerning the intestinal tract of the fetal and suckling animals can be suggested. Some of these suggestions are listed below:

1. Administer colchicine or other drug serving to arrest mitosis to suckling animals. In essence, the rate of mitosis of the crypt epithelium would be reduced which would have a direct reflection on the turn-over rate of the intestinal epithelium. Theoretically, this would cause "ageing" of the epithelial cells since they would remain on the villi for longer than normal periods of time. This would be done in an attempt to learn whether ageing of the cells would have any affect on the inclusion bodies.

2. Determine what effect, if any, the feeding of various diets to suckling animals would have on the intracellular inclusion bodies. In this procedure, diets could be altered to include high and low protein diets, liquid diets, diets with high residue and with low residue, etc. In addition, periods of fasting for various lengths of time could be included.

3. Do more studies on the intestinal epithelium of the early fetal stages. Small acid phosphatase containing inclusion bodies have been described in the stratified epithelium of 16 and 17 day old inclusion bodies by Behnke (26) and it would be interesting to determine what other enzymes, if any, are contained within these structures. To achieve proper resolution in this tissue, it may be necessary to experiment with some of the embedding rosins used in electron microscopy for light microscopic sections.

4. Perform electron microscopic histochemistry of the intestinal epithelium of the suckling and fetal animals. This would be done in an attempt to learn whether the cytoplasmic enzyme activity localized around the inclusion bodies was a manifestation of diffuse cytoplasmic activity or of enzyme containing vesicles or "storage granules." At the same time, insight into the origin of the intravesicular enzymes might be gained. If the proper methods of demonstration can be devised, it would be of interest to study the esterase positive areas in the basal cytoplasm of the crypt epithelium of the late fetal and early postnatal stages.

5. Administer isotope-labelled compounds to the digestive tract of suckling animals and follow the course taken by the ingested materials with autoradiography. In this procedure the use of both

digestible and indigestible labelled materials would be useful for study in an attempt to see: (1) the intracellular route taken by digestible materials following digestion; (2) the speed of absorption and digestion; and (3) the fate of indigestible residues within the inclusion bodies.

LITERATURE CITED

1. HEIDENHAIN, R. Beitrage zur Histologie und Physiologie der Dunndarmschleimhaut. Arch. f. d. ges. Physiol. 43 suppl: 1-46. 1888.

2. SCHMIDT, J. E. Beitrage zur normalen und pathologischen Histologie einiger Zellarten der Schleimhaut des menschlichen Darmkanales. Arch. f. mikr. Anat. u. Entwickl. ges. 66: 12-40. 1905.

3. PATZELT, B. Der Darm. Handbuch der mikroskopischen Anatomie des Menschen. Edited by W. von Möllendorff. Berlin: Springer-Verlag. V(3): 1-448. 1936.

4. PARAT, M. C. R. Soc. Biol. 87: 1273. 1922. cited by F. Bierring, et al. On the nature of the meconium corpuscles in the human fetal intestinal epithelium. I. Electron microscope studies. Acta Path. Microbiol. Scandinav. 61: 365-376. 1964.

5. PATTEN, B. M. Human Embryology. 2nd ed. New York: McGraw-Hill Book Co., 1964.

6. MAXIMOW, A. A. and BLOOM, W. A Textbook of Histology. 7th ed. Philadelphia: Saunders and Co., 1957.

7. TOBECK, A. Ueber angeborene Verschlüsse (Atresien) des Darmrohres. Virchow's Arch. 265:330-353. 1925.

8. PARAT, M. Le Meconium es-il un dechet? Bull. Histol. Appl. 1:268-278. 1924.

9. EHRLICH, P. Ueber immunität durch vererbung und säugung. Z. Hyg. InfektKr. 12:183-203. 1892. cited by F. W. R. Brambell et al. Antibodies and Embryos. London: Althone Press. 1951.

10. MÖLLENDORFF, von W. Ueber die Antilnahme des Darmepithels an der verarbeitung enteral und parenteral Zugefuhrter saurer Farbstoffe. Münch. med. Woch. 71:569. 1924. cited by S. L. Clark. The ingestion of proteins and colloidal materials by columnar absorptive cells of the small intestine in suckling rats and mice. Jour. Biophys. Biochem. Cytol. 5:41-50. 1959.

11. COMLINE, R. S., ROBERTS, H. E., and TITCHEN, D. A. Histological changes in the epithelium of the small intestine during protein absorption in the newborn animal. Nature 168:84-85. 1951.

12. SMITH, T. Hydropic stages in the intestinal epithelium of new-born calves. Jour. Exp. Med. 41:81-88. 1925.

13. COMLINE, R. S., POMEROY, R. W., and TITCHEN, D. A. Histological changes in the intestine during colostrum absorption. Jour. Physiol. 122:6P. 1953.

14. HILL, K. J. and HARDY, W. S. Histological and histochemical observations on the intestinal cells of lambs and kids absorbing colostrum. Nature 178:1353-1354. 1956.

15. HILL, K. J. Gastric development and antibody transference in the lamb, with some observations on the rat and guinea pig. Quart. Jour. Exp. Physiol. 41:421-432. 1956.

16. LEISSRING, J. C. and ANDERSON, J. W. The transfer of serum proteins from mother to young in guinea pig. III. Postnatal studies. Am. Jour. Anat. 109:175-181. 1961.

17. ANDERSON, J. W. and LEISSRING, J. C. The transfer of serum proteins from mother to young in guinea pig. II. Histochemistry of tissues involved in prenatal transfer. Am. Jour. Anat. 109:157-173. 1961.

18. LEISSRING, J. C. and ANDERSON, J. W. The transfer of serum proteins from mother to young in guinea pig. I. Prenatal route. Am. Jour. Anat. 109:149-156. 1961.

19. WRIGHT, G. H. and NIXON, D. A. Absorption of amniotic fluid in the gut of fetal sheep. Nature 190:4778. 1961.

20. WISLOCKI, G. B. Experimental studies on fetal absorption. I. The vitally stained fetus. Contributions to Embryology. Washington, D. C.: Carnegie Institution of Washington 11:47-53. 1920.

21. CLARK, S. L. The ingestion of proteins and colloidal materials by columnar absorptive cells of the small intestine in suckling rats and mice. Jour. Biophys. Biochem. Cytol. 5:41-50. 1959.

22. TAYLOR, J. J. and FLAA, R. C. Histogenesis of the small intestine of the rat. Anat. Rec. 145:353. 1963.

23. OWMAN, C. Prenatal changes in epithelium of small intestine of rat fetus pinealecomized in utero. Quart. Jour. Exp. Physiol. 48:408-422. 1963.

24. OWMAN, C. Communic. Dept. Anat. Univ. Lund, Sweden 2:1, 1962. cited by C. Owman. Prenatal changes in epithelium of small intestine of rat fetus pinealectomized in utero. Quart. Jour. Exp. Physiol. 48:408-422. 1963.

25. MOE, H. and BEHNKE, O. Cytoplasmic bodies containing mitochondria, ribosomes, and rough-surfaced endoplasmic membranes in the epithelium of small intestine of newborn rats. Jour. Cell Biol. 13:168-171. 1962.

26. BEHNKE, O. Demonstration of acid phosphatase-containing granules and cytoplasmic bodies in the epithelium of fetal rat duodenum during certain stages of differentiation. Jour. Cell Biol. 18:251-265. 1963.

27. GLUCKSMANN, A. Cell deaths in normal vertebrate ontogeny. Biol. Rev. 26:59. 1951. cited by R. Bellairs. Cell death in chick embryos as studied by electron microscopy. Jour. Anat. 95:54-60. 1961.

28. NOVIKOFF, A. B. Lysosomes and the physiology and pathology of cells. Biol. Bull. 117:385. 1959.

29. BELLAIRS, R. Cell death in chick embryos as studied by electron microscopy. Jour. Anat. 95:54-60. 1961.

30. HALLIDAY, R. The absorption of antibodies from immune sera by the gut of the young rat. Proc. Roy. Soc. Series B. 143:408-423. 1955.

31. DEUTSCH, H. F. and SMITH, V. R. Intestinal permeability to proteins in the newborn herbivore. Am. Jour. Physiol. 191:271-276. 1957.

32. PAYNE, L. C. and MARSH, C. L. Gamma globulin absorption in the baby pig. The nonselective absorption of heterologous globulins and factors influencing absorption time. Jour. Nutr. 76:151-158. 1962.

33. BIERRING, F., ANDERSEN, H., EGEBERG, J., BRO-RASMUSSEN, F., and MATTHIESSEN, M. On the nature of the meconium corpuscles in the human fetal intestinal epithelium. I. Electron microscopic studies. Acta Path. Microbiol. Scandinav. 61:365-376. 1964.

34. deDUVE, C. The lysosome. Sci. Amer. 208:64-72. 1963.

35. deDUVE, C., PRESSMANN, B. C., GIANETTO, R., WITTLAUX, R., and APPELMANS, F. Tissue fractionation studies, 6. Intracellular distribution patterns of enzymes in rat-liver tissue. Biochem. Jour. 60:604-617. 1955.

36. ESSNER, E. and NOVIKOFF, A. B. Human hepatocellular pigments and lysosomes. Jour. Ultrastructure Res. 3:374-391. 1961.

37. ASHFORD, T. P. and PORTER, K. R. Cytoplasmic components in hepatic cell lysosomes. Jour. Cell Biol. 12:198-202. 1962.

38. SHARMA, D. R. and MATZKE, H. A. Histochemical contributions to the study of lysosomes in the rat spinal cord. Anat. Rec. 151:59-68. 1965.

39. BRANDES, D., BUETOW, D. E., BERTINI, F., and MAKLOFF, D. B. Role of lysosomes in cellular lytic processes. I. Effects of carbon starvation in Euglena-gracilis. Exp. and Molec. Path. 3:583-609. 1964.

40. BRANDES, D., BERTINI, F., and SMITH, E. W. Role of lysosomes in cellular lytic processes. II. Cell death during holocrine secretion in sebaceous glands. Exp. Molec. Path. 4:245-265. 1965.

41. HSU, L. and TAPPEL, A. L. Lysosomal enzymes of rat intestinal mucosa. Jour. Cell Biol. 23:233-240. 1964.

42. NOVIKOFF, A. B. Biochemical and staining reactions of cytoplasmic constituents. Developing Cell Systems. Edited by D. Rudnick. 18th Growth Symposium. New York: Ronald Press Co., 167-204. 1960.

43. ANDERSEN, H., BIERRING, F., EGEBERG, J., BRO-RASSMUSSEN, R., and MATTHIESSEN, M. On the nature of the meconium corpuscles in human fetal intestinal epithelium. II. Cytochemical study. Acta. Path. Microbiol. Scandinav. 61:377-393. 1964.

44. KOLDOVSKY, O., FATOVA, E., HAHN, P., and VACEK, Z. The functional development of the gastrointestinal tract in rats. The Development of Homeostasis. CSAV Symposium. Prague: Czechoslovak Academy of Sciences. 155-163. 1961.

45. FARRIS, E. J. and GRIFFITH, J. Q. The Rat in Laboratory Investigation. 2nd ed. New York: Hafner Pub. Co., 1962.

46. SABATINI, D. D., BENSCH, K., and VARNETT, R. J. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. Jour. Cell Biol. 17:19-58. 1963.

47. LILLIE, R. D. Histopathologic Technique. 2nd ed. New York: Blakiston and Co., 1954.

48. MOWRY, R. B. Revised method providing improved coloration of acidic polysaccharides with Alcian blue 8GX supplied currently. Jour. Histochem. Gytochem. 8:323. 1960.

49. McMANUS, J. F. A. Histological and histochemical uses of periodic acid. Stain Technol. 23:99-108. 1948.

50. BARKA, T. and ANDERSON, P. J. Histochemistry, Theory, Practice and Bibliography. New York: Hoeber Medical Div., Harper and Row Publishers, Inc. 1963.

51. BURSTONE, M. S. Histochemical demonstration of acid phosphatases with Naphthol AS phosphatases. Jour. Nat. Cancer Inst. 21:523-540. 1958.

52. PEARSE, A. E. G. Azo dye methods in enzyme chemistry. Int. Rev. Cytol. 3:329. 1954.

53. NACHLAS, M. M., MONIS, B., ROSENBLATT, D., and SELIGMAN, A. M. Improvement in the histochemical localization of leucine animopeptidase with a new substrate L-leucyl-4-methoxy-2-naphthylamide. Jour. Biophys. Biochem. Cytol. 7:261-264. 1960.

54. BARNETT, R. J. and SELIGMAN, A. M. Histochemical demonstration of protein-bound sulfhydryl groups. Science. 116:323-327. 1952.

55. YASUMA, H. and ICHAKAWA, T. Ninhydrin-Schiff and Alloxan-Schiff staining: A new histochemical staining method for protein. Jour. Lab. Clin. Med. 41:296-299. 1953.

56. LILLIE, R. D. A Nile blue staining technique for the differentiation of melanin and lipofuscins. Stain Technol. 31:151-153. 1956.

57. PEARSE, A. E. G. Histochemistry, Theoretical and Applied. 2nd ed. Boston: Little, Brown and Co. 1961.

58. NACHLAS, M. M., TSOU, K. C., DeSOUZ, E., CHENG, C. S., and SELIGMAN, A. M. Cytochemical demonstration of succinic dehydrogenase by the use of a new p-nitrophenol substitute ditetrazole. Jour. Histochem. Cytochem. 5:420-436. 1957.

59. JOHNSON, F. P. The development of the mucous membrane of the esopahagus, stomach, and small intestine of the human embryo. Am. Jour. Anat. 10:521-561. 1910.

60. COULOMBRE, A. J. and COULOMBRE, J. L. Intestinal development. I. Morphogenesis of the villi and musculature. Jour. Embryol. Exp. Morph. 6:403-411. 1958.

61. BRAMBELL, F. W. R. and HALLIDAY, R. The route by which passive immunity is transmitted from mother to fetus in the rat. Pro. Roy. Soc. Series B. 145:171-178. 1956.

62. MOOG, F. The functional differentiation of the small intestine. II. The differentiation of alkaline phosphomonoesterase in the duodenum of the mouse. Jour. Exp. Zool. 118:187-207. 1951.

63. MOOG, F. The functional differentiation of the small intestine. I. The accumulation of alkaline phosphomonoesterase in the duodenum of the chick. Jour. Exp. Zool. 115:109-129. 1950.

64. RICHARDSON, D., SERKOWITZ, S.D., and MOOG, F. The functional differentiation of the small intestine. V. The accumulation of non-specific esterase in the duodenum of chick embryos and hatched chicks. Jour. Exp. Zool. 130:57-69. 1955.

65. HARTMANN, J. F. and WELLS, L. J. Fate of food introduced directly into the fetal stomach. Proc. Soc. Exp. Biol. Med. 68:327-330. 1948.

66. WEST, E. S. and TODD, W. R. Textbook of Biochemistry. 3rd Edition. New York: MacMillan Co. 1961.

67. CORNELL, R. and PADYKULA, H. A. A cytochemical study of intestinal absorption in the postnatal rat. Anat. Rec. 151:339. 1965.

68. HOLT, J. H. and MILLER, D. The localization of phosphomonoesterase and aminopeptidase in brush borders isolated from intestinal epithelial cells. Biochim. Biophys. Acta. 58:239-243. 1962.

69. SHNITKA, T. K. Enzymatic histochemistry of gastrointestinal mucous membrane. Fed. Proc. 19:897-904. 1960.

70. deDUVE, C. The lysosome concept, Lysosomes; Ciba Foundation Symposium. Edited by A. V. S. deReuck and M. Cameron. Boston: Little, Brown and Co. 1963. 1-29.

71. BRANDES, D. Observation on the apparent mode of formation of pure lysosomes. Jour. Ultrastruct. Res. 12:63-80. 1965.

72. MOE, H., ROSTGAARD, J., and BEHNKE, O. On the morphology and origin of virgin lysosomes in the intestinal epithelium of the rat. Jour. Ultrastruct. Res. 12:396-403. 1965.

73. HALLIDAY, R. The termination of the capacity of young rats to absorb antibody from the milk. Proc. Roy. Soc. London Series B. 145:179-185. 1956.

74. MOOG, F. and THOMAS, E. R. The influence of various adrenal and gonadal steroids on the accumulation of alkaline phosphatase in the duodenum of the suckling mouse. Endocrinol. 56:187. 1955.

75. HALLIDAY, R. The effect of steroid hormones on the absorption of antibody by the young rat. Jour. Endocrinol. 18:56-66. 1959.

76. MOOG, F. The functional differentiation of the small intestine. III. The influence of the pituitary adrenal system on the differentiation of phosphatase in the duodenum of the suckling mouse. Jour. Exp. Zool. 124:329-346. 1953.

77. DEMPSEY, E. W. Electron microscopy of the visceral yolk sac epithelium of the guinea pig. Amer. Jour. Anat. 93:331-363. 1953.

78. WEISS, J. M. The role of the Golgi complex in fat absorption as studied with the electron microscope with observations on the cytology of duodenal absorptive cells. Jour. Exp. Med. 102:775-782. 1955.

79. BENNETT, H. S. The concepts of membrane flow and membrane vesiculation as mechanisms for active transport and ion pumping. Jour. Biophysic. Biochem. Cytol. Suppl. 2:99-103. 1956.

80. MOOG, F. The functional differentiation of the small intestine. VIII. Regional differences in the alkaline phosphatases of the small intestine of the mouse from birth to one year. Devel. Biol. 3:153-174. 1961.

81. RUYTER, J. H. C. Histochemically detectable alkaline phosphatase in the cuticular border of the epithelium of the small intestine. Acta. Anat. 16:209-220. 1952.

82. ZAMCHECK, N. Dynamic interaction among body nutrition, gut mucosal metabolism, and morphology and transport across the mucosa. Fed. Proc. 19:855-864. 1960.

83. SPENCER, R. D. and KNOX, W. E. Comparative enzyme apparatus of the gut mucosa. Fed. Proc. 19:886-897. 1960.

84. PADYKULA, H. A. Recent interpretations of intestinal morphology. Fed. Proc. 21:873-879. 1962.

85. BRAMBELL, F. W. R., HALLIDAY, R., and MORRIS, I. G. Interference by human and bovine serum and serum protein fractions with the absorption of antibodies by suckling rats and mice. Proc. Roy. Soc. London Series B. 149:1-11. 1958.

86. COMLINE, R. S., ROBERTS, H. E., and TITCHEN, D. A. Route of absorption of colostrum globulin in the newborn animal. Nature 167:561-562. 1951.

87. HALLIDAY, R. Prenatal and postnatal transmission of passive immunity to young rats. Proc. Roy. Soc. London Series B. 144:427-430. 1955.

88. BRAMBELL, R. W. R., HEMMINGS, W. A., and HENDERSON, M. Antibodies and Embryos. London: Althone Press. 1951.

89. BRAMBELL, F. W. R., BRIERLEY, J., HALLIDAY, R., and HEMMINGS, W. A. Transference of passive immunity from mother to young. Lancet 266:964-965. 1954.

90. BRAMBELL, F. W. R., HEMMINGS, W. A., HENDERSON, M., OAKLY, C. L., and ROWLANDS, W. T. The accumulation of antibodies in the stomach contents of foetal rabbits. Proc. Roy. Soc. London Series B. 138:195-204. 1951.

91. ABBAS, T. M. and TOVEY, J. E. Proteins of the liquor amni. Brit. Med. Jour. 1:476-479. 1960.

92. ROULET, D. L. A. and von MURALT, G. Antigenanalytische Untersuchungen an Fruchtwasser und Meconiumprotein. Schweiz. Med. Wschr. 3:74-79. 1961.

93. GUYTON, A. C. Textbook of Medical Physiology. 2nd ed. Philadelphia: W. B. Saunders Co. 1956. 1106-1107.

PLATE I

Figure 1. Photomicrograph showing transverse sections of intestinal tube of 16 day old fetal animal. E) stratified epithelium; M) smooth muscle fibers. H & E; 200x.

Figure 2. Photomicrograph of transverse section of intestine of 17 day old fetus showing the morphology of the stratified epithelium and of the intestinal wall. Arrows indicate small buds of mesenchyme which project into the epithelial layer. F) cleft in epithelium; S) scattered mesenchymal zone. H & E; 200x.

PLATE II

Figure 3. Photomicrograph of transverse section of cephalic portion of small intestine at 18 days gestation. Observe numerous villi which project into the intestinal lumen (L). M) muscularis. H & E; 200x.

Figure 4. Photomicrograph of proximal intestine of 18 day old fetus demonstrating single layer of epithelial cells on villus (A) and stratified epithelium in crypt areas (P). H & E; 504x.

PLATE IV

Figure 7. Photomicrograph of transverse section of intermediate segment of small intestine of 18 day old fetus. Observe intraepithelial "blisters" (V) and finger-like projections of built up cells (arrows). H & E; 200x.

Figure 8. High power photomicrograph of intestinal villus of 19 day old fetus. Arrows indicate small PAS-positive droplets in the cytoplasm of the epithelial cells and within the lamina propria of villus. (E) Epithelium, (S) Stroma of villus. Gluteraldehyde fixation; PAS; 1120x.

PLATE V

Figure 9. High power photomicrograph of intestinal villus of 22 day old fetus showing numerous PAS-positive droplets and "clear" vacuoles in the supranuclear epithelial cell cytoplasm. (D) PAS-positive droplets; clear vacuoles indicated by arrows. For further orientation refer to Figures 8 and 10. Formalin; PAS-Alcian blue; 1120x.

Figure 10. Photomicrograph of intestinal mucosa of 22 day old fetus. For purposes of orientation the following are labelled: (M) microvillus or striate border; (A) apical or supranuclear cytoplasm of epithelium; (N) columnar cell mucleus; (B) basal cytoplasm of epithelium and (S) connective tissue stroma of villus. Formalin; H & E; 1120x. The arrow indicates a large unstained cytoplasmic vacuole.

PLATE VI

Figure 11. Photomicrograph of distal segment of intestine of 22 day old fetus showing accumulation of mass of mucus like material (M) within intestinal lumen. PAS-Alcian blue; 200x.

Figure 12. Photomicrograph of section of small intestine of 18 day old fetus incubated for alkaline phosphatase showing concentration of activity in the striate border of the columnar cells (see Figure 10). The arrows indicate the negative image produced by the muclei. 504x.

PLATE III

Figure 5. Photomicrograph of intestinal mucosa of cephalic portion of intestine of 18 day old fetus. Arrows indicate goblet cells in the differentiating epithelium. PAS-Alcian blue; 504x.

Figure 6. Photomicrograph of transverse section of distal segment of intestine of 18 day old fetus showing folds and furrows in the epithelium. H & E; 200x.

PLATE VII

Figure 13. Photomicrograph of intestinal villus of small intestine of 22 day old fetus. Observe moderate to strong alkaline phosphatase activity in the cuticular border of the villus epithelium. 504x.

Figure 14. High power photomicrograph of transverse sections of intestinal villi of 22 day old fetus. Observe accumulation of alkaline phosphatase in the supranuclear cytoplasm of the columnar cells (arrows). orientation, (S) represents stroma forming the core of the villus which is surrounded by radially arranged columnar cells. 1120x.

PLATE VIII

Figure 15. Photomicrograph of section of intestinal villi of 18 day old fetus incubated for acid phosphatase. Sites of acid phosphatase activity are indicated by dark precipitate in the cuticular border of the epithelium (see Figures 10 and 14). 504x.

Figure 16. Photomicrograph showing transverse sections of intestinal villi of 22 day old fetus incubated for acid phosphatase. Observe concentration of activity in the striate border and in the Golgi zone of the cells (arrows). For orientation, see Figure 14. 504x.

PLATE IX

Figure 17. High power photomicrograph of intestinal villus of small intestine of 22 day old fetal rat demonstrating accumulation of acid phosphatase in the apical cytoplasm of the columnar cells. (U) supranuclear cytoplasm; (N) area occupied by nucleus. 1120x.

Figure 18. Photomicrograph of transverse section of intestine of 17 day old fetus demonstrating non-specific esterase in the basal portions of the epithelium (arrows). (L) intestinal lumen; (T) tear splitting tissue; (S) mesenchymal stroma; (E) epithelial layer. 504x.

PLATE X

Figure 19. Photomicrograph of intestinal villi of 22 day old fetus incubated for the demonstration of non-specific esterase activity. Arrows indicate sites of enzyme activity in the apical cytoplasm of the columnar cells. 504x.

Figure 20. High power photomicrograph of intestinal mucosa of 22 day old fetus showing distribution of non-specific esterase activity around vacuoles (V) in the supra-nuclear cytoplasm of the columnar cells. The arrows indicate granules of dye indicating esterase activity. Observe absence of activity in the striate border. 1120x.

PLATE XI

Figure 21. Photomicrograph of unfixed section of intestine of 19 day old fetus incubated for leucirs aminopeptidase. Observe distribution of enzyme in apical cytoplasm (arrows) and in nuclei of cells (N). 504x.

Figure 22. Photomicrograph of fixed section of small intestine of 22 day old fetus. Observe distribution of activity (demonstrated by dark coloration) in the striate border and apical cytoplasm of epithelial cells. 504x.

PLATE XII

Figure 23. Photomicrograph of intestinal villus of 22 day old fetus which had received iron by the intra-amniotic route. Arrows indicate sites of the positive Prussian blue reaction in the apical portions of the epithelial cells. (N) epithelial cell nucleus. 1120x.

Figure 24. Photomicrograph of villus of ileum of newborn animal demonstrating PAS-positive droplets and empty vacuoles in the apical cytoplasm of the columnar cells. For orientation refer to Figure 9. PAS-Alcian blue; 1120x.

PLATE XIII

Figure 25. Photomicrograph of ileum 12 hour old rat demonstrating PAS-positive bodies in columnar cells (arrows). PAS-Alcian blue; 1120x.

Figure 26. Photomicrograph of mucosa of ileum demonstrating large PAS-positive inclusion bodies in the apical cytoplasm of the columnar cells. (I) inclusion body; arrows indicate vacuoles in apical cytoplasm and within inclusion bodies. 3 days post partum; PAS-Alcian blue; 1120x.

PLATE XIV

Figure 27. Photomicrograph of transverse sections of villus located at junction of duodenum and jejunum. Arrows indicate small PAS-positive droplets in the apical cytoplasm of the columnar cells. 3 days post partum; PAS-Alcian blue; 1120x.

Figure 28. Photomicrograph of villus epithelium of jejunum of 3 day old animal. (Arrows) PAS-positive droplets in apical cytoplasm of the columnar cells. PAS-Alcian blue; 1120x.

PLATE XV

Figure 29. Low power photomicrograph of ileum at 3 days of age showing distribution of the inclusion bodies in the villus epithelium. Observe large bodies at apical end of villus (at arrow) whereas at the proximal end, the bodies fade out (F). PAS-Alcian blue; 225x.

Figure 30. Photomicrograph of villus epithelium of ileum demonstrating large inclusion bodies filling apical portion of cell. Observe shrinkage of intravesicular material leaving clear spaces (indicated by arrows). Note that the epithelial cell nuclei are compressed at the basal ends of the cells. 5 days post partum; H & E; 504x.

PLATE XVI

Figure 31. Photomicrograph of transverse section of villus of ileum showing homogeneous inclusion bodies (I) and "clear" vacuoles (V) in apical cytoplasm. Observe small PAS-positive droplets in tunica propria of villus (arrow). 8 day old animal. Gluteraldehyde fixation; PAS-Alcian blue; 1120x.

Figure 32. Photomicrograph of gluteraldehyde fixed mucosa of ileum of 2 day old animal. Arrows indicate large clear spaces in the inclusion bodies and PAS-positive droplets in the basal cytoplasm of the columnar cells. PAS-Alcian blue; 900x.

PLATE XVII & XVIII

Figure 33. Photomicrograph of gluteraldehyde fixed mucosa of ileum of 14 day old animal. The arrows demonstrate weakly colored Alcian blue positive "rims" in the inclusion bodies. Alcian blue - hemotoxylin counterstained; 1120x.

Figures 34-37. Figures 34-37 represent diagramatic pictures of the columnar cells showing distribution of enzymes and level of activity at selected stages of postnatal development. The numbers 1, 2, 3 indicate the epithelium of the duodenum, jejunum and ileum, respectively. The block labelled "10 days" is representative of the typical picture obtained in the suckling animals. Similarly "24 days" is typical of the weanling animals. Criteria used for the classification of the enzyme activity is described at the beginning of Chapter III.

PLATE XIX

Figure 37. See Figure 34.

Figure 38. Photomicrograph showing transverse sections of villi of duodenum demonstrating distribution of alkaline phosphatase in epithelium. Observe activity in cuticular border of epithelium with weak concentration in supranuclear Golgi zone of cells (arrows). For orientation refer to Figure 14. 6 days post partum; 1120x.

PLATE XX

Figure 39. Photomicrograph showing transverse sections of villi of candal segment of gut of 12 hour old animal. Observe accumulation of alkaline phosphatase in supranuclear cytoplasm (arrows) of columnar cells; 1120x.

Figure 40. Photomicrograph showing intestinal villus of jejunum incubated for alkaline phosphatase. Observe accumulation of enzyme in cuticular border and apical cytoplasm. Arrows indicate small enzyme containing inclusion bodies. 6 days post partum; 504x.

PLATE XXI

Figure 41. Photomicrograph showing villi of ileum of 1 day old animal incubated for alkaline phosphatase. In the epithelium, observe unstained supranuclear inclusion bodies (V) surrounded by sites of weak cytoplasmic enzyme activity; 504x.

Figure 42. High power photomicrograph showing mucosa of ileum at 6 days demonstrating distribution of alkaline phosphatase activity. Observe strong activity in inclusion body (I) surrounded by a zone of diffuse activity in cytoplasm; 1120x.

PLATE XXII

Figure 43. Photomicrograph showing villus epithelium of jejunum incubated for acid phosphatase. Observe weak activity in cuticular border (K) and inclusion body demonstrating activity (arrow). 6 days post partum; 504x.

Figure 44. Photomicrograph showing villus epithelium of ileum from 12 hour old animal incubated for acid phosphatase. Observe extensive enzyme activity in supranuclear cytoplasm; 504x.

PLATE XXIII

Figure 45. Photomicrograph showing transverse sections of villi of ileum from 1 day old animal incubated for acid phosphatase. Observe unstained inclusion bodies in apical zone of epithelium surrounded by sites of cytoplasmic enzyme activity; 504x.

Figure 46. Photomicrograph showing distribution of acid phosphatase in mucosa of ileum of 6 day old animal. Observe strong activity in large supranuclear inclusion bodies (arrow) and surrounding cytoplasm; 504x.

PLATE XXIV

Figure 47. Photomicrograph showing section of intestine of newborn animal incubated for non-specific esterase. Observe sites of enzyme activity in apical portions of the cells of the villus epithelium (A). Activity in the basal cytoplasm of the crypt epithelium is demonstrated by the arrows; 310x.

Figure 48. Photomicrograph showing transverse sections of villi of ileum incubated for non-specific esterase. Positive reactions demonstrated by coarse, dense precipitate in the apical cytoplasm (arrows). Observe absence of activity in cuticular border. 12 hours post partum; 1120x.

PLATE XXV

Figure 49. Photomicrograph showing intestinal mucosa of ileum from 1 day old animal incubated for non-specific esterase. Observe weak activity in supranuclear inclusion bodies (arrows); 504x.

Figure 50. Photomicrograph of ileum of 8 day old animal showing distribution of non-specific esterase. Observe strong activity in inclusion bodies (I) and scattered sites of cytoplasmic activity in adjacent cytoplasm. Note absence of activity in cuticular border; 900x.

PLATE XXVI

Figure 51. Photomicrograph of unfixed section of jejunum of 18 day old animal demonstrating distribution of aminopeptidase activity in villus epithelium. Observe activity on proximal portions of villi (W) whereas at the distal ends (A) activity disappears; 200x.

Figure 52. Photomicrograph of fixed section of ileum of 15 day old animal showing distribution of aminopeptidase activity. Note the distribution is similar to above; 390x.

PLATE XXVII

Figure 53. Photomicrograph of transverse sections of villi of ileum of 5 day old animal showing the distribution of aminopeptidase activity. For orientation (K) represents cuticular border demonstrating weak activity and (S) represents the core of the villus. No activity is demonstrated in the inclusion bodies (arrows); 504x.

Figure 54. Photomicrograph showing transverse section of villus of ileum of 15 day old animal. The arrow demonstrates disintegration of the intravesicular material. Gluteraldehyde; PAS; 1120x.

PLATE XXVIII

Figure 55. Photomicrograph showing villus epithelium of 21 day old animal. Observe "empty" inclusion bodies at apex of villus. Formalin; PAS-hemotoxylin; 504x.

Figure 56. Photomicrograph of gluteraldehyde fixed section of ileum of 20 day old animal showing "empty" vacuoles in epithelium (arrows). PAS; 504x.

PLATE XXIX

Figure 57. Photomicrograph showing villus epithelium of ileum incubated for acid phosphatase. Empty inclusion body demonstrated by arrow. 20 days post partum; 504x.

Figure 58. Photomicrograph showing section of duodenum incubated for alkaline phosphatase. Observe strong activity in striate border of epithelium and a weak zone of activity in the supranuclear Golgi zone of the columnar cell (arrows). 22 days post partum; 225x.

PLATE XXX

Figure 59. Photomicrograph showing section of duodenum incubated for acid phosphatase. Observe strong activity in striate border of villus epithelium. Arrows demonstrate weak activity in Golgi zone of epithelium. 23 days post partum; 225x.

Figure 60. Photomicrograph showing section of duodenum incubated for non-specific esterase. Observe course precipitate in cells at villus apex (arrow) whereas in more proximal portions more extensive activity exists (H). Observe absence of activity in striate border (K). 21 days post partum; 340x.

PLATE XXXI

Figure 61. Photomicrograph showing distribution of leucine aminopeptidase activity in villus epithelium (arrow). 19 days post partum; 400x.

Figure 62. Photomicrograph showing distribution of alkaline phosphatase in ileum of 23 day old animal. Arrow indicates sites of activity; 225x.

PLATE XXXII

Figure 63. Photomicrograph of section of ileum demonstrating positive reactions for lipofuscins in inclusion bodies (arrows). Dark precipitate demonstrates artifact produced by migration of dye. Nile blue sulfate; 15 days post partum; 225x.

Figure 64. Photomicrograph of section of ileum treated with Ziehl-Neelsen method for lipofuscins. Observe acid fastness in large inclusion bodies (arrow). Toluidine blue counterstain; 3 days post partum; 504x.

PLATE XXXIII

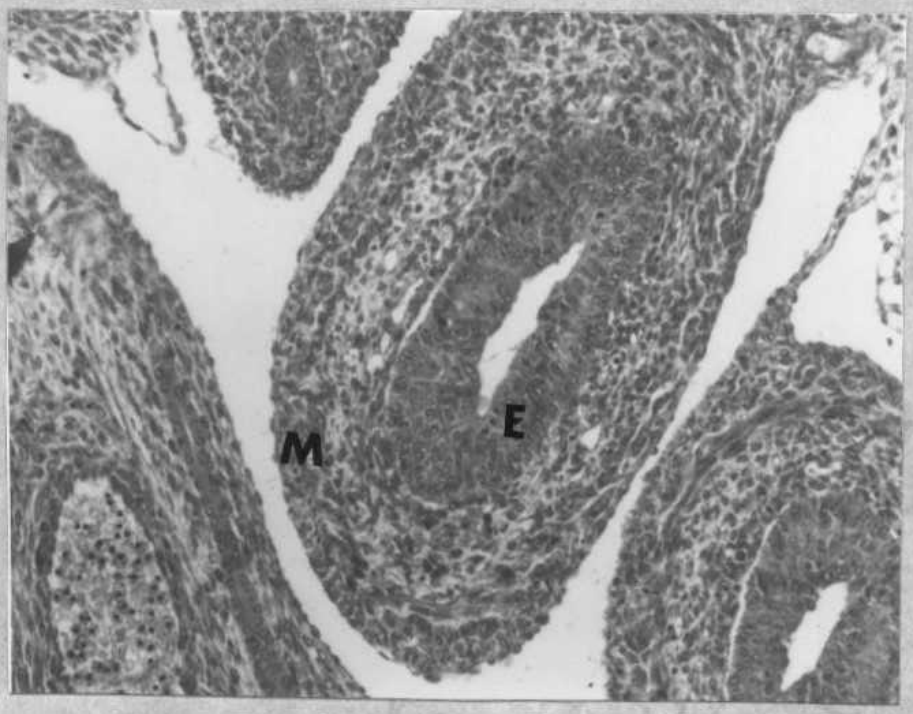
Figure 65. Photomicrograph showing action of ileum demonstrating positive reactions for sulfhydryl groups (arrows). Observe positive reactions in erythrocytes in core of villi. DDD reaction; 3 days post partum; 400x.

Figure 66. Photomicrograph showing section of ileum demonstrating positive reactions for amino groups (arrow). Alloxan-Schiff reaction; 6 days post partum; 504x.

PLATE XXXIV

Figure 67. Photomicrograph of section of ileum treated with Feulgen reaction. Observe positive reaction in nuclei and negative reaction in inclusion bodies (arrows). 10 days post partum; 504x.

Figure 68. Photomicrograph of section of ileum treated with succinic dehydrogenase reaction. Arrows indicate negative image created by inclusion bodies. (N) nucleus. Unfixed frozen section; 9 days post partum; 504x.



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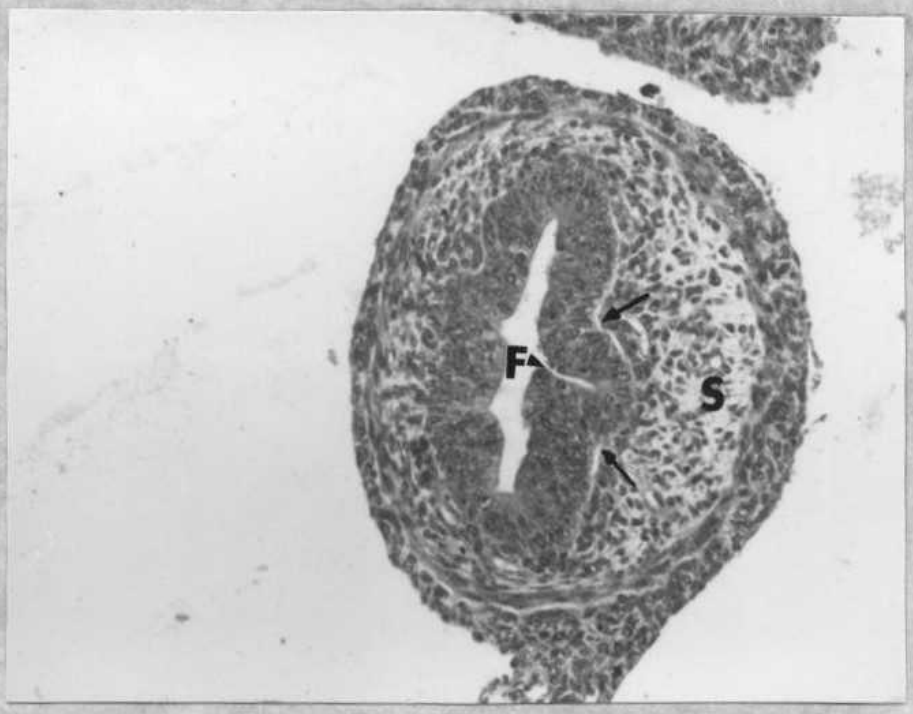
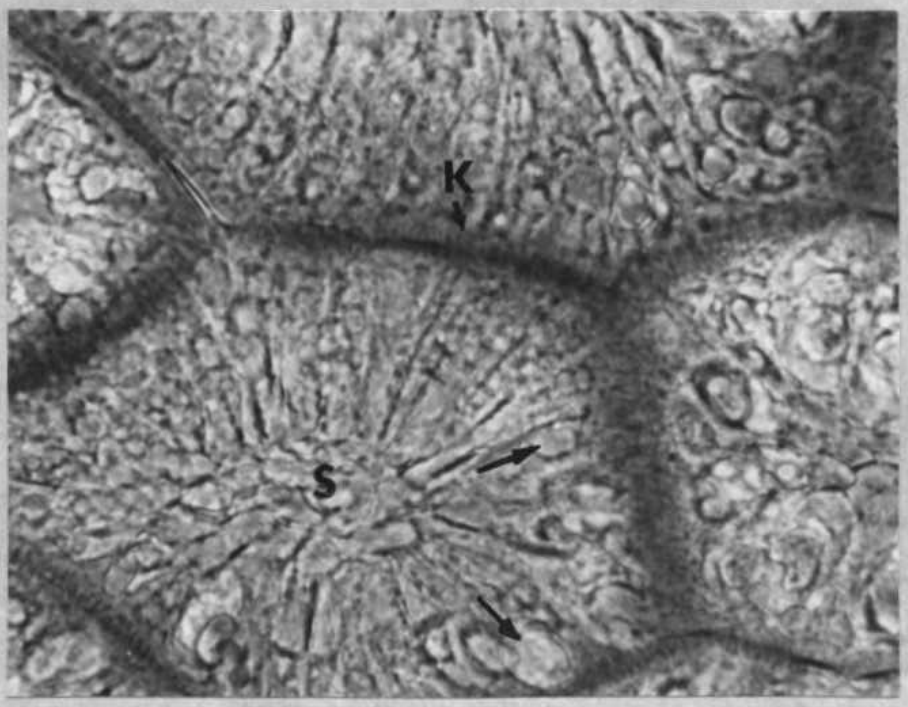


Figure 2

PLATE I



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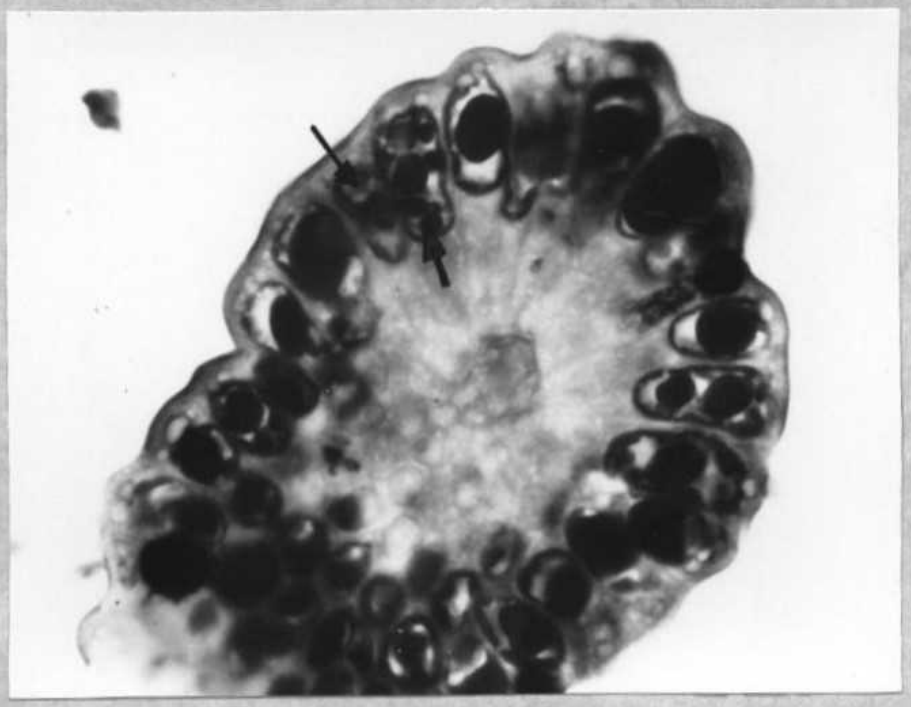
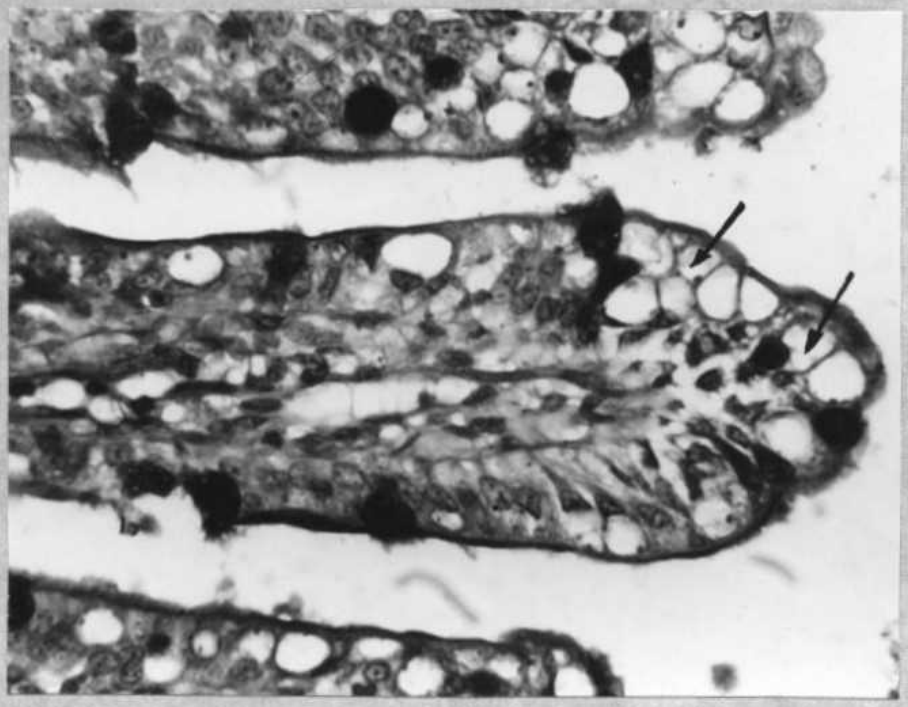


Figure 54

PLATE XXVII



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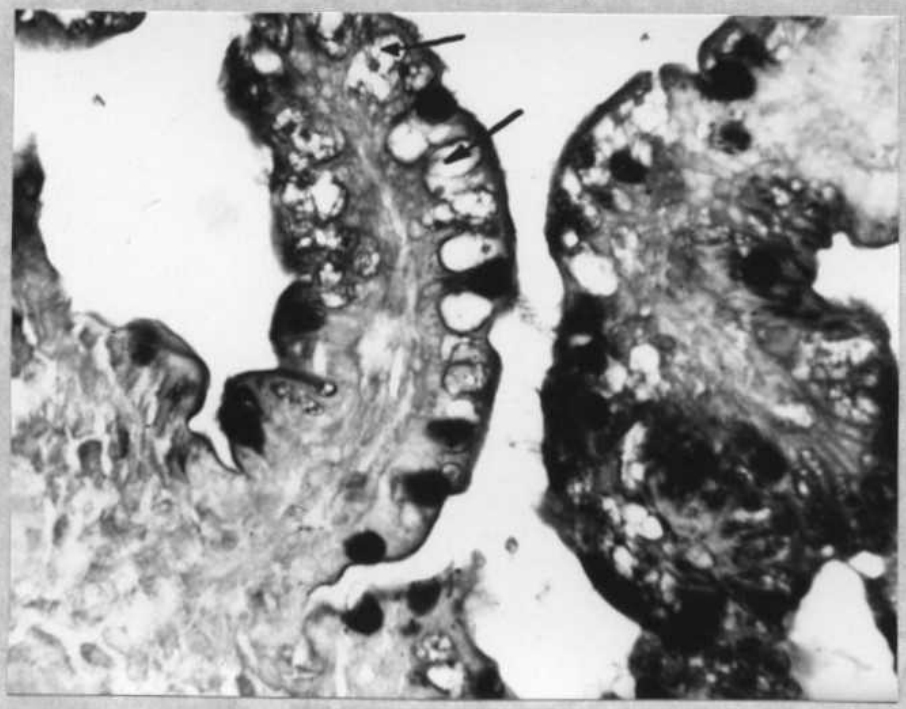


Figure 56

PLATE XXVIII

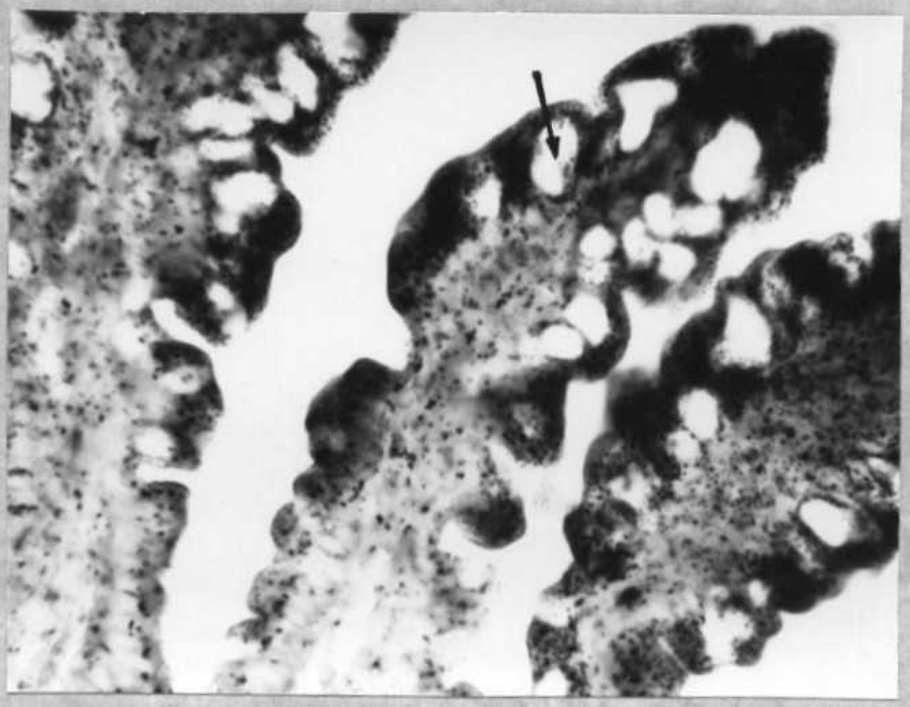
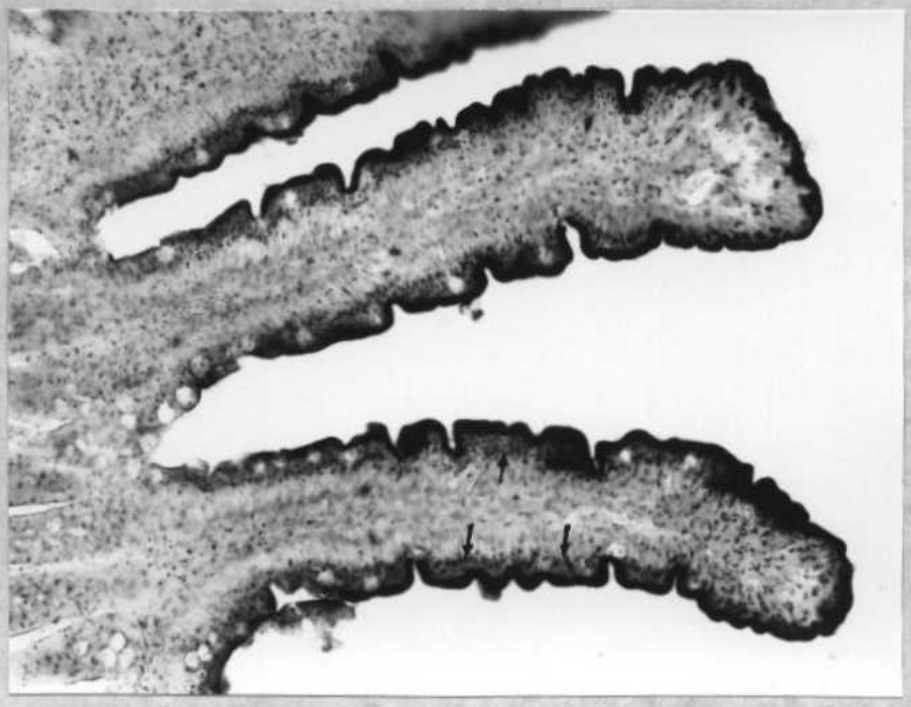


Figure 57

Figure 58

PLATE XXIX



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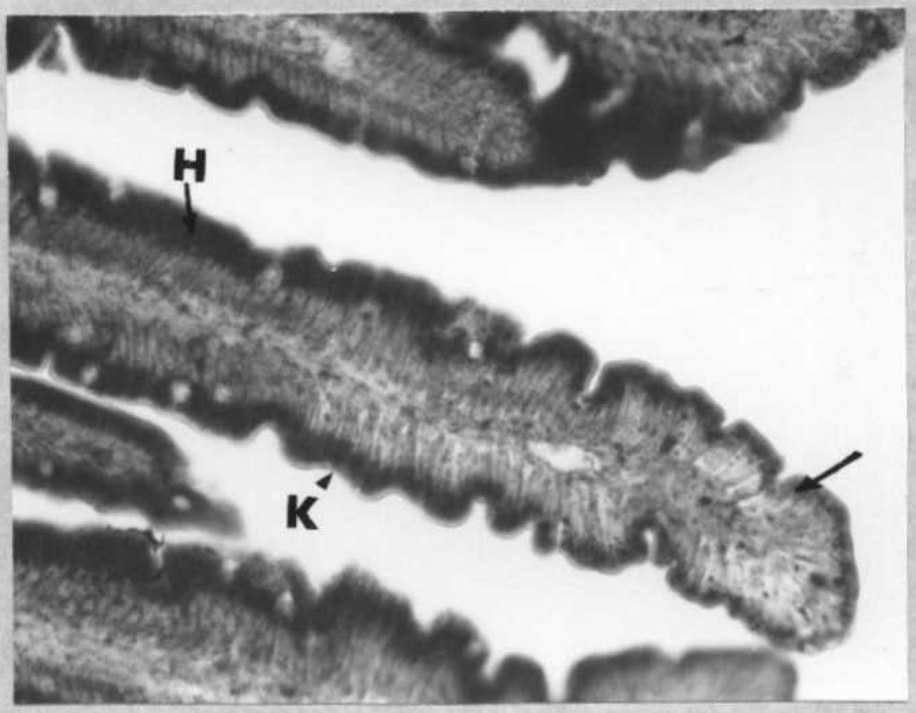
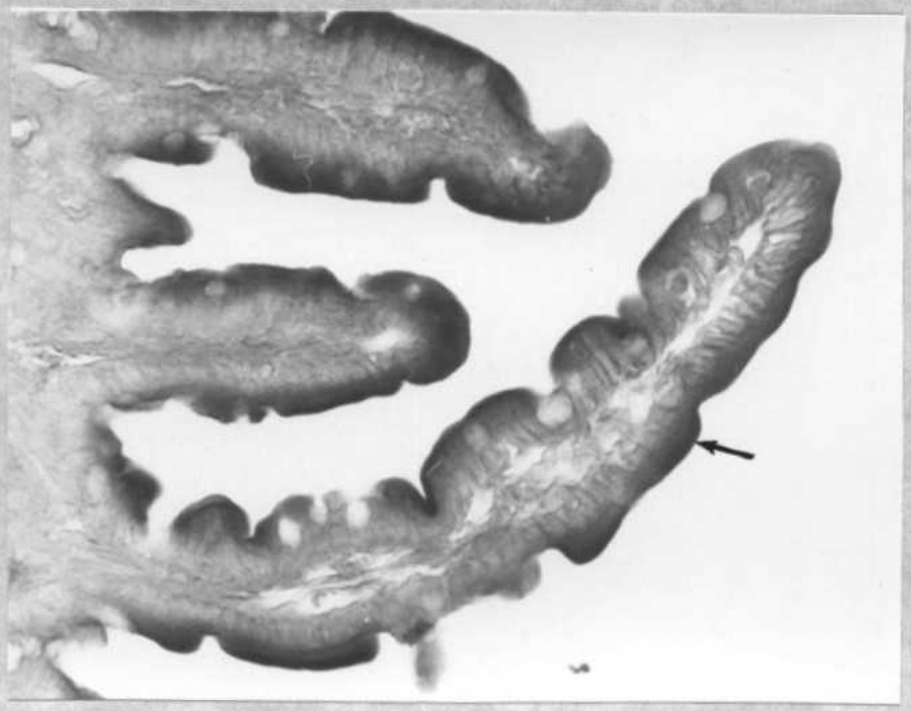


Figure 60

PLATE XXX



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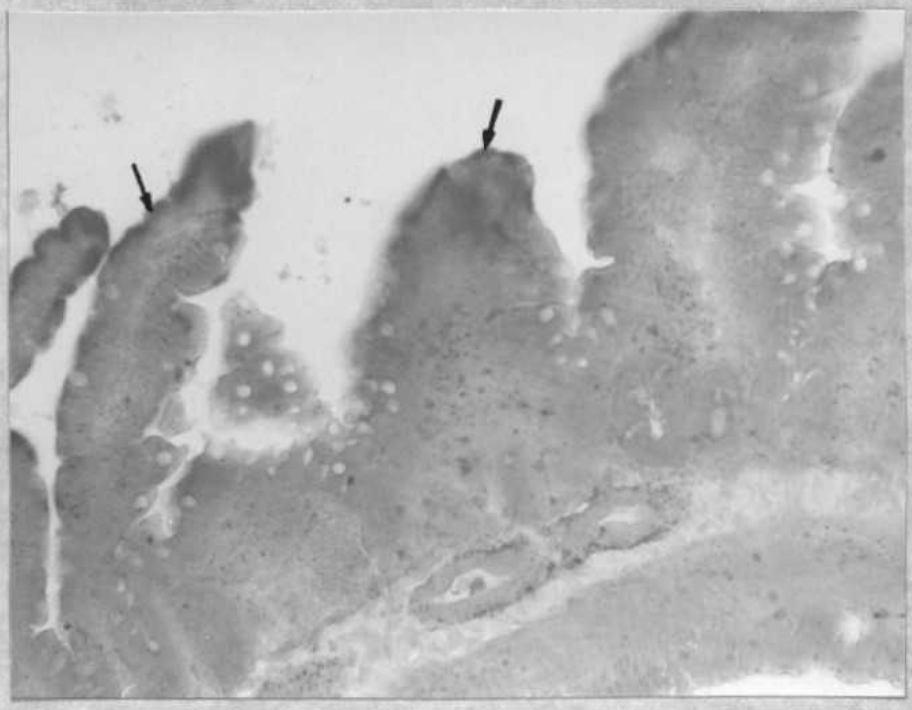
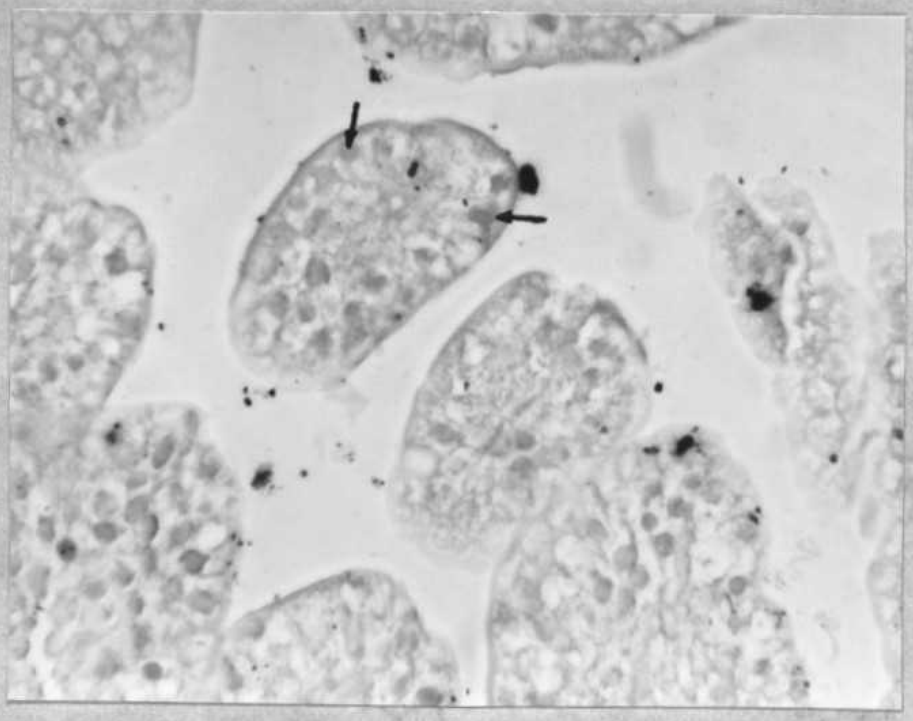


Figure 62

PLATE XXXI



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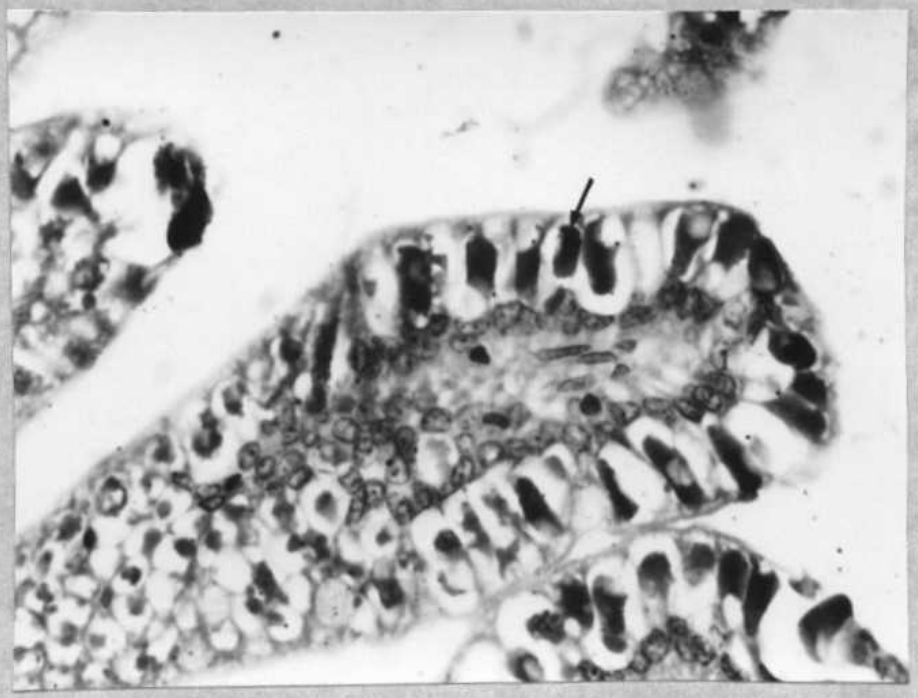

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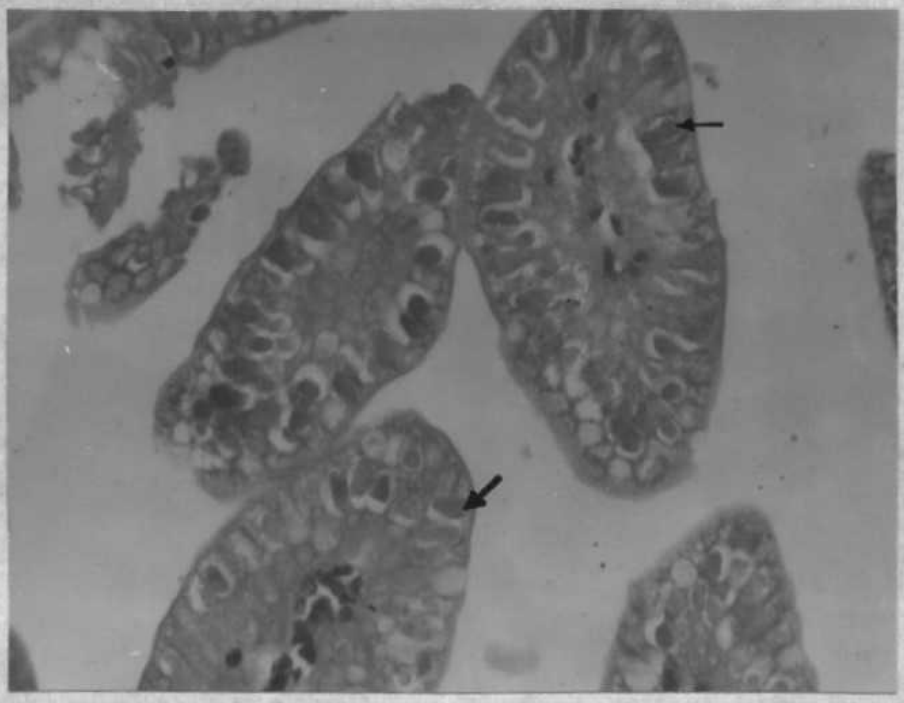
PLATE XXXII



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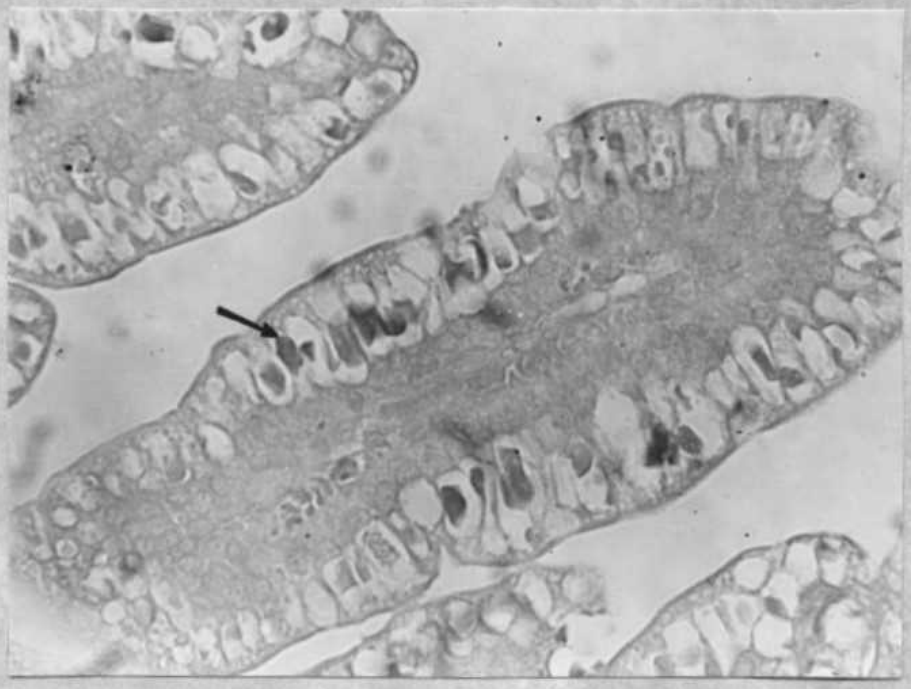
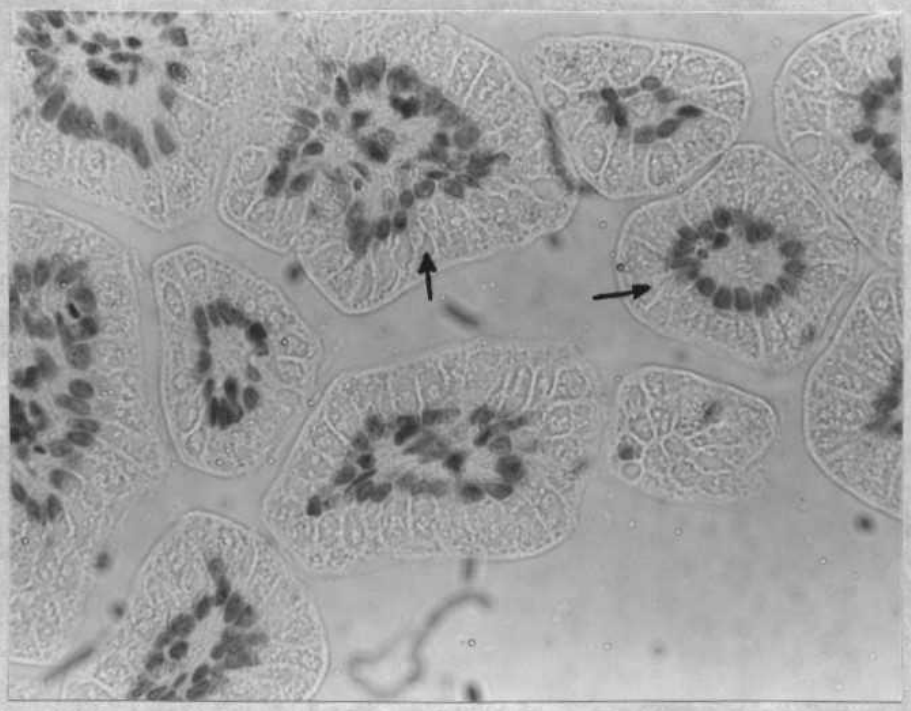


Figure 66

PLATE XXXIII



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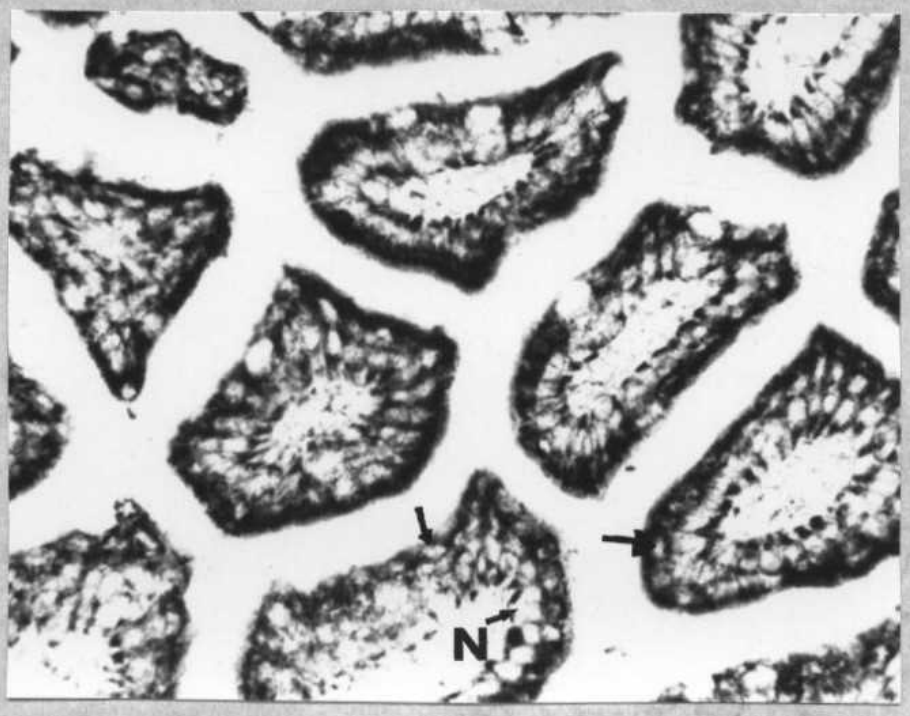

Figure 68

PLATE XXXIV