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THE EFFECT OF GASTRIN ON THE SMALL INTESTINE

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THE EFFECT OF GASTRIN ON

THE SMALL INTESTINE

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W H O A R E B O T H

B I G A N D S M A L L

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THE EFFECT OF GASTRIN ON THE SMALL INTESTINE

CHAPTER I

INTRODUCTION

The intestinal epithelial cells represent one of the most dynamic cell populations of the body. Their cellular properties are highly variable and change with respect to their location on the villus surface, the position along the length of the intestine, the quantity, form, and nutritive value of ingested food, and the age of the animal. The cellular properties which are affected by these conditions include the characteristics of division in the crypts of Lieberkuhn, rate of migration up the villi, level of differentiation, and the rate of cellular extrusion at the villus tip. Consequently, the entire cell renewal process is usually affected and as is usually the case, all properties are changed in parallel so that synchrony of cellular events is not disrupted.

The nature of the agent, or agents regulating this complex cellular renewal mechanism are presently being investigated in many laboratories. Although some progress has been made in this area, the extreme sensitivity of the intestinal tract to changing local conditions is still unexplained. Isolated changes in intestinal properties have been observed in response to various types of hormone therapy (i.e., growth

hormone, thyroxin, insulin, glucagon, corticosteroids). However, few, if any, of these responses can be explained teleologically, and the physiological importance of these hormones in the regulation of intestinal structure and function is presently uncertain.

Strangely missing from this long list of hormones, which have been implicated in the control of intestinal cell renewal, is gastrin. Gastrin is a hormone which is released from the antral region of the stomach and most probably from the small intestine as well. Gastrin's role as a secretagogue is now well established; however, recent experiments have indicated that it may have an equally important second function. There is evidence that it may have a potent trophic influence on gastro-intestinal tissue. It is also known that gastrin release is extremely variable and can be influenced by many of the aforementioned conditions that stimulate changes in intestinal structure and function. For this reason we have decided to investigate the effect of gastrin on intestinal properties both at the gross and cellular levels.

This introductory chapter will be outlined as follows: 1) Brief History of Gastrin (1900-1960); 2) Normal Intestinal Properties in the Adult; 3) The Effects of Starvation on Intestinal Properties; 4) The Effects of Gastrointestinal Surgical Procedures on Intestinal Properties; 5) The Ontogenic Development of Intestinal Properties; 6) Effect of Hormones (other than Gastrin) on Intestinal Properties; 7) Gastrin as a Trophic Agent; 8) Possible Regulatory Role of Gastrin on Intestinal Property Changes; and 9) Hypotheses.

The majority of experiments to be discussed in this section were performed on rodents (either rats or mice), and for the sake of

simplicity, the species will not be specified. Experiments on other species will be referred to accordingly. In addition, we will limit the scope of this dissertation to the properties of the principal epithelial cells.

Brief History of Gastrin (1900-1960)

The gastric secretory nature of gastrin was first revealed by the classical experiments of Edkins (10). In this study, it was found that injections of homogenates of pyloric regions of the stomach into a dog stimulated a gastric secretory response in the recipient. Edkin hypothesized that a hormonal mechanism most probably was involved, similar to the mechanism disclosed by Bayliss and Starling, who had demonstrated that injections of duodenal mucosal homogenates stimulated a secretory response from a denervated pancreas (4). Later, of course, it was demonstrated that this crude homogenate contained the hormone, secretin. In the same sense, Edkins postulated the presence of a hormone, gastrin, in the pyloric region of the stomach. The importance of this pioneering experiment was then minimized during the Pavlovian period when the neurological regulation of the stomach was emphasized. The effect of histamine on gastric secretion was then revealed, and since histamine was known to be present in the gastric mucosa, Edkins' hormonal theory seemed to be dead (101, 106). It was not until Komarov's experiment in the 1930's that the gastrin theory was revived (128). In this experiment, a histamine-free gastric mucosal proteinaceous fraction was injected into an animal and successfully stimulated a gastric secretory response. This experiment was followed by the classical study of Grossman, Robertson, and Ivy who disspelled any doubt concerning the presence of gastrin (43).

They successfully induced a gastric secretory response in a denervated transplanted fundic pouch by mechanically distending the pyloric glandular region of the stomach. The final proof of the presence of gastrin was made when Gregory and Tracy synthesized the hormone and, upon injection, mimicked the gastric secretory effect of the endogenous agent (39). Since these classical papers were published, numerous other studies have been made elucidating the actions and properties of gastrin. For the sake of brevity, we will only list some of the major findings in this area:

1) gastrin release is triggered by vagal stimulation (125)

2) gastrin release is stimulated by mechanical distension of the antrum (43)

3) other stimulants of gastrin release are simple structured amino acids and Ca^{++} (41, 102)

4) gastrin is synthesized and released in the antrum and the duodenum (41, 42)

5) gastrin's active site for gastric secretion is found in the C-terminal tetrapeptide (40)

6) gastrin is believed to act directly on the parietal cell to stimulate HCl secretion (57)

7) gastrin release is inhibited by acid in the antrum and duodenum (the latter mechanism may be due to the stimulation of secretin release) (41, 59)

In the remainder of this chapter we will discuss the properties of the intestinal epithelium under normal and changing conditions; then we will discuss our present inadequate knowledge of the mechanisms that may be involved in the control of these properties. The more recent findings on the second action of gastrin, as a G.I. trophic agent, will then be introduced, and an argument will be made implicating gastrin as a major intestinal regulatory factor.

Normal Intestinal Properties in the Adult

Brief Discussion of Intestinal Physiology

The structure of the small intestine is divided into three major regions: the duodenum, the jejunum, and the ileum. The duodenum receives food from the stomach and is the region where the pancreatic juice mixes with the food bolus and where the bulk of hydrolysis of lipids, proteins, and carbohydrates is believed to occur. The hydrolyzed food particles are propelled into the jejunum where the final stages of digestion and absorption of proteins and carbohydrates occur. These digestive and absorptive processes are believed to occur sequentially on the brush border membrane of the intestinal epithelial cell (16). This unique spatial relationship of these two processes has been referred to as demonstrative of the intestinal property of "kinetic advantage" (16). It is believed that the ileum is a major site of fatty acid and bile salt absorption, and it is here that micelles come in contact with the brush border membrane and the contents are discharged and transported into the cell for processing. It should be emphasized that the three major regions of the small intestine are not as functionally compartmentalized as one might suspect from the description given here; digestion and absorption of protein, lipids, and carbohydrates occur along the entire length of the small intestine, but the optimal activity of these processes occurs in the regions just specified.

General Discussion

If one places a cross section of a region of the small intestine under the light microscope, the gross mucosal structure can be examined. The mucosa of the small intestine consists primarily of two

areas: a crypt region at its base in which cellular proliferation is believed to occur, and finger-like villus structures onto which the immature, non-proliferative epithelial cells migrate and eventually differentiate into functional digestive epithelial cells (76). Consequently, both the crypt and villus are studded by epithelial cells at different levels of maturation and functional potential. In the remainder of this section the discussion will deal with our present knowledge of the cellular properties of epithelial cells located on the crypt and the villus and the transitional changes that occur as migration proceeds.

The average height of an intestinal crypt in the rat has been found to be approximately 35 cells high (13). It has also been demonstrated that crypt height decreases linearly as one moves distally along the small intestine (1). It has been shown by Carnie, et al., employing radioautography to monitor the incorporation of ^{3}H-thymidine into DNA, that approximately 60% of the cells near the base of the crypt are active in DNA synthesis (13). However, as one moves towards either the very base of the crypt or its top, the incidence of cells active in DNA synthesis markedly decreases. It was calculated that only approximately 30% of the total cells lining the crypt participate in DNA synthesis. Not surprisingly, the incidence of cells observed in mitosis follows a similar distribution but in this case the optimal region of activity is shifted slightly towards the top of the crypt. This indicates that the cells have migrated between the time of DNA synthesis (S) and mitosis (M) of the cell cycle. (A diagrammatic representation of the cell cycle can be found in the Appendix, page 153.)

It was also found that several hours after the injection of

^{3}H-thymidine, approximately 95% of the crypt cells that were dividing carried label indicating that the cells undergoing DNA synthesis (as indicated by incorporating ^{3}H-thymidine) were preparing for cell division.

In order to understand the cellular processes in the crypt further, Carnie, et al. designed a series of theoretical model systems which would account for their experimental findings (13). Their steady state model which best approximated their experimental results was based on the following assumptions: 1) the crypt is a blind ending tube with no influx of precursor cells, 2) all cells, whether proliferative or non-proliferative, migrate to the mouth of the crypt in columnar fashion, 3) proliferative cells are present in the bottom third of the crypt, 4) the frequency of appearance of non-proliferative cells increases linearly as the cells move up the middle third of the crypt, and 5) only non-proliferative cells are present in the top third of the crypt. It is these non-proliferative cells which will ultimately differentiate into the digestive epithelium. The model is also based on the assumption that there are two possible routes which a cell may take to become "non-proliferative" (Q):

(1) by symmetrical division

$$P \longrightarrow Q + Q$$

(2) by asymmetric division

$$P \longrightarrow Q + P$$

While asymmetric division is impossible genetically, the authors have argued that a non-proliferative nature can be induced by environmental factors.

Let us now briefly examine the biochemical properties of

proliferative crypt epithelial cells.

The epithelial cells located in the crypt are programmed for proliferation. As just described, DNA synthesis occurs exclusively in the crypt. This property has been demonstrated both by radioautography and by measuring DNA synthesis directly by scintillation counter determinations on serial transverse sections of frozen intestinal tissue (13, 21). Similarly, it has been demonstrated that RNA and protein synthesis takes place primarily in the crypt region, although significant activity has been demonstrated to spread to the villus base, and to some extent, to the body of the villus itself (81). In order to perform these biosynthetic processes, the immature crypt epithelial cells contain highly active enzymes which catalyze the manufacture of these cellular components. The enzymes located in these cells are too numerous to be listed here, but the major ones are the purine and pyrimidine biosynthetic enzymes, RNA polymerase, and the mitochondrial and pentose shunt enzymes which provide the cofactors and high energy compounds needed for the biosynthetic reactions to proceed to completion (32, 98, 128). It is also interesting that significant activity of several digestive enzymes, notably alkaline phosphatase, esterase, and acid phosphotase have been found in epithelial cells just below the mouth of the crypt (26).

The ultrastructure of epithelial cells in the crypt has been studied extensively by several investigators (99, 122). At the periphery, these immature epithelial cells are studded by short microvilli, which are relatively few in number and oriented in a haphazard manner. Core structures are found within the cylindrical microvilli, which are covered by a very thin, transparent fuzzy coat. The cells are

cuboidally shaped and have a predominant nucleus (constituting approximately 30% of the cellular volume). The endoplasmic reticulum is poorly defined and polysomal clusters are frequently found. The latter is a common characteristic of immature cells of many tissues (99). The Golgi complex is present and intricately formed in these young cells. Consistent with this finding is the frequent occurrance of zymogen-like vesicles throughout the cytoplasm (for it is believed that zymogen granules are packaged in the Golgi) (56). One fascinating characteristic of these cells is that it appears that they tend to release giant cytoplasmic blebs (several micra in diameter) into the lumen. These cytoplasmic regions which are secreted by an exocytotic process may contain ribosomes, secretion granules, or an occasional membraneous cisterna.

As alluded to previously, the epithelial cells at the base of the crypt migrate towards its mouth. It has been determined that the rate of migration increases as the cell ascends, until it attains a maximum velocity in the upper third of the crypt (13). Once a cell reaches this position, it is invariably non-proliferative and is thought to be locked into the G_1 phase of the cell cycle (13). It has been observed that it takes approximately 12.5 hours for a cell to migrate onto the villus base (13). Once the cell reaches the villus base, it begins to ascend up the villus at a constant rate. It has recently been demonstrated that these two migratory processes are independent and can be altered separately under differing conditions (51). Villus migration has been determined to take between one and two days under normal conditions depending on the region of intestine studied (one day in the

duodenum and two days in the ileum). As the mature cell reaches the villus apex it is extruded into the intestinal lumen where it becomes part of the "succus entericus" or intestinal secretion. The relationships between cellular division in the crypt, migration of the cells up the crypt, villus migration and the eventual cellular desquamation is presently unclear.

As the cell migrates up the villus, it begins to differentiate into a mature digestive intestinal epithelial cell (33). Its biosynthetic potential as indexed by the quantity of RNA, DNA, and biosynthetic enzymic activity is diminished in the upper villus regions (21, 32, 128). In contrast, its digestive enzyme potential increases markedly as the cell ascends the villus. It has been demonstrated by several diverse techniques that all brush border enzymes increase in stepwise fashion as the cell moves up the villus (20, 98, 128). Included among these enzymes are lactase, maltase, and alkaline phosphatase, levels of which will be reported in the "Results" section of this dissertation. It is presently not known whether the increase in digestive enzymatic activity is due to activation of preexistent enzymes or the initiation of their synthesis.

Concomitant with the increase in digestive enzymatic activity are the ultrastructural changes that correspond with the transition from immature cells to mature epithelial cells. A multitude of long thin microvilli cover the surface of the cell. It has been calculated that their number increases approximately ten fold from the sparsely covered crypt cells (122). Their presence increases the absorptive digestive cell surface which is exposed to food particles in the lumen. Since it

has been established that many of the digestive enzymes (including maltase, lactase, and alkaline phosphatase) are located on the brush border membrane (91), it follows that there would be an expected increase in enzyme activity.

The microvilli of the mature cell contain core filaments of unknown function, although recently it has been postulated that they may have a contractile nature, serving as a microvillus pumping mechanism (54). The core filaments rest on a filamentous structure, known as the terminal web, which runs perpendicular to them. It is this structure that adds rigidity to the brush border complex and accounts for its intact isolation upon fractionation of the intestinal mucosae (97). It should be noted that the terminal web is absent in the immature crypt epithelial cells (122).

The microvillus membranes of mature intestinal epithelial cells are covered with a thicker, less filamentous fuzzy coat than is found in the crypt. Some other ultrastructural characterisitcs of a mature intestinal villus epithelial cell in contrast to an undifferentiated crypt cell is its columnar shape, its increase in cytoplasmic mass, and in turn, its less predominant nucleus (14% of the cellular volume) and its more extensive and better developed endoplasmic reticulum (122).

The mechanisms regulating the processes of cell division and migration in the crypt, villus migration, differentiation, and extrusion just described in the above section are not understood. The interrelationships and interdependence of these cellular events is also unclear at the present time. Each of these cellular processes is highly variable and can be readily changed by altering environmental conditions.

The Effect of Starvation on Intestinal Properties

Starvation is known to have severe atrophic effects on the intestinal mucosa. It has been demonstrated that both villus and crypt height are markedly reduced by food deprivation. Steiner and co-workers have demonstrated that the marked reduction of intestinal wet weight, RNA, DNA, and protein content is disproportional to the loss of body weight during the starvation period (116). This finding has recently been verified by McManus, et al. who measured dramatic decreases in intestinal DNA content during short periods of starvation (88). As far as is known, no other organ is as sensitive to food deprivation as is the small intestine.

Earlier experiments have shown that starvation retards the mitotic activity in the crypt to approximately half its normal frequency (23, 50). More recently, Hopper has performed cell cycle analyses on crypt cells and demonstrated that the cell cycle changed very little during a period of starvation. It was found, however, that the phases of the cycle did change markedly during this period, with G_1 being significantly shortened and S being significantly prolonged (103).

The effect of starvation on cellular migration rate is presently quite confusing. Brown, et al. have demonstrated that starvation of mice from one to four days retards the migration rate to approximately half its normal value (9). This would, in turn, double the villus residence time of each epithelial cell. This determination was performed by injecting ^{3}H-thymidine into two groups of mice (starved and fed controls) sacrificing the animals at different times, removing a jejunal segment, performing autoradiography on a section of it, and measuring

the progression of the label up the villus with time. However, Hopper, et al. have shown in a recent paper that the effect of starvation on the migratory process is more complex in the rat (and possibly in the mouse as well) (51). They found that the migration rate in the crypt is significantly reduced during a ten day starvation period, increasing the time it takes a crypt cell to ascend to the villus base from 12.5 (control) to 16.3 hrs (starved). However, once a crypt cell (from a starved animal) reaches the villus base, its rate of migration is markedly accelerated so that it reaches the villus tip in a shorter period of time than do the cells of the fed control. The reason for the different effects of starvation on crypt and villus migration rates are unexplained at present. There are two possible explanations for the increase in villus migration rate. First, it is known that starvation is associated with a marked shortening of the villus height (119). Consequently, if the villus migration rate is unaltered by starvation, one would expect the cell to reach the villus tip in a shorter period of time than is the case in the fed control. This argument brings up the question of the proper use of the term "migration rate". Does it mean cell velocity, or percentage of villus height covered in a period of time? The use of the term is extremely vague in the literature. The second possible explanation for the "increased villus migration rate" involves the effects of starvation on the last phase of the cell renewal system, cellular desquamation at the villus apex. Hooper and colleagues observed a marked reduction in the number of epithelial cells lining the villus during periods of starvation (49). They also determined that the diminished villus cell number could not be attributed totally to the reduced

mitotic activity in the crypt, and concluded that cellular desquamation must be increased during starvation. This in turn may stimulate the villus migration rate so that villus integrity is preserved. In a later paper, however, they failed to find a difference in the number of extrusion zones on villi of fed and starved animals (50). Consequently, the tentative conclusion of his previous paper is presently dubious.

The effects of starvation on intestinal epithelial cell differentiation is also quite confusing at the present time. Histological stains for acidophilic granules in the cytoplasm (usually a sign of differentiation of cells from a proliferative to a secretory nature) indicate that starvation may cause an impairment of cellular maturation (77). This observation was then supported by the finding of Blair, et al. and Deren, et al. who found significant decreases in the specific activity of the brush border enzyme, sucrase during a starvation period (5, 22). On the other hand, Levin, et al. found no significant difference in the specific activity of maltase (a brush border enzyme which has biochemical properties similar to sucrase, and which actually may be part of the same protein molecule) and recorded a significant increase in intestinal specific peptidase activity (79, 108). There have recently been several papers that support the theory of increased biochemical differentiation during starvation. McNeil, et al. have demonstrated significant increases in specific lactase activity during starvation (89). This was supported by the experiments of Troglia, et al. who demonstrated dramatic increases of intestinal disaccharidase activity (sucrase, maltase, and lactase) during periods of chronic undernutrition (127). Increased differentiation of intestinal epithelial cells has also been demonstrated by

Kershaw, et al. who showed marked increases in active transport of amino acids and glucose, employing both in vitro and in vivo techniques (62). Thus, in summary, the effects of starvation on intestinal epithelial differentiation are quite confusing.

Cell cycle analysis can also be used as supportive evidence that differentiation is increased during starvation. Hopper, employing the method of Carnie, et al., has measured the maturation phase of crypt intestinal epithelial cells by using the following formula (13, 51): (Time 50% of villi are labelled after ^{3}H-thymidine injection) - (1/2 S + G_2 + M) = maturation phase. They have found a significant lengthening of the maturation period during starvation.

The morphologic changes occurring during starvation are quite apparent. As mentioned previously, there is a striking diminution of villus height, and the number of epithelial cells lining the crypt and the villus is markedly reduced (119). These findings have recently been supported by a study involving long term starvation of frogs (100). In addition, these investigators recorded a two-fold decrease in the number of villi per intestinal ring section after a four month starvation period.

There have been no electron microscopic studies of the effects of starvation on epithelial structure. However, the following high power light microscopic findings have been made. There is an increase in the relative size of the nucleus of crypt epithelial cells during a short period of starvation. In addition, these cells show a marked increase in vacuolization (9). The villus epithelial cells assume a more cuboidal configuration and also have an unusually high number of vacuoles

in their cyotplasm (9, 100). The brush borders of the epithelial cells are also only faintly visible during starvation (119). Whether this is due to diminuition in the size and number of microvilli or to the absence of stainable or dense substance within them can only be answered by a comprehensive electron microscopic study.

In conclusion, it appears that starvation has broadly based effects on the cell renewal system of the intestinal epithelium. Cell division, maturation, migration, and extrusion all seem to be highly sensitive to the amount of food ingested by the animal. Some of the findings discussed above have added to our knowledge of the oell renewal mechanism, i.e., that crypt and villus migrations are independent of one another, and that villus migration is not merely a result of the pressure of cells emerging from the crypt; other findings have added a certain amount of confusion to the problem. It is our desire in the experimental section of this dissertation to investigate further areas that are presently under contention. We will also investigate a possible endocrine mechanism responsible for the supersensitivity of intestinal tissue to the absence or presence of food in the intestinal lumen.

Effects of GI Surgical Procedure on Intestinal Properties

It has been found that epithelial cell dynamics can be radically altered by surgical procedures of the small intestine. Loran, et al. have demonstrated that surgical resection of 10 cm of ileum (approximately 10% of the length of intestine) from rats stimulates rapid proliferation of cells in the crypts (82, 83). Normally approximately 30% of the crypt epithelial cells are labelled within one hour after injection of ^{3}H-thymidine (indicating active DNA synthesis) (13, 83). The

percentage of labelled cells in the crypt then increases for several hours after the injection and eventually plateaus at approximately 65% (the time it takes to plateau is shortest in the duodenum and longest in the ileum). The period of time it takes to plateau has been interpreted by the authors to represent the transit time of an epithelial cell from the proliferative zone in the crypt to the base of the villus. After ileal resection, however, this situation changes dramatically. One hour after injection of ^{3}H-thymidine approximately 65% of the crypt cells are labelled in all regions of the intestine. This percentage remains constant for several hours after injection unlike the increasing percentage of the controls. This finding was interpreted to indicate that crypt transit time of epithelial cells in these resected intestines is so rapid that accumulation of labelled cells in the crypt fails to occur and no additive effect is recorded. These conclusions were supported by the finding that the villi of the resected group were labelled at much earlier time periods than in the control population. The regional variation in migration rate was also abolished in these rats, as the ileal villi were labelled approximately at the same time as duodenal villi. It was also reported that after ileal resection, labelled epithelial cells had migrated further along the villi a day after injection of ^{3}H-thymidine than control cells. This may be interpreted as an increased villus migration rate due to ileal ablation, but it may also be a result of the increased proliferate activity in the crypts. The epithelial cells that move onto the villi have been demonstrated to be relatively immature, having many of the characteristics of undifferentiated crypt cells (82).

Cell cycle analyses were also performed on crypt epithelial

cells of the two groups of animals. It was found that cellular generation time was significantly shortened by resection. This was attributed primarily to a decrease in the S phase.

It thus appears that ileal resection somehow stimulates proliferative activity in the crypts and possibly the cellular migration rate up the crypt. The authors hypothesized that ileal resection somehow triggers the release of an "intestinal epithelial growth hormone", which exerts its effects on the cell population in the crypt. The nature of this hormone was unknown.

Almost identical changes in intestinal epithelial cellular dynamics are found after total abdominal vagotomy in the dog (112). It has been demonstrated that vagotomy stimulates proliferative activity in the crypts and speeds up the migration of the cells from the crypt to the villus. The cell cycle time was also shortened due to the operation and this was primarily attributed to a decrease in both the S and G_1 phases of the cell cycle (although the evidence implicating G_1 was indirect and not very convincing). The major differences between the effects of vagotomy and ileal resection on the intestinal epithelium was the fact that the ileal cells were unresponsive to vagotomy, and a significant shortening of the height of the crypts was observed after the vagal operation (whereas crypt size was said to remain constant after ileal resection). The authors of this paper were cognizant of the "ileal resection" study and also noted the similarity of the results.

Altmann and Leblond performed a comprehensive study on the effects of surgical transposition of intestimal segments on villus height which yielded interesting results (1). As alluded to briefly before,

the villus height decreases as one moves from the proximal duodenum to the distal ileum. The villus height, whether measured in millimeters or in the number of epithelial cells lining a villus wall, is approximately three times greater in the duodenum than in the ileum. With this in mind, and with expectations of gaining insight into what regulates this villus size gradient, these investigators performed a series of experiments which will be outlined below.

In the first series, the following transposition experiments were performed: 1) an ileal segment was transplanted to the jejunum, 2) a jejunal segment was transplanted to the ileum, and 3) a duodenal segment was transplanted to the ileum.

The following observations on villus height were made two to three months after the above operations:

1) the ileal segments transplanted to the jejunum increased in villus height until they equalled the size of a normal jejunal villus,

2) the villi of the jejunal segment transplanted to the ileum decreased in height approaching, but not attaining, the short ileal villus size, and

3) the villus height of the duodenal segment transplanted to the ileum maintained its large size. It had, in fact, a positive trophic influence on the ileal tissue distal to it, causing a significant increase in the height of the villi.

In the next series of experiments, proximal duodenal segments (including the duodenal papilla), distal duodenal segments, jejunal segments, and ileal segments were resected, made into blind sacs, and anastomosed onto the colon. These sacs were oriented in such a way that intestinal chyme would not enter into them. At autopsy several months later, it was found that only the proximal duodenal segment maintained

its normal villus structure. Evidence of the normal villus height gradient was also seen along the length of this segment. Changes in villus height in the other blind sacs were seen, with the distal duodenal and jejunal villus height decreasing and the ileal villus height increasing, so that the villi in these three blind sacs approached the same height. Another interesting finding was that the proximal--distal villus height gradient was abolished in the three blind sacs.

In the last series of experiments, various types of side to side connections were established, namely, between the ileum on the one hand and the proximal duodenum, antrum, oxyntic gland, or squamous part of the stomach on the other hand. It was found that ileal connections with both the proximal duodenum and antrum dramatically stimulated the ileal villi to increase in size, whereas there was little or no effect on villus height after oxyntic gland or squamous connections were established. The authors of this eloquent study then designed a model system to describe their results. They hypothesized that the duodenum and antrum contain "villus enlarging factors" which are secreted into the lumen of the gastrointestinal tract and mix with the chyme. They also theorized that the ileum secretes a "villus reducing" factor which similarly is released into the chyme in the distal end of the small intestine. It is the resulting balance between these two factors which account for the villus gradient in height as one moves down the gut.

The results of these surgical procedures (ileal resection, vagotomy, and transposition of intestinal segments) are quite fascinating; the conclusions reached by the various authors are quite interesting. We will refer to them again at the close of this chapter and

discuss them at some depth in the "Discussion" section of this dissertation, and, at that time, we will consider another possible interpretation of their results involving the subject of this dissertation, gastrin.

Ontogenic Development of Intestinal Properties

The small intestine is known to undergo a dramatic series of changes the first month of life. At birth, the intestine has well-defined crypts and villi. However, upon closer inspection, one finds marked differences between the neonatal and adult small intestine. The villi are slightly shorter in the neonate, and the crypts are very shallow, so that the villus/crypt ratio actually is greater in the newborn rodent (47). It is also found that the intestinal weight/body weight ratio and the jejunal RNA/DNA ratios are much less after birth than they are in the adult (47, 120). These gross changes in intestinal properties led investigators to study the ontogenic development of the intestine in more detail and their findings will be reported below. The intestinal mucosa of suckling rats (days 1-15 after birth) has an extremely slow rate of proliferation. Several hours after an injection of ^{3}H-thymidine only 27% of the crypt cells were labelled in the 1-15 day age bracket. This is in contrast with approximately 40% of the crypt cells being labelled under the same conditions in 21 + day old rats (47). Coincident with these findings the mitotic index in crypts was found to increase approximately 80% between day 15 and day 21. Crypt height is markedly altered between the 15th and 21st day of life, increasing approximately three-fold during this period (47). The villus height has also been shown to increase slightly during the third

week of life, approaching normal adult values (47). Further, villus migration has been demonstrated to be quite retarded the first few weeks of life. As discussed previously in earlier sections, in the adult rodent it takes 1.5-2 days for an epithelial cell to migrate from the crypt to the villus apex. In the suckling rat, however, only 1/4 to 1/8 of the villus is covered in this period of time. To our knowledge, no study has been undertaken to differentiate the crypt migration rate from the villus migration rate during ontogenic development, so further interpretation of the above results is impossible at the present time. The investigators studying these changes noted that an overshoot of several of these intestinal properties occurs at the end of the third week and beginning of the fourth week of life, so that the values attained during this time exceed the known adult values. This was found in the intestinal weight/body weight and jejunal RNA/DNA ratio, as well as in the greatly accelerated migration rates of the epithelial cells up the villi (47).

It is known that between the third and fourth week of life, suckling rodents begin to wean. With this in mind, Herbst, et al. studied the effects of early weaning by gavage feeding of nine day old rats (44). It was their hope to find whether it is the change from a liquid to solid food diet which induces the above developmental changes in intestinal properties. The results demonstrated that gavage feeding induced a precocious maturation of the small intestine, in that the relative weight of the intestine, the crypt height, and the mitotic index all increased towards adult values.

The ontogenic development of enzymic activity of intestinal

epithelium follows an interesting pattern in rodents. At birth there is a relatively high lactase activity and alkaline phosphatase activity and low or negligible sucrase and maltase activity (64, 105). As the suckling period proceeds into the second week, there is either an increase or no change in intestinal lactase and alkaline phosphatase activity. During this period, sucrase and maltase remain at their low neonatal values. A drastic change in enzyme levels is initiated in the third week of life; both lactase and alkaline phosphatase activities begin to decrease, whereas sucrase and maltase activities increase. These changes in enzymatic activities proceed in a direction towards adult levels.

Upon closer inspection, it was found that the ontogenic development of both alkaline phosphatase and lactase varies, depending upon which region of the intestine is studied. It was found that the alkaline phosphatase activity is highest in the ileum and low in the duodenum in suckling rats. After the third week of life, however, the situation is reversed and alkaline phosphatase activity decreases as one moves down the small intestine (35, 64). Nevertheless, alkaline phosphatase activity of the whole small intestine markedly decreases during this period. A similar situation exists in the case of lactase (66). It has been demonstrated that the ileum of suckling rats has maximal lactase activity in comparison to other regions. The jejunal lactase of these young rats is also found in relatively high activity. In the third week of life, ileal lactase activity decreases in a dramatic fashion, whereas jejunal activity decreases slightly, the result being the attainment of the adult gradient of lactase activity (decreasing in activity from jejunum to ileum). Once again, the enzymic activity of the whole intestine is

reduced in the process. It has also been noted by Koldovsky, et al. that in the suckling rat there are two separate lactase enzymes, one having a pH optimum of 3.5 ("acidic" lactase) and the other having a pH optimum of 5.6 ("neutral" lactase). It is the latter enzyme which is found on the brush border, the former being most probably of lysosomal origin (66, 67). Both of these enzymes are significantly reduced in the third week of life.

The mechanism involved in the dramatic changes in the activity of the digestive enzymes has been studied in several laboratories. Herbst, et al. have demonstrated that gavage feeding of nine day old rats stimulates the precocious development of intestinal sucrase activity (47). These findings were supported by Rosensweig and Herman's studies which demonstrated that both sucrase and maltase activities can be induced by the presence of increased quantities of sucrose and maltose in the diet (as in the case when a suckling animal begins to wean) (104).

Consequently, the presence of sucrose and maltose in relatively high concentrations in solid food may be the major stimulant for the increase in activity of their respective hydrolases. This theory, however, has been questioned by Rubino, et al. who attempted but failed to stimulate the precocious development of sucrase by oral administration of sucrose to suckling rats (105). It was also found that sucrase and maltase activity developed normally when suckling was prolonged by feeding three week old rats goat's milk (35). Consequently, the reason for the development of sucrase and maltase in the third week is rather unclear. It appears that ingestion of solid food stimulates early

appearance of these enzymes. The question of whether it is due to the dietary content of the disaccharides in the food has yet to be settled. It also appears that normal development of these enzymes can occur without weaning. This experiment, too, will have to be verified in the future.

The reason for the decrease in lactase activity during the third week of life is also unknown. Since lactose is the major disaccharide in "mother's milk" it was thought that its disappearance from the diet at the onset of weaning may be the causative factor. However, it was found that the decrease in lactase activity was not prevented when weaning rats were given a special solid high lactose diet (66, 113). Therefore, at this stage it appears that the decrease in lactase activity is independent of the composition of the young rat's diet.

The mechanism involved in the developmental changes of alkaline phosphatase have not been studied in great detail from the dietary perspective (mainly because its physiological role is unknown). It has been shown, however, to undergo normal developmental activity changes earlier than usual if rats are weaned in the second week instead of the third (35).

The ultrastructural maturity of an intestinal epithelial cell in the neonate and suckling rats varies along the length of the small intestine. The villus epithelial cells in the duodenum and the jejunum are relatively immature, as indicated by a cuboidal shape, predominant nuclei and poorly developed microvilli. The cells develop a mature ultrastructure only after weaning (15). In contrast, the ileal epithelial cells are mature, and have a highly organized brush border, and

a well developed endoplasmic reticulum and Golgi complex (15, 37). If a section is taken for ultrastructure after milk enters the ileum, the electron micrograph will reveal the presence of vesicular bodies within the cytoplasm, along with the presence of a large supranuclear body. This is a representation of the ileal tissue's ability to absorb milk proteins by the process of pinocytosis. In a detailed ultrastructural study, Graney demonstrated that the vesicle originally forms in an inter microvillus space by the pinching off of the surrounding surface membrane, thus encompassing the protein molecules (37). It is believed that the protein laden vesicle then travels to the supranuclear body to unload the protein. It also should be noted that there are a large number of lysosomal bodies in the cytoplasm and they may play a role in the processing of the protein. This pinocytic property of the ileal intestinal epithelium of the suckling rat is thought to be important in the maintenance of the immunity of the young animal, for it is believed that maternal antibodies present in maternal milk can pass into the suckling rat's circulation via this route. This pinocytic property is quite transient and is abolished upon the onset of weaning.

In conclusion it appears that many of the biochemical and morphological properties of the intestinal epithelium undergo a dramatic change between the second and third week of life, which is coincident with weaning. The actual physiological agent which triggers these changes is unknown. Although some answers to these questions will be provided in the next section, it will become apparent that we are still very far from understanding the true nature of the regulatory mechanism involved.

The Effects of Hormones (Other than Gastrin) on Intestinal Properties

The influence of hormones on intestinal properties is presently being studied in many laboratories throughout the world (78). Because of the extreme sensitivity of the intestinal epithelium to changing conditions (as outlined in the preceding sections), it was thought that the endocrine system may play a major role. In the discussion presented below the action on the intestine of the following hormones will be discussed: 1) growth hormone and thyroid hormone, 2) adrenal corticosteroids, and 3) insulin and glucagon. Also, the effects of vitamin D will be discussed in this section. The nature of the actions of these hormones on intestinal properties has to be interpreted carefully. As Levin pointed out in his review of the subject, the hormones can induce changes in the intestinal epithelium in many ways, only one of which is a primary interaction with an epithelial cell (78). The presence or absence of a hormone can affect intestinal properties in a variety of ways, notably by changing the appetite of the animal, by changing gastrointestinal motility and/or secretion, by changing the electrolyte balance of the animal, by stimulating the release of another hormone which, in turn, acts on the intestine, or by playing a permissive role by making the cell susceptible to the action of other hormones. These possibilities are difficult to discern when working *in vivo*, and it is always necessary to demonstrate a specific action *in vitro*, if the primary action of the hormone on the cell is to be verified.

Growth Hormone and Thyroxin

It is known clinically that hyperthyroidism is associated with

a marked increase in intestinal weight (78). This has been verified in the laboratory by the induction of experimental hyperthyroidism and by the chronic injection of thyroxin in rats (78). The increase in weight is primarily due to an increase in villus height and total mucosal thickness (126). It was also noted that the microvillus border is hypertrophied, and there is an enhancement of brush border enzyme activity under these conditions (126). Consistent with these findings, there is a decrease in weight and development of intestinal structure and function in the hypothyroid animal (8). Leblond, et al. demonstrated that the mitotic index of crypt epithelial cells is reduced approximately 50% upon the removal of the thyroid (74). It was then discovered that the mitotic index of these hypothyroid rats can be increased to normal values with the administration of thyroxin.

A similar type of atrophy of small intestinal structure occurs in hypophysectomized animals. It has been demonstrated that removal of the pituitary gland causes marked decreases in the height and diameter of villi and in the general weight of the small intestine (46). In the study cited before, Leblond, et al. demonstrated that hypophysectomy, like thyroidectomy, markedly reduces the mitotic index in the crypt. This change was then shown to be reversed by "replacement" therapy with growth hormone (74). These investigators then proceeded to study the inter-relationships of the actions of thyroid hormone and growth hormone on the small intestine. In this study, they injected thyroidectomized animals with growth hormone, hypophysectomized animals with thyroxin, and both groups of surgically ablated animals with both hormones at the same time. Their results demonstrated that the thyroidectomized animals

were highly responsive to growth hormone (with the mitotic index returning to normal), whereas the hypophysectomized animals failed to respond to thyroxin treatment. When both hormones were injected simultaneously to both pituitary and thyroid ablated animals, mitotic activity increased in both cases. Since thyroxin has no effect on intestinal mitotic activity when the pituitary is removed, the authors concluded that thyroxin was acting indirectly on the intestinal epithelium by stimulating the release of endogenous growth hormone which has a mitogenic effect on the intestinal epithelium.

The physiological importance of these findings (the effect of growth hormone and thyroxin on the intestine) is presently uncertain. It is known that thyroidectomy and hypophysectomy are accompanied by a marked loss in appetite of the animal, as well as a retardation in gastrointestinal motility. On the other hand, increasing the levels of these hormones has the reverse effect (78). Since in none of the above studies the experimental and control groups were pair fed, one is hard pressed to conclude whether the changes in intestinal properties were due to a varied amount of food ingested or a direct hormonal action on the intestinal epithelium.

If one does make the assumption that growth hormone (whether stimulated by thyroxin or by other means) does have a direct mitogenic effect on the crypt epithelial cells, the question arises whether this is representative of a specific effect on the intestine or a general effect on all the tissues in the body. This questions can be partially answered if growth hormone or thyroxin can be implicated in the highly variable growth properties of the intestine discussed in the above

sections. In starvation, for instance, it is known that growth hormone levels rise to a high level, whereas blood thyroxin levels decline (12, 107). This increase in growth hormone levels seems inconsistent with the fact that the intestine undergoes atrophy during starvation, thus undermining the possibility that growth hormone plays a role in the sensitization of the intestine to the absence or presence of food. Similarly, it is known that both growth hormone and thyroxin are found in relatively high concentrations in the blood after birth and are not dramatically changed by weaning (107). Consequently, the possibility that these hormones play an important role in the modification of intestinal cell renewal during these periods must be tentatively ruled out.

Adrenal Corticosteroids

Adrenal corticosteroids have been found to stimulate precocious development of adult intestinal properties in newly born rodents. This action was originally revealed by Moog, et al. who demonstrated that a single injection of cortisone induces the precocious development of duodenal alkaline phosphatase in two week old mice (94). Since that pioneering experiment, the development of several other brush border enzymes has been shown to be sensitive to the adrenocorticosteroids. Several investigators have demonstrated that hydrocortisone injections into three to nine day old rats stimulates the precocious development of intestinal sucrase and maltase (24, 47). The stimulatory action of these hormones on the early development of the enzymes has been demonstrated in vitro in organ culture systems containing strips of intestinal tissue from young animals (24, 48, 93). These latter experiments definitively

prove that hydrocortisone directly interacts with intestinal epithelial cells. Until recently, no effect of the corticosteroids on lactase activity could be found. However, in 1971 Koldovsky, et al. successfully stimulated the precocious decline of "acid" lactase in the ileum by daily injections of cortisone into rats nine to fifteen days old (68). Therefore, the corticosteroids seem to have a major influence on the development of most of the intestinal enzymes with the notable exception of brush border (neutral) lactase.

The adrenal corticosteroids may also play an important role in the changes in cellular proliferative activity occurring at weaning. It has been demonstrated that rats receiving injections of hydrocortisone beginning on the third day of life have significantly greater mitotic activity and crypt height than control rats when they are sacrificed at the end of the second week of life (47). Migration rate has also been shown to be affected by steroid injection, increasing approximately four-fold over the controls. It thus appears that the steroids induce cells of the villus and crypt to develop properties characteristic of mature epithelial cells.

The effects of steroids on ultrastructural development have also been studied (15). It has been demonstrated that within forty-eight hours after an injection of cortisone into an eight day old rat the immature epithelial cells lining the villi are replaced by differentiated cells identical to the villus epithelial cell of an adult. In addition, the hormone inhibits the pinocytic activity of ileal epithelial cells, so that intact proteins can no longer be absorbed by this route. Once again, the hormone injection appeared to accelerate the

normal adult development of these properties.

Consequently, there is a great deal of evidence linking corticosteroid secretion with the normal ontogenic development of intestinal structure and function. This theory is strengthened somewhat by the fact that the steroids are initially synthesized and released by the immature adrenal cortex between the fourteenth and twenty-first day of life which is the period in which the intestine begins to develop adult properties (24, 94). There are, however, several weaknesses in this theory; first, the rat intestine is only sensitive to steroid injection between the third and ninth day of life. If the intestine is insensitive to steroid treatment after day nine and the steroids are first endogenously released at day fourteen (which corresponds with the onset of adult development), one wonders if the steroids really do play a major role in the development of the intestine. The second argument that can be used against the theory is based on the fact that gavage feeding of young rats stimulates precocious development of intestinal properties (47); the relationship between this finding and stimulation of steroid secretion is presently obscure.

As alluded to above, the adult small intestine is relatively unresponsive to the adrenal corticosteroids. This conclusion was arrived at by looking throughout the literature and finding approximately 50% of the papers arriving at opposite conclusions. Adrenalectomy has been reported to reduce maltase and alkaline phosphatase activity, but both activities return to normal when the condition is compensated for by adding sodium chloride to the animal's drinking water (21, 22).

The adrenal corticosteroid levels in the blood do not seem to

be affected during periods of starvation or undernutrition (12). For this reason it is not likely that corticosteroid secretion (or lack of secretion) has a major regulatory influence on the intestinal changes that occur during periods of food deprivation.

Insulin and Glucagon

Several laboratories in recent years have demonstrated that relative intestinal weight (intestinal weight/body weight) is markedly increased in alloxan diabetes (109, 110). The mucosa seems to be specifically affected by this condition, increasing in both the relative wet and dry weights. Another interesting finding by these investigators is that the duodenal tissue is initially affected a short period after the diabetic condition is induced, and with time the whole length of the intestine becomes trophically involved.

These findings are interesting because they lead one to assume that insulin may be an inhibitor of intestinal growth. However, the investigators of this study brought up the interesting point that diabetes is known to be associated with a marked increase in food consumption. In an earlier paper, it was demonstrated that rats made hyperphagic by hypothalamic lesions have an increase in intestinal weight similar to that reported in this paper (7). The major difference between these conditions is the inability of the diabetic animal to utilize or store the ingested nutrient, hence, a loss in body weight (which, in turn, increases the relative intestinal weight). Consequently, one is unable to definitively state at this point whether an insulin deficiency does have a specific and direct trophic influence on the small intestine. Support for this possibility was recently provided in a clinical report which

described the effects of a glucagonoma on the small intestine (10). It was shown that abnormally high levels of glucagon in the blood are associated with a marked increase in intestinal weight. Upon closer inspection, it was noted that villus height was dramatically increased above normal values in this patient. These findings, therefore, leave the possibility open that the balance between insulin and glucagon may be critical for intestinal growth. In one case, a diabetic condition (in which the effects of glucagon are not counterbalanced) may induce intestinal epithelial hypertrophy, and, in the other, a glucagon excess induces the development of a similar condition. Further studies in this area are needed to clarify the physiological importance of these findings.

The effects of alloxan diabetes on intestinal enzymatic capacity have also been studied (96). In pair-fed control and diabetic rats, it has been demonstrated that sucrase specific activity is doubled (in the diabetic rat) both in mucosal homogenate and brush border preparations. Enzyme kinetic studies disclosed that the diabetic condition is associated with an increase in Vmax but not in the Km of sucrase. These results suggest that diabetes stimulates an increase in the quantity of enzymatic protein molecules per cell but not in their biochemical properties. These results are supported by the previous findings of Hossain, et al. who demonstrated an increase in maltase activity in diabetes (52).

The effects of insulin on intestinal structure and function are quite interesting. Of all the hormones discussed so far, insulin and its antagonist, glucagon, are most sensitive to the consumption of food. It

is known that insulin levels rise shortly after a meal, as the blood sugar levels rise (107). Similarly, periods of starvation decrease insulin levels and increase glucagon levels in the blood (12, 107). From the marked intestinal atrophy that occurs during starvation, it appears that these values should be going in the other direction if the hormones would have physiological importance at this time. The increased enzyme levels during starvation (as reported by some investigators), however, may be due to the changes in insulin and glucagon levels.

The changes in insulin and glucagon levels at the onset of weaning have not been studied yet. It would be of interest to see if these could have any role in the intestinal development that occurs at this time.

Vitamin D

Vitamin D recently has been implicated as being important in the maintenance of intestinal structure and function. It has been demonstrated to have a significant stimulatory effect on Ca^{++} and amino acid transport in the small intestine (107, 118). Consistant with the above findings is the known fact that vitamin D deficient animals have abnormalities in Ca^{++} metabolism and frequently suffer from rickets or osteomalacia. This may be due to the inability of the small intestine to absorb $Ca^{++}$and the resistence of bone to undergo calcification in the absence of Vitamin D (107). The intestinal structure is also very sensitive to the presence of vitamin D. It has been demonstrated that vitamin D is a stimulant for RNA synthesis in the intestinal epithelium (117). In another study, it has been shown that in vitamin D depleted rats, there is a marked decrease in duodenal mucosal wet and dry weight,

which can be completely reversed in the forty-eight hours after vitamin D repletion (124).

Gastrin as a Trophic Agent

The first indication that gastrin may have a trophic influence on gastrointestinal tract tissue was found in patients suffering from the Zollinger-Ellison syndrome (130). In this disease, a gastrin-producing pancreatic tumor forms, releasing the hormone in high concentration in the blood. Upon autopsy of patients suffering from this gastrinoma, it was found that there was marked hyperplasia of the gastric and duodenal mucosa.

These observations were immediately followed by laboratory experiments in which high levels of gastrin were maintained in rats by different means. In the first experiment, Crean, et al. chronically injected a group of rats with supramaximal doses of pentagastrin and during the same time interval, injected a control group with saline and another with histamine (18). After twenty-one days, the animals were sacrificed and the gastric mucosa was removed for observation. These investigators found significant increases in fundic weight, fundic mucosal thickness, and the number of parietal cells in the fundic region of the stomach in the pentagastrin-injected rats in comparison to the other two groups. The cells of the antrum and the squamous region of the stomach did not appear to be affected by the pentagastrin treatment. In addition, no significant difference in the chief cell population was induced by the treatment. In an accompanying paper, Crean, et al. attempted to stimulate endogenous gastrin release by partially obstructing the pyloric-

duodenal bulb junction (this was accomplished by tying a ligature around the junction) (17). This concept was based on the belief that the ligature would markedly retard gastric emptying, causing food to accumulate in the antrum, resulting in its distension, which in turn stimulates chronic release of gastrin. Their results showed a general increase in all gastric morphological properties, notably, an increase in stomach weight, mucosal thickness (both fundic and antral), mucosal height (both fundic and antral), and in the total number of parietal and chief cells. Because the gastrin radioimmunoassay was not perfected at this time, it was not known whether the trophic changes were due to hypersecretion of gastrin or a local inflammatory reaction which would be caused by the obstructed condition.

These earlier findings have been supported more recently by Stanley, et al., who chronically injected rats with a maximal dose of pentagastrin for fourteen days and demonstrated dramatic increases in both basal and stimulated gastric secretory output over a control group of rats (114). This increase in gastric secretion after chronic pentagastrin treatment was closely related to an increase in the total number of parietal cells.

Another perspective in studying the trophic effects of gastrin on the gastric mucosa was pursued by Martin, et al. (85). These investigators removed the source of gastrin by performing an antrectomy followed by Billroth I gastroduodenostomy on a group of rats. Upon sacrifice of the animals six to nine weeks after the operation, they removed the oxyntic gland region and made morphological comparisons between them and those of sham-operated controls. Their results revealed dramatic

atrophy of the oxyntic mucosa in the gastrin-depleted animals. There was a significant reduction of mucosal height, thickness, and in the number of parietal and chief cells in the oxyntic gland area of the antrectomized animals in comparison to the controls.

Detailed biochemical studies on the effect of pentagastrin on the gastric and duodenal mucosae have accompanied these morphological and physiological findings. Johnson, et al. have demonstrated that pentagastrin stimulates protein synthesis in both oxyntic and duodenal mucosae, employing both in vivo and in vitro techniques (58). In a more recent study, Chandler, et al. demonstrated that pentagastrin also stimulates RNA synthesis in both the oxyntic and duodenal mucosae (14). Mayston and Barrowman have been studying the effects of chronic pentagastrin administration on the pancreas in comparison to the effects of histamine and saline (86). They have found that the pancreas of the pentagastrin-treated rats weighs significantly more than that of the controls. They also noted that the acinar cell density and concentration of DNA was decreased by this treatment, whereas RNA concentration increased. It was also found that total pancreatic DNA was unchanged, and that total RNA was increased in the pentagastrin injected rats. These authors concluded that pentagastrin induced acinar cell hypertrophy and that the differences in tissue weight was primarily due to an increase in tissue H_2O. This is an extremely interesting theory, but the necessary wet weight-dry weight comparison determinations were not performed.

Gastrin has also been shown to affect enzymic activity of gastrointestinal tissues. Kahlson, et al. demonstrated in the early 1960's

that gastrin stimulated histidine decarboxylase activity in the stomach (61). Johnson, et al. then demonstrated that antrectomy markedly reduces histidine decarboxylase activity and that this decline in activity can be completely reversed by injections of pentagastrin (60).

The pancreatic enzymes lipase, amylase, and esterase have also been shown to be gastrin sensitive. Mayston, et al. have shown reductions of all three enzymes after chronic pentagastrin treatment, with significant differences being found in the latter two activities (86).

It thus appears that gastrin (or its derivative, pentagastrin) has a significant effect on the structure and function of gastrointestinal tissues. The epithelial cells of the gastric and duodenal mucosal seem to be sensitive to gastrin, readily increasing in cellular mass in the presence of high levels of the hormone, or degenerating in its absence. The acinar cells of the pancreas also are affected by this gastrointestinal hormone, although the action here may be of a different nature. As described above, gastrin is also known to influence the enzymic capacity of tissues on the digestive system.

Possible Regulatory Role of Gastrin in Intestinal Property Changes

With all these known actions and properties of gastrin in mind, we began to explore the possibility of whether a "gastrin effect" could account for some of the variable intestinal properties discussed at length in the above sections. It is also known that within minutes of eating a meal blood gastrin levels begin to rise, peaking at a value five times the basal level between forty-five and sixty minutes after the completion of the meal. The gastrin concentration in the blood then

begins to decline, reaching a stable postprandial level between three to four hours after the meal (73). During periods of starvation, this marked stimulation of gastrin levels would be absent. If gastrin trophically affects the intestinal mucosa, its absence during starvation could be the cause of the mucosal atrophy. The changes in enzyme levels during starvation may also be attributed to this gastrin deficiency during starvation.

Gastrointestinal surgical procedures markedly changes the properties of the intestinal epithelium. Ileal resection stimulates proliferative activity in the crypt. This could be attributed, in part, to the removal of sites of a gastrin antagonist, secretin, which is thought to be located along the entire length of the small intestine (90). The authors concluded that ileal resection somehow released an "intestinal epithelial growth hormone". Could it actually be explained by a condition in which the trophic action of gastrin is unchecked by low levels of secretin? Vagotomy also was shown to stimulate proliferation of intestinal epithelial cells of the crypt. It is also known that vagotomy stimulates the release of high levels of gastrin along with super sensitizing hormonal receptor sites (87, 127). Consequently, these results too, can be explained as a response to high levels of the hormone. Altman, et al.'s elegant transposition experiments revealed that there was possibly a "villus enlarging factor" located in the antrum and duodenum, and an antagonistic "villus reducing factor" located in the ileum. The locations of the "villus enlarging factor" coincide perfectly with our present knowledge of the location of gastrin along the gastrointestinal tract, and the "villus reducing factor" may correspond to secretin sites

in the ileal wall (although their experimental results do not contain convincing evidence that a "villus reducing factor" exists at all).

The possible relationship between gastrin and ontogenic development of the small intestine is quite interesting. Recent experiments by Zelenkova, et al. reveal that gastrin activity initially appears during the third week of life (129). This corresponds very well with the time of normal development of the intestine both structurally and functionally. One would also assume that gastrin release would be highly sensitive to the change from suckling to weaning (or liquid food to solid food), since the major stimulant for gastrin release is thought to be the mechanical distension of the antrum (43).

Lastly, the effects of vitamin D on the small intestine are quite interesting when one considers a possible gastrin relationship. Recent experiments in several laboratories have demonstrated that Ca^{++} (when administered intravenously) is a potent stimulator of gastrin release (3). With this in mind, along with the knowledge that vitamin D is needed for Ca^{++} to be transported into the body, it follows that vitamin D deficiency is associated with low blood Ca^{++} levels and quite possibly low basal gastrin levels as well. If this is true, many of the trophic effects thought to be directly due to vitamin D may actually be attributable to gastrin.

Hypotheses

For the reasons stated above, I feel that the effects of gastrin on the intestinal epithelium need to be investigated. At this time, therefore, I wish to state several hypotheses that will be tested in the course of this dissertation research. Each of these is based on

the premise that gastrin has a trophic influence on the intestinal epithelium. We also believe that, in turn, the hormone will change the functional capacity of the intestine. The exact nature of this latter action cannot be predicted at the present time.

(1) The functional and atrophic structural changes of the small intestine during starvation are in part due to a gastrin deficiency.

(2) Gastrin plays a major role in the intestinal developmental changes that occur at the onset of weaning.

(3) High and low endogenous gastrin levels result in trophic and degenerative intestinal structural changes, respectively. Diverse functional changes are also elicited by these two contrasting conditions.

(4) Gastrin has a direct trophic action on the intestinal epithelium, which can be demonstrated in an *in vitro* system.

A more detailed description of the experiments which will be employed to test these theories will be presented in the following chapter.

CHAPTER II

METHODS AND MATERIALS

Experimental Design

In order to investigate the possible relationships between the concentration of gastrin in the blood and the changes in intestinal properties occurring during starvation and ontogenic development, as well as the role of gastrin in the maintenance of intestinal properties under normal adult conditions, the following series of experiments was performed.

Study I - The Effects of Pentagastrin Administration on the Changes in Intestinal Properties During Starvation

In this study, the effects of different periods of starvation (0, 1, 3, 5 days) on the rat small intestinal structure and function were studied in the presence and absence of pentagastrin treatment. Control groups were injected with either saline or histamine.

Study II - The Effects of Gastrin (or Its Pentagastrin Derivative) on the Ontogenic Development of Intestinal Properties

This study was divided into three parts:

(a) The effects of prolonged suckling periods (in comparison to rats suckled and weaned at normal periods) on the development of the structure and function of the small intestine.

(b) The effects of pentagastrin on intestinal development in rats (injected between the ages of 15-22 days old) which are suck-

ling for an extended period of time.

(c) The effects of pentagastrin treatment on intestinal development in suckling rats (injected between the ages of 9-14 days old).

Study III - The Effects of High Endogenous Levels of Gastrin on the Structure and Function of the Small Intestine

In this study, partial obstruction of the pyloric-duodenal junction was created surgically as described by Crean, et al. (17). This is believed to cause prolonged antral distension after meals, resulting in hypersecretion of gastrin. Various intestinal properties such as nucleic acid and protein content, as well as enzymatic avtivity, were studied approximately five weeks after surgery. The results were compared to the intestinal properties of sham-operated controls.

Study IV - The Effects of Low Endogenous Levels of Gastrin on the Structure and Function of the Small Intestine

In this study, rats were antrectomized; this was followed by a Billroth I gastroduodenostomy as described by Hakanson, et al. (44). Controls were sham-operated at the same time period. Intestinal properties were studied approximately five weeks after surgery. A pilot morphological study was also performed on rats which underwent antrectomy followed by a Billroth II gastrojejunostomy. Sham-operated rats served as controls in this experiment.

Study V - The Effect of Pentagastrin on the Ultrastructure and Growth Properties of Duodenal Cells in Culture

This in vitro study measured the proliferative potential, cell cycle components, and enzymatic and morphological properties of duodenal cells in the presence and absence of pentagastrin.

Analytical Methods

Studies I-IV

The following parameters of intestinal structure were measured by the methods noted below.

1. Wet weight of tissue, on a Mettler balance.
2. Dry weight of tissue, by a modified method of Schedl, et al. (110) (see Appendix, page 147).
3. Protein by the method of Lowry, et al. (84) (see Appendix, page 143).
4. DNA by the method of Giles, et al. (a modification of the Burton method) (11, 34) (see Appendix 145).
5. RNA by the method of Fleck, et al. (30) (see Appendix, page 146).
6. Villus and crypt heights by the methods described by Altman, et al. (1) (see Appendix, page 150).

The intestinal functional parameters were measured by the methods of analysis listed below.

1. "Neutral" lactase activity was measured by the methods of Dahlqvist, et al. and Koldovsky, et al. (19, 65) (see Appendix, page 147).
2. "Acidic" lactase activity by the method of Koldovsky, et al. (65) (see Appendix, page 147).
3. Maltase activity by the method of Dahlqvist, et al. (19) (see Appendix, page 147).
4. Alkaline phosphatase activity by the method of Klein, et al. (63) (see Appendix, page 149).

Study V

The following functional and structural properties were measured, using the techniques as identified below.

1. Percentage of cells in the proliferation pool by the method of Kollmorgen, et al. (70, 71) (see Appendix, page 151).

2. Cell doubling time by a modification of the method described by Kollmorgen, et al. (70) (see Appendix, page 150).

3. Crude approximation of generation time by the modified method of Kollmorgen, et al. (69) (see Appendix, page 153).

4. Ultrastructural analysis by the method of Miller, et al. (92).

In the Section below, I will initially outline the general experimental procedure followed in Studies I-IV (in vivo experiments) and those followed in Study V (in vitro experiment).

General Experimental Procedure

Animal Selection (Studies I-IV)

Littermate King-Holtzman inbred cream-colored rats were used in all experiments. Littermate rats (both male and female) were divided equally into the groups of rats used in each experiment. An attempt was also made to distribute the sexes equally among the groups. The adult males and females, of course, were always kept separate. This equalization of the sexes in each group was just a precautionary measure because studies of Blair, et al., as well as unreported studies by us, have demonstrated essentially no differences in rat intestinal structural and functional properties due to sex (5).

If more than one litter of rats were needed for the study, a second or third litter from the same strain, which was born within several days of the others, was used. The same methods of distribution were used in these experiments.

Animal Housing and Diet

All the animals were housed in the animal quarters of the University of Oklahoma Health Sciences Center from a week before the

experiment until its termination. Due to the nature of the experiments different diets and cages were used for the different studies. These will be listed below.

Study I. Littermates approximately 150-200 gms were used for this study. They were fed Teklad rat pellets prior to the commencement of the experiment. Animals which were in the 0 day starvation group (fed group) were allowed to feed and drink water ad libitum. Animals that were starved for varying periods of time (1, 3, 5 days) were allowed to drink water ad lib. They were housed in individual wire mesh cages (1 rat/cage). The wire mesh grating was large enough to allow fecal material to fall through. This housing system served to prevent cannibalism and coprophagia.

Study IIa and IIb. Young littermate rats which were in the "prolonged suckling" groups were placed along with their mother (or foster mother) in large wire mesh bottomed cages. The rats (including the mother) were allowed to drink water ad lib and were given no access to solid food. After approximately twelve hours, the fasted mother was removed and placed in a cage with weaning rats with access to food and water, while a recently fed foster mother (a lactating rat which gave birth to another litter at approximately the same date) was placed in the young rats' cage for the next twelve hour period. This rotation process was continued throughout the period of the experiment. It was found to work quite successfully for the mothers rarely rejected or cannabalized the suckling rats during this period. In addition, the young rats looked healthy and gained weight during this period (although it was significantly less than the weight gain of weaning rats).

The rats that were allowed to wean were placed in plastic cages with their mother and at an age of 15 days were given access to both water and food (broken Teklad pellets were placed on the bottom of the cage to facilitate the weaning process). Their lactating mother was rotated to the "prolonged suckling" group after twelve hours and the fasted mother from the above group was placed in their cage for the next twelve hours. These weaning rats were also intermittently suckling and no maternal rejection was observed.

Study IIc. In this experiment, suckling rats were placed in plastic cages with lactating mothers. The mothers were allowed food and water ad lib. Every twelve hours the mothers of the experimental and control group were exchanged, so that the conditions of the two groups could be better controlled. No evidence of weaning was observed in the suckling rats, and their eyes were still closed at the time of sacrifice.

Study III and IV. In this experiment, adult rats, weighing 150-200 gms, were fasted a day before the operation. After the operation, both experimental and sham-operated rats were placed in individual wire mesh cages (1 rat/cage) and were given no water or food for two days. During this time, fluid and nutrient were supplemented by subcutaneously injecting 15 ml of sterile, 5% dextrose solution twice a day. On the third day they were given access to water and clysed as described above. On the fourth day the animals were given 3-4 Teklad pellets and subcutaneous feeding was terminated. Their diet was increased steadily until the end of the week. At this time, they were allowed to feed ad lib. Approximately two weeks after the operation, the experimental (either obstructed or antrectomized) animals were ear marked and placed

in large metal cages with the controls for the duration of the experiment.

Processing of Intestinal Tissue (Studies I-IV)

The rats were weighed initially and then sacrificed by cervical transection. A midline incision was immediately made exposing the entire length of the small intestine. The duodenal bulb-pyloric and ileal-cecal junctions were located and cut. The small intestine was then removed from the abdominal cavity and flushed with a cold isotonic saline solution. The intestine was then placed on a cold tray where it was trimmed of connective tissue (small tissue rings were then removed at this point if histology was desired) and slit longitudinally. The intestine was then washed free of any residual fecal material and secretion by rinsing it in a series of beakers containing cold isotonic saline solution. Next, the tissue was then blotted on filter paper and weighed on a Mettler balance. The intestine was then wrapped in a labelled piece of aluminum foil and quickly frozen in a dry ice-acetone bath. The frozen sample was then stored in a freezer at $0^{o}C$ and processed within the week.

At the time of processing, the tissue was removed from the freezer, unwrapped, and placed in a Waring blender. It was then homogenized for one minute in deionized distilled cold water (approximately 40 ml water/gm tissue). At the end of a minute, 5.0 ml of the homogenate was pipetted into duplicate funnels which contained porcelin filter discs (perforated with 1 u diameter holes). This technique tended to remove the larger uncut pieces of tissue and produced a uniform and

homogenous suspension. The intestinal homogenate was collected in a set of chilled test tubes from which aliquots were removed for biochemical determination. A flow sheet of this procedure can be seen in Figure 1.

Aliquots were removed and diluted in the fashion outlined below for each biochemical determination.

1. 0.1 ml for protein determination.

2. 0.1 ml for "neutral" lactase assay (except in Study II where a 1:4 dilution of the homogenate was initially made, from which a 0.1 ml aliquot was removed).

3. 0.1 ml for "acidic" lactase assay (same modifications as above were made in Study II).

   A 1:4 dilution of the homogenate was made from which the following aliquots were removed.

4. 0.1 ml for alkaline phosphatase assay (a 0.1 ml aliquot from a 1:8 dilution was removed in Study II).

5. 0.1 ml for sucrase assay (a 0.1 ml aliquot was removed from the undiluted homogenate in Study II).

6. 5.0 ml for RNA-DNA extraction procedure.

   A 1:30 dilution of the homogenate was made, from which the following aliquot was removed.

7. 0.1 ml for maltase assay (a 0.1 ml aliquot was removed from the undiluted homogenate in Study II).

Initiation and Maintenance of Duodenal Cultures (Study V)

In this study a single male adult rat from the King-Holtzman strain was used. The rat was fasted overnight and injected with a 250 ug/kg body weight dose of pentagastrin (to produce maximal gastric secretion) three hours before sacrifice. The rat was killed at this time by cervical transection, and the abdominal cavity was opened by a midline incision under sterile conditions. A region of proximal duodenum was

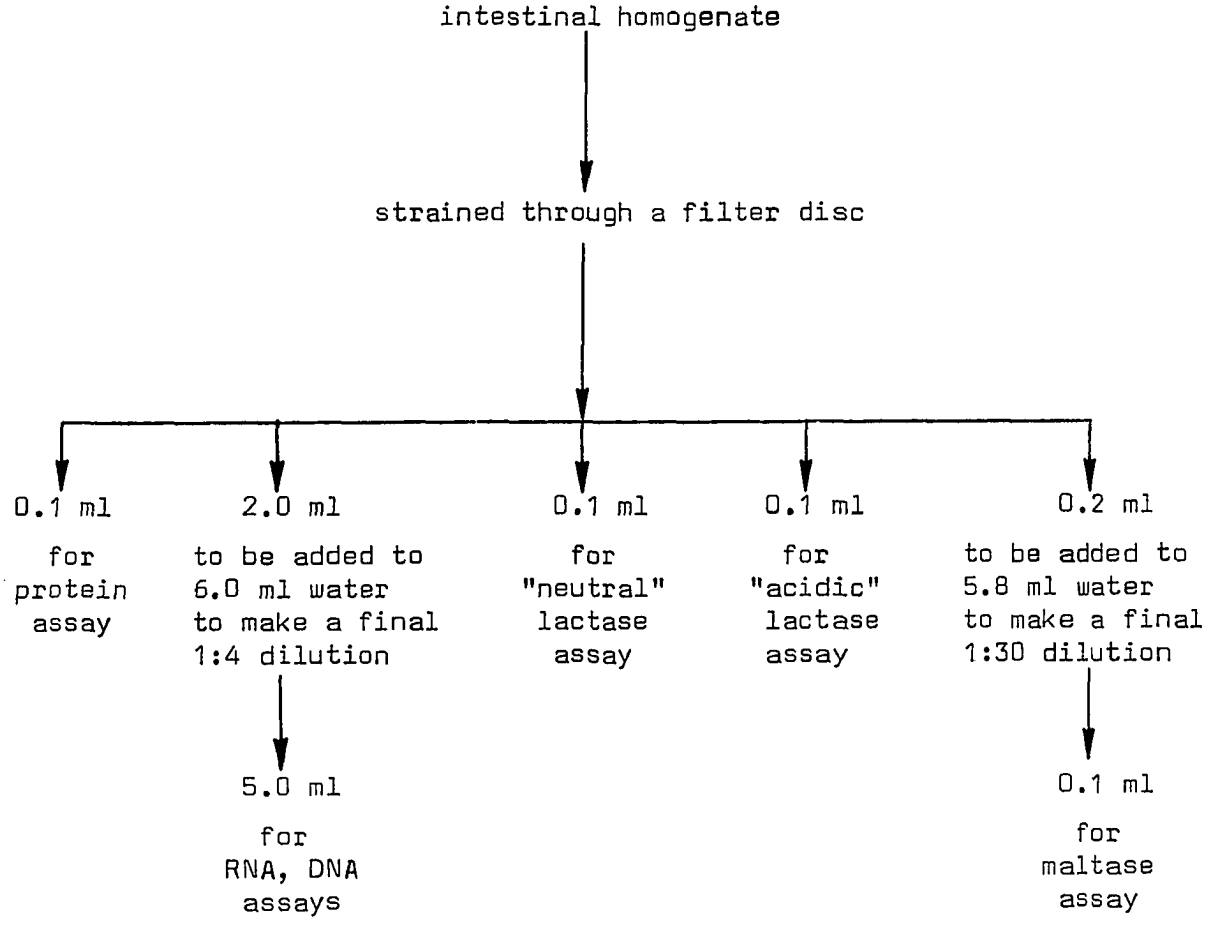

Figure 1.--Processing of intestinal tissue.

exposed, and a 2 centimeter section was resected and placed into a series of petri dishes containing a solution of Minimum Essential Medium for washing purposes. After it was placed in the third petri dish, the full thickness of the tissue was cut into numerous 1-2 mm pieces. The duodenal tissue pieces were then placed in a set of four T_{60} tissue culture flasks containing fetal calf tissue culture medium to which a fungizone was added. After the addition of these potential explants, the flasks were gassed with 8% CO_2 in air, tightly stoppered, and placed in an incubator set at 37.5°C. All these steps were performed under rigorously sterile conditions. The cultures were then left undisturbed for approximately a week during which time growth was observed in all flasks. At the end of the week two of the cultures were randomly designated "saline controls" and the others were labelled "pentagastrin treated". At this time the original medium was replaced with fetal calf medium (without fungizone). Pentagastrin was then added daily to the appropriate flasks at a concentration of 0.5 ug/ml medium. An equal volume of saline was added to the control flasks at similar time intervals.

When the cultures became confluent (cells completely covered the bottom of the flask), subculturing was performed. This was accomplished by treating the cultures with a 0.05% trypsin solution (made up in Earle's balanced salt solution) for approximately 5-10 minutes, as described by Evans, et al. (28). It is thought that trypsin cleaves the bonds between the cell surface membrane and the glass, thus disrupting the cellular attachment to the flask and releasing viable cells in suspension. This tryptic suspension was then diluted by the addition of

fetal calf medium and an inoculum was added to a second T_{60} flask containing the necessary volume of medium. Approximately two hours after subculture, the trypsin-containing medium was removed and replaced with fresh medium. This completed the subculturing procedure.

Sacrifice of Cultures for Ultrastructural Examination (Study V)

The duodenal cells were grown in plastic T_{75}'s for morphological determinations. At the time of sacrifice, the plastic T-flasks were removed from the incubator. The cells were rinsed several times with isotonic saline, and a trypsin solution (0.05%) was added to remove the cells from the glass. The cell suspension was then poured into a 40 ml centrifuge tube and prepared for ultrastructure by the method of Miller, et al. (92).

Solubilization and Injection Procedures for Pentagastrin, Histamine, and Saline (Studies I-V)

When it was desired to increase the gastrin levels in the blood by exogenous means, the pentapeptide derivative of gastrin, pentagastrin, was injected into the animal as a gastrin substitute. The structure of the pentagastrin molecule is as follows: β-Ala-Try-Met-Asp-Phe-NH_2. This molecule contains the C-terminal tetrapeptide (underlined), which has been found to be the active site of the larger gastrin molecule (in regard to its ability to stimulate gastric secretion) (40). It has yet to be definitively proven that the active sites of gastrin for gastric secretory and G.I. trophic stimulation are identical. Support for this contention was provided by Crean, et al., who demonstrated that pentagastrin injections stimulated parietal cell growth in the rat fundus (18). These results have been supported by Stanley, et al. (114).

Based upon these findings, we have made the assumption that the active sites for the two functions are one and the same; therefore, we have used pentagastrin as an exogenous substitute for gastrin.

Solubilization. A certain quantity of pentagastrin powder was weighed out on a Mettler balance and poured into a beaker. A small volume of isotonic saline was then added. In order to solubilize the pentagastrin, a small volume of 0.1 N $(NH_4)_2SO_4$ was added, which subsequently increased the pH of the solution. This is performed because pentagastrin is readily soluble under alkaline conditions. After the alkaline solution was formed, 0.1N HCl was added to neutralize the solution. Once a pH of 7 was attained, the necessary volume of isotonic saline was added to make a concentration of 250 ug pentagastrin/ml. Small volumes (1-3 ml) of this solution were pipetted into plastic vials and stored at $0^{o}C$ for Studies I-IV. For Study V, this 250 ug/ml solution was diluted 1:10 and sterile filtered. The filtrate (having a concentration of 25 ug/ml) was pipetted in small volumes (3.0 ml) into small sterile glass vials and stored at $0^{o}C$. Histamine solutions were made at a concentration of 20 mg/ml in saline immediately before injection.

Injection Procedure. For the in vivo studies (I-IV), the pentagastrin solution was thawed and was injected subcutaneously into the rats at a concentration of 250 ug/kg body weight (which would induce a maximal gastric secretory response). This was performed three times daily for the length of the experimental period (between 1-7 days). An equivalent volume of commercially prepared sterile saline was injected subcutaneously into the controls at similar time intervals.

Histamine was injected subcutaneously at a concentration of 20 mg/kg body weight three times a day.

For the in vitro study (V), the pentagastrin solution was added daily to the culture media at a 1:50 (pentagastrin: medium) dilution ratio. The final concentration of pentagastrin in the tissue culture flasks was 0.5 ug/ml medium. A similar volume of sterile isotonic saline was added daily to the control flasks.

Surgical Procedures (Studies III and IV)

Antrectomy (Billroth I). This procedure was performed as described by Johnson, et al. and Hakanson, et al. (60, 44). Rats were initially anesthetized with ether which was administered throughout the operation whenever necessary. A midline incision was made exposing the stomach. The connective tissue around the stomach was carefully removed and the major blood vessels leading to the antrum and duodenal bulb were ligated. The antrum was then resected by making a cut extending from the greater to the lesser curvature, so that the entire lesser curvature was removed up to the limiting ridge. A few millimeters of duodenal bulb were also surgically resected. Gastrointestinal continuity was then reestablished by anastomasing the duodenum to the fundic region of the stomach (Billroth I).

Antrectomy (Billroth II). The same procedure as described above, was followed up to the point of anastomosis. At this time the open end of the proximal duodenum was sewn closed and a longitudinal incision was made in the proximal jejunum. A Billroth II end to side anastomosis of the stomach and the jejunum was then constructed. In both antrectomy procedures simple laparotomies were performed on control

rats. These will be referred to as sham operated.

Pyloric Obstruction as Described by Crean, et al. (17). In this procedure the animals were anesthetized as described above, and a midline incision was made exposing the stomach. In order to standardize the degree of obstruction a bougie was placed beside the duodenal pyloric junction. A ligature was then tightened around the tissue at this point and the bougie was slipped out.

A control group of rats was sham operated as described above.

Statistical Analysis

The vast majority of experimental results in this dissertation were based upon the comparison of experimental and control groups. The statistical differences between the results obtained from these groups were calculated using a Student's t test for unpaired values (80). A regression coefficient determination was also employed in the analysis of the data where an r value of 1.0 represented a linear relationship (80). The 5% probability level was chosen as an indication of significance. The calculations were performed on a computerized Olivetti 101 calculator.

CHAPTER III

RESULTS

Study 1. The Effect of Pentagastrin Administration on the Changes of Intestinal Properties during Starvation

The intestine undergoes severe atrophy during periods of starvation (88, 116). This well-documented finding was verified both morphologically and biochemically in this study. Histological sections of mid-jejunum were studied in rats which were allowed to feed ad lib and rats which were fasted for a period of three days. As can be seen in Fugures 2a and 2b, there is a marked reduction in many parameters of intestinal structure after a short period of starvation. A highly significant difference in crypt and villus height between the fed and starved animals (see Table 1) was recorded.

Intestinal RNA was measured at various periods of starvation. As shown in Figure 3, the RNA content is markedly reduced during starvation, and its decrease is disproportionate to the loss in body weight. A similar reduction was seen in the changes of intestinal DNA content during prolonged periods of food deprivation (see Figure 4). Intestinal protein content and wet weight were also studied and both decreased in relationship to body weight (see Table 2). Significant differences in intestinal protein content were found between all the various stages of starvation. The intestinal wet weight was significantly lower in the

Fig. 2a.--Jejunal structure in rat feeding ad lib. (X 106)

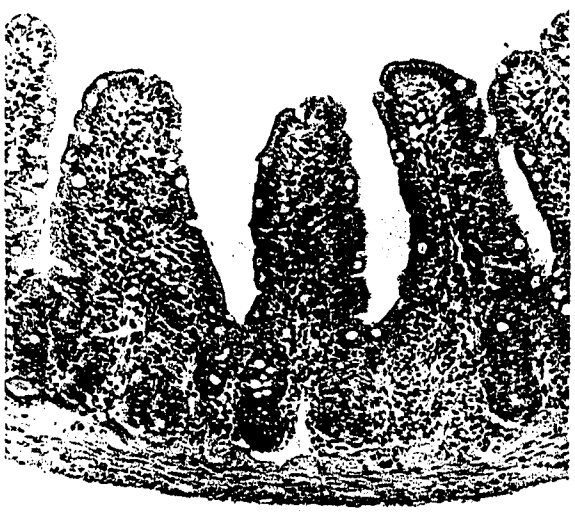

Fig. 2b.--Jejunal structure of rat starved for three days. (X 106)

TABLE 1

JEJUNAL CRYPT AND VILLUS HEIGHT
IN FED AND 3-DAY STARVED RATS

Group	Crypt Height (mm)[a]	Villus Height (mm)[a]
Fed n=2	0.1920 ± 0.0120	0.3838 ± 0.0337
Starved n=3	0.1015 ± 0.0064	0.2598 ± 0.0053
p	<0.005	<0.005

[a]Mean Value ± S.E.

$$\text{where S.E.} = \sqrt{\frac{\Sigma(y - \bar{y})^2}{n}}$$

y = sample

$\bar{y}$ = sample mean

n = sample size

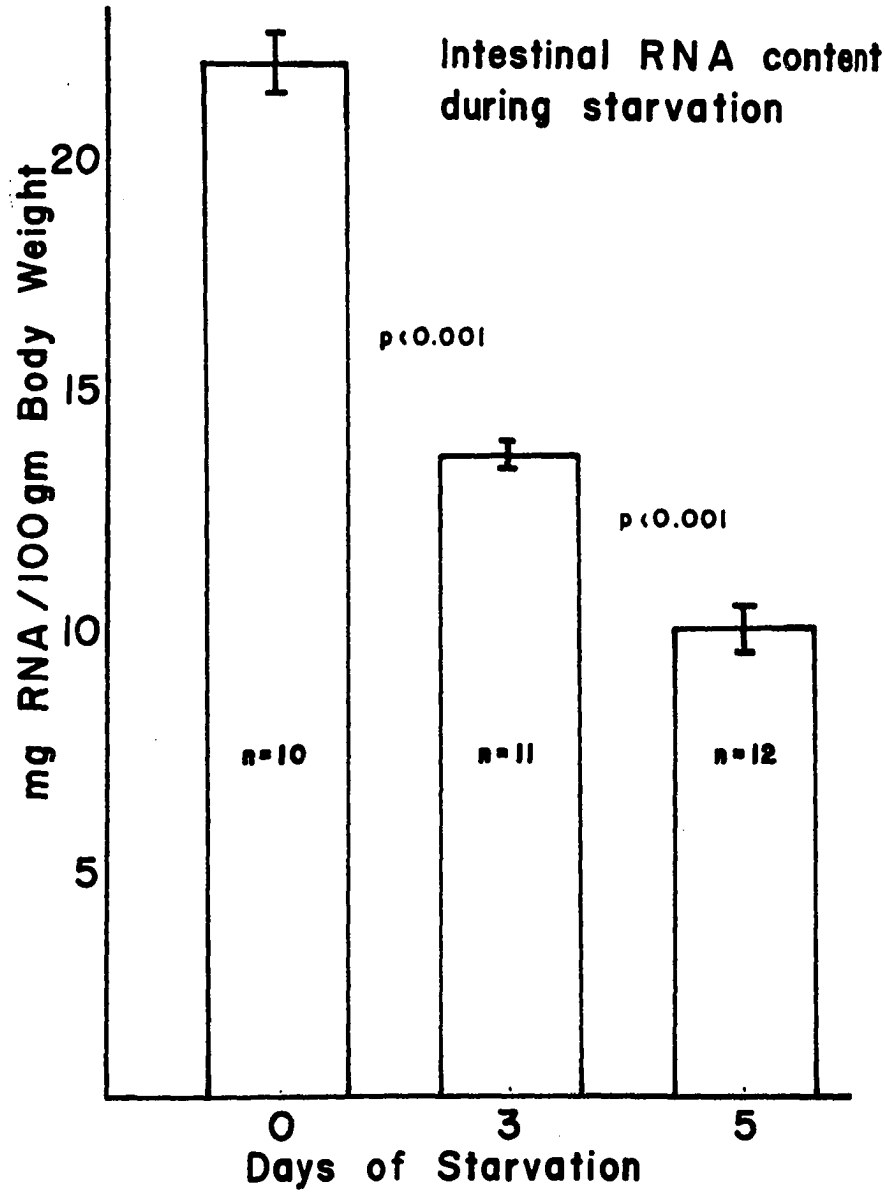

Fig. 3.--Values expressed are mean ± S.E. The probability levels represent the statistical differences of the nucleic acid content between the preceding and succeeding starvation periods.

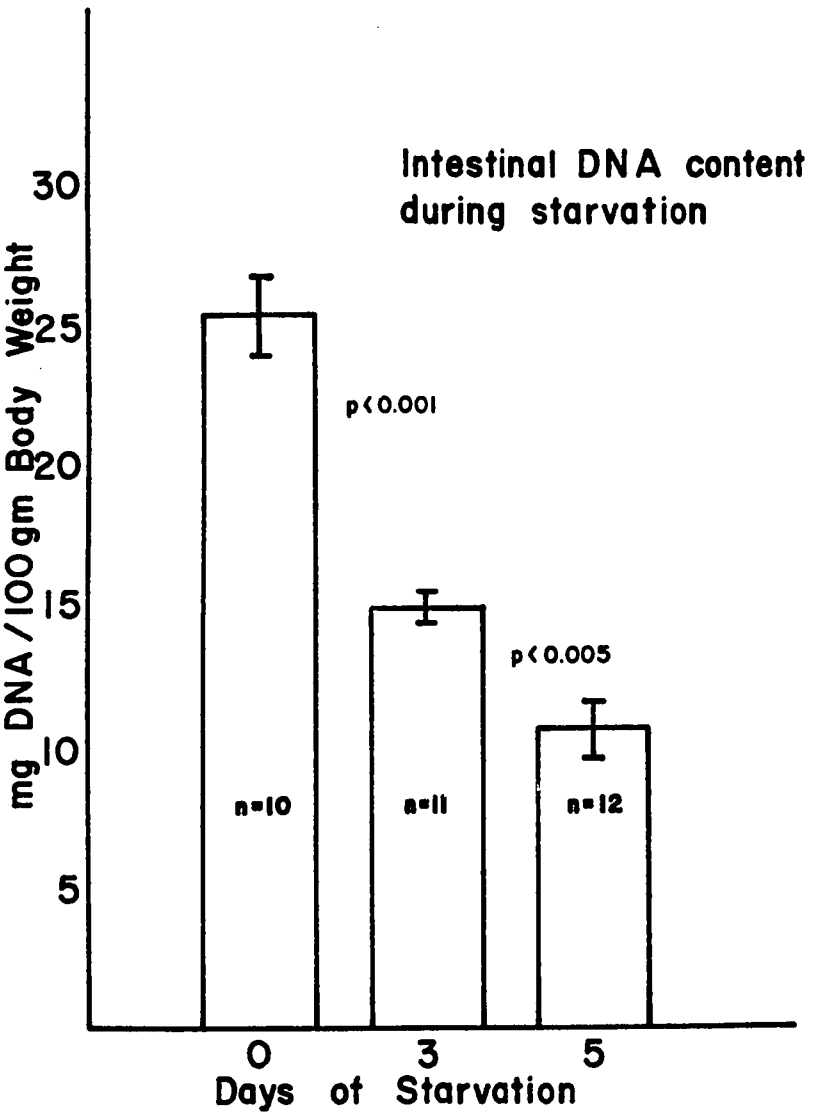

Fig. 4.--Values expressed as mean ± S.E. The probability levels represent the statistical differences of the nucleic acid content between the preceding and succeeding starvation periods.

TABLE 2

INTESTINAL PROTEIN AND WET WEIGHT CHANGES DURING STARVATION

Days Starved	mg. protein[a] / 100 gm. body wt.	gm. wet wt.[a] / 100 gm. body wt.
0 n=10	522.26 ±32.77	3.614 ±0.070
	*	*
1 n=4	385.51 ±31.69	3.284 ±0.235
	*	n.s.
3 n=19	294.94 ±7.73	2.932 ±0.125
	*	n.s.
5 n=10	257.46 ±7.95	2.709 ±0.113

[a]Mean Values ± S.E.

*Represents a significant difference at the 0.05 level between the preceding and succeeding values.

n.s. - Represents nonsignificance between the preceding and succeeding values.

1-day fasted group in comparison to the fed rats (indicated as 0-day starved in Table 2) and in the 5-day starved group in comparison to the 1-day fasted group.

It thus appears that, during starvation, the structural constituents of the intestine are degenerating at a faster rate than are the other tissues of the body.

The changes in the digestive capacity of the small intestine were then studied during periods of starvation. The activity measurements of three different enzymes--lactase, maltase, and alkaline phosphatase--are listed in Table 3. Rats fasted 1-day (instead of fed rats) were used in these comparisons of enzymatic levels, because this eliminated the possibility of residual food contaminating the assay solutions, which could occur when using fed animals. In addition, lactase activity was determined at pH 5.6 only; this was done, because it was demonstrated in a pilot study that brush border lactase activity changed in parallel fashion to the lactase activity measured at pH 5.6, and that lysosomal activity made no significant contribution to the changes seen.

As can be seen in Table 3, both lactase and maltase steadily increased in activity during starvation. Lactase activity appeared to be increasing at a faster rate than maltase activity. A regression coefficient analysis was performed on lactase specific activity changes during starvation and, as can be seen in Figure 5, the increase in activity with time had characteristics of a linear relationship.

In contrast with the disaccharidase activities, alkaline phosphatase followed a different activity pattern during starvation. As can be seen in Table 3, the activity at day-1 and day-5 were not signifi-

TABLE 3

LACTASE, MALTASE, AND ALKALINE PHOSPHATASE ACTIVITY DURING STARVATION

Days Starved	Lactase Activity[a,b] gm. wet weight	Maltase Activity[a] gm. wet weight	Alkaline Phosphatase[c] gm. wet weight
1 n=4	0.5376 ±0.0728	8.893 ±1.124	24.820 ±3.29
	*	*	n.s.
3 n=10	1.1341 ±0.0655	15.056 ±0.867	30.94 ±5.15
	*	*	*
5 n=12	1.6580 ±0.0851	20.267 ±0.966	20.92 ±1.33

*and n.s. have the same meanings, as described in Table 2.

[a]Activity expressed as mean ± S.E. in uM disaccharide hydrolyzed/ min. at 37°C. at optimum pH.

[b]Enzyme analysis was performed only at pH 5.6 for starvation studies, since the lactase activity changes at this pH were demonstrated to directly reflect the changes in "neutral" or brush border lactase activity.

[c]Activity expressed as mean ± S.E. in mg. of phenolphthalein released/30 min. at 37°C. at optimum pH.

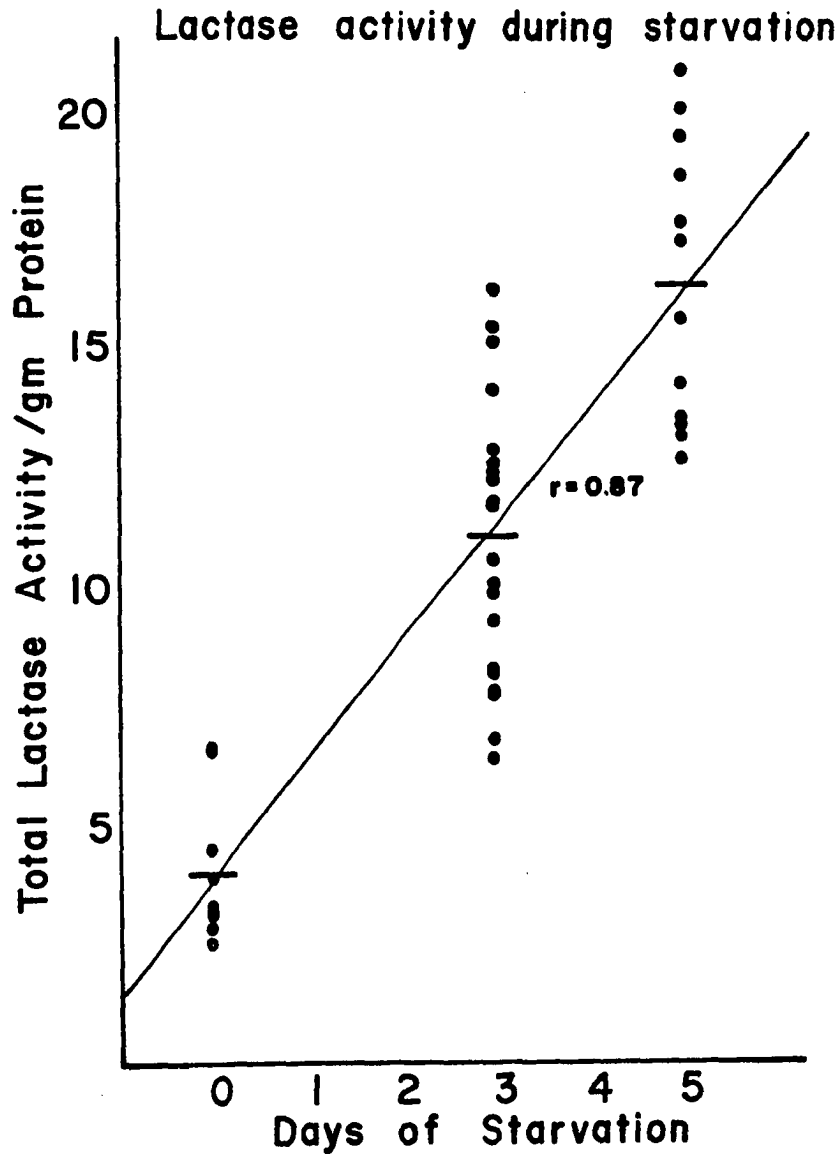

Fig. 5.--Lactase activity expressed as uM of lactose hydrolyzed/min. at 37°C. at pH 5.6, "r" represents the regression coefficient of the proportionality (where in a linear relationship r = 1).

cantly different.

The effects of pentagastrin treatment on these intestinal changes during starvation were then studied. The 3-day starvation period was chosen as the optimal time to investigate this problem, because the starved animals are still in a relatively good state of health at this time (in comparison to the sickly condition of rats starved for five days).

In order to study this problem, half of the starved animals were injected subcutaneously with a maximal dose of pentagastrin (250 ug/kg body weight) three times a day, and a control group was injected with an equal volume of saline at the same time periods. At the end of the third day, the animals were sacrificed and the intestinal tissue was removed and processed for analytical and morphological determinations.

Crypt and villus height measurements were made on the control and treatment groups. A slight increase in villus height and a more noticeable increase in crypt height (10%) was seen in the pentagastrin-treated rats. However, both these changes were insignificant.

The intestinal nucleic acid contents were then determined, and the values of the two groups were compared. As can be seen in Figure 6, there was a small increase in both RNA and DNA content after pentagastrin treatment. A significant difference was recorded only in RNA at this time; however, in another study (which will not be reported here) a significant increase in total intestinal DNA/body weight was found after pentagastrin treatment during a 5-day starvation period. Intestinal protein content was also significantly increased by pentagastrin

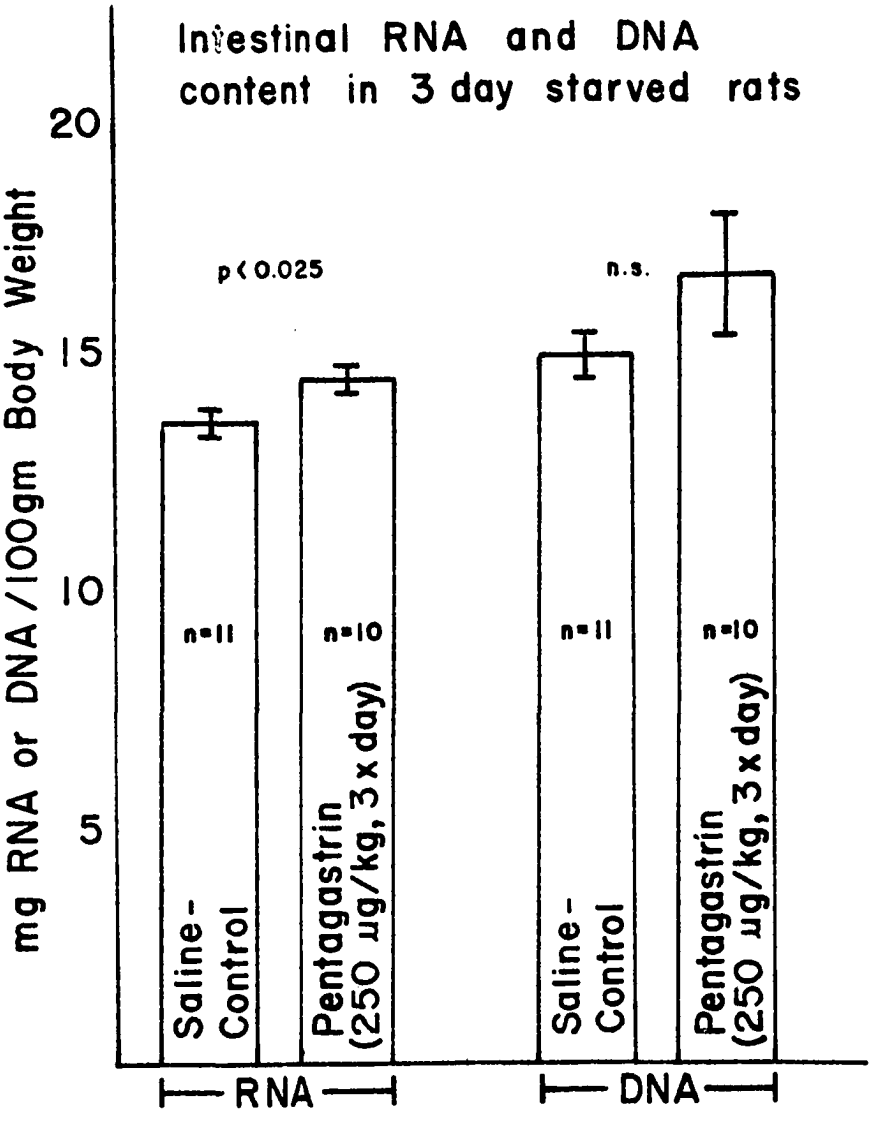

Fig. 6.--Values expressed as mean ± S.E.

treatment in the 3-day starvation experiment.

The effect of pentagastrin treatment on the changes occurring in enzymatic activity during starvation were then investigated. It was found that pentagastrin treatment significantly reduced lactase activity (approximately 30%) in comparison with saline injected 3-day starved controls. As graphically demonstrated in Figure 7, this reduction in activity was evident no matter how the activity was expressed. It was also found in accompanying studies that the decrease in enzyme activity in response to pentagastrin treatment occurred only after three days, for no significant differences were found in 1- and 2-day starvation studies. This change, occurring after three days, was persistent, for a significant difference in enzymatic activity was also measured between a 5-day starved pentagastrin group and a 5-day starved control group.

Maltase activity also was found to be influenced by hormonal treatment during starvation (see Table 4). The significant difference in maltase activity between pentagastrin and control groups initially appeared after three days of treatment and was present at the 5-day periods as well. This decrease in maltase activity, like its increase in activity during starvation, is less dramatic than the changes in lactase activity during these periods.

Slight and insignificant changes in alkaline phosphatase were measured in response to pentagastrin at the 3-day starvation interval (see Table 4). At the 5-day starvation period, however, the enzyme activity was not significantly higher in the hormonally treated group. From this information we concluded that intestinal alkaline phosphatase was not responsive to pentagastrin injections during starvation.

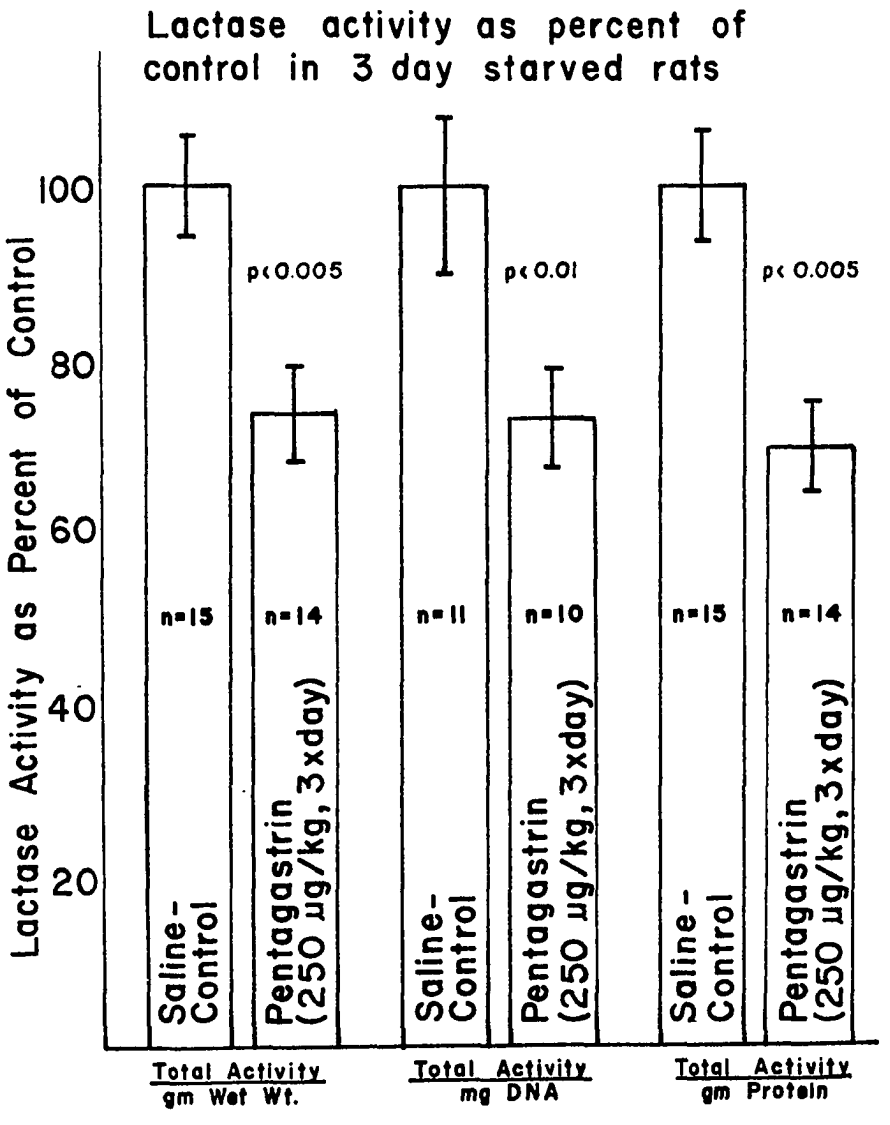

Fig. 7.--Lactase activity, measured at pH 5.6, expressed as mean ± S.E. in per cent of control values.

TABLE 4

MALTASE AND ALKALINE PHOSPHATASE ACTIVITY
IN 3-DAY STARVED RATS IN RESPONSE
TO PENTAGASTRIN TREATMENT

Group	Maltase Activity[a] gm. wet weight	Alkaline Phosphatase Activity[a] gm. wet weight
Control n=20	100.00% ±4.47%	100.00% ±3.74%
Pentagastrin n=18	86.10% ±6.24%	91.18% ±6.48%
p	<0.05	n.s.

[a]Activity expressed as mean ± S.E. in per cent of 3-day starved control values.

In order to determine whether the responses to pentagastrin treatment could be attributed to an enhanced gastric secretory activity (which results from pentagastrin injections) or to a more direct action of the hormone on the intestinal epithelium, the following experiment was performed. Two groups of rats were starved for a 3-day period, one group receiving injections of a dose of histamine (20 mg/kg body weight) three times a day, and the other group being injected with saline at the same time periods. This experiment was performed, because histamine is a potent secretagogue, and when injected at this dosage, it simulates the gastric secretory conditions prevailing after an injection of pentagastrin.

No significant differences in intestinal RNA, DNA, and protein content, or enzyme activity were found between the two groups (histamine and control). Consequently, one can conclude that histamine treatment was ineffective in reproducing the structural and/or functional changes which are seen after pentagastrin administration.

Some of the changes found after pentagastrin treatment may be attributable to a change in cellular hydration. In order to investigate this possibility, wet-weight:dry-weight determinations were performed. It was found that the intestine of both the 3-day starved control and pentagastrin treated group consisted of 81-82% water (no significant difference between groups being found), and thus changes in tissue hydration could not be considered a serious factor which might influence the results.

Study 2. The Effects of Gastrin (or Its Pentagastrin Derivative) on the Ontogenic Development of Intestinal Properties

The immature intestine of suckling rats undergoes dramatic changes in properties during the third and fourth week in life. As described in the introductory section, many of these developmental changes actually overshoot the normal adult values in the first week of weaning. This physiological phenomenon was recorded in our preliminary ontogenic developmental studies as can be seen in Figure 8. On day 14 of life, the wet weight, DNA, RNA, and protein contents of the small intestine are all less than their respective adult values (with the RNA and protein content in the suckling rats being significantly lower). At day 24, the rats have been weaning for over one week, and there is a significant increase in all structural parameters (in comparison to both suckling and adult values). These relative values then decrease towards the adult levels.

Dramatic changes also occur in the enzymatic capacity of the small intestine in the first month of life (with both lactases, and alkaline phosphatase decreasing and maltase increasing in activity). As mentioned earlier, the reason for these functional and structural changes is presently unclear, and in the studies which follow, a possible gastrin influence will be investigated.

Study 2a. The Effect of Prolonged Suckling

The effects of a prolonged suckling period were studied, in order to determine which developmental changes are initiated by the act of weaning. Suckling littermates were divided into two groups; one was allowed to wean and the other was deprived of access to solid food.

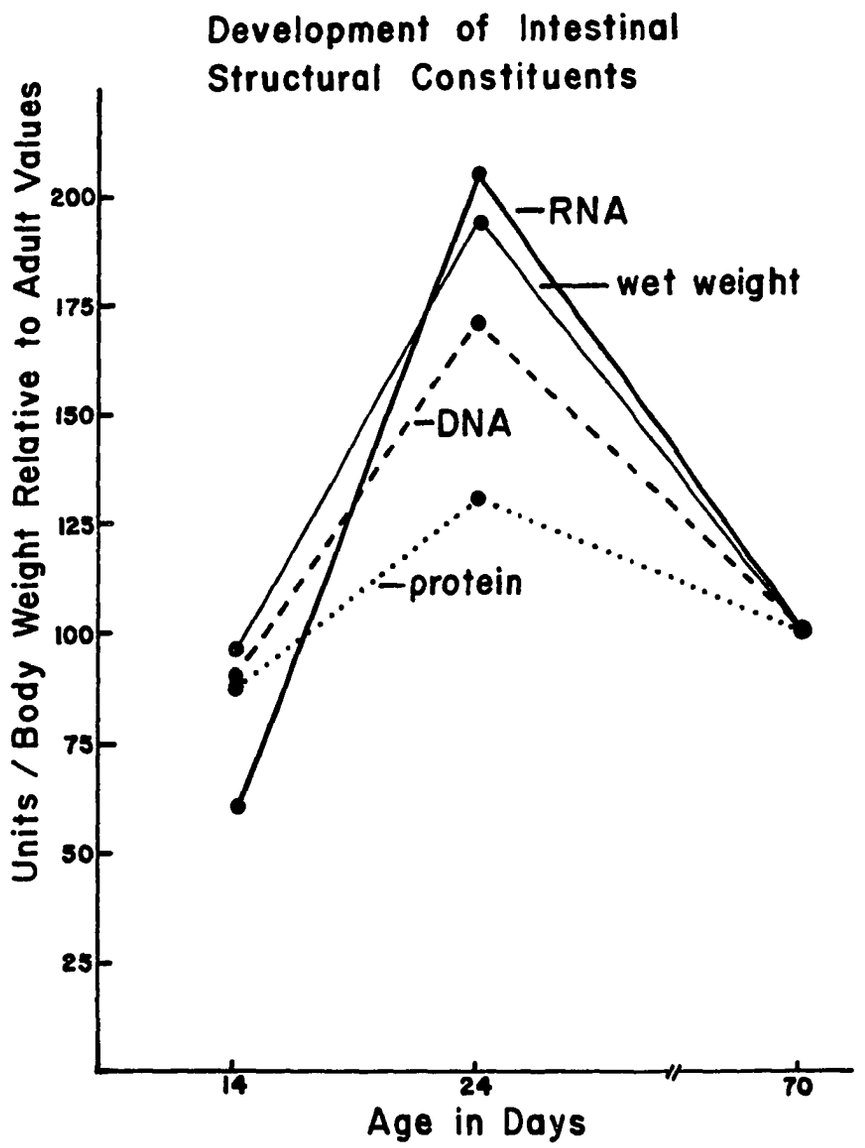

Fig. 8.--Expressed as mean per cent of adult values.

The differences in intestinal RNA and DNA content between the two groups can be seen in Figure 9. In spite of the marked increase in body weight which occurs during weaning, the total intestinal RNA and DNA content when expressed per unit of body weight are higher in this group than in the "suckling only" rats. The increase in RNA content was highly significant, whereas the apparent increase in DNA levels was not significant. The weaning group also had a small increase in both intestinal weight and protein content relative to body weight, but these changes did not reach the level of significance.

Possible differences in the development of enzymatic activity were then studied. Normal development of adult values of alkaline phosphatase and maltase was evident in both groups of rats. Alkaline phosphatase activity approached the low adult values, decreasing to 1/3 of its activity at 14 days, and there was absolutely no difference discernible between the groups. The maltase activity had increased from low values at 14 days of age to levels surpassing known adult values. This overshoot has been previously reported in the literature (46). There was a slightly greater maltase activity in the weaning group, but this difference proved to be nonsignificant.

Lactase activity was then measured in these 24-day-old rats. Because of the high levels of both "acidic" and "neutral" lactase activity in suckling rats, these two enzyme activities were separated by the method of Koldovsky, et al. (65) as described in the Appendix (page 148). It was found that while "acidic" lactase activity declined normally to low adult values in both groups, marked differences existed between the groups in the development of "neutral," or brush border

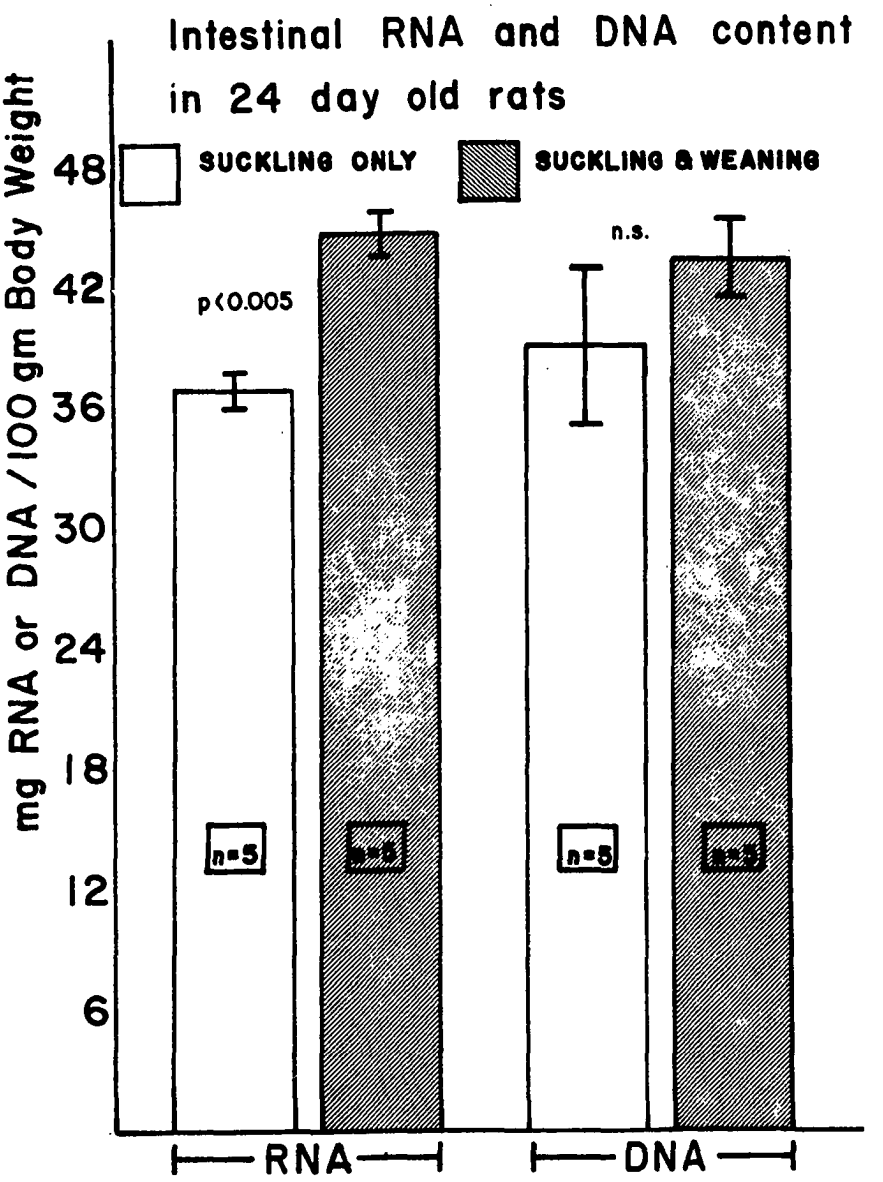

Fig. 9.--Values expressed as mean ± S.E.

lactase (see Figure 10). The weaning rats demonstrated the normal decrease in lactase activity to adult values (approximately 15-fold), whereas the rats deprived of solid food had only a 3-fold decrease in "neutral" lactase activity from the high suckling values. As can be seen, the differences between the groups were highly significant.

Study 2b. The Effect of Pentagastrin on Intestinal Properties of 22-Day-Old Rats

In this study, littermate rats were divided into two groups on day 14. The treatment group received pentagastrin injections (250 ug/kg, 3 X day) and the control group was injected with saline. Both groups were deprived of solid food and were dependent on suckling as the sole source of nourishment. On day-22, the rats were sacrificed.

Table 5 lists the results obtained. Significant increases were found in the "pentagastrin-treated" group (in comparison to the controls) in relative intestinal wet weight, protein, and RNA content, while an insignificant increase in the DNA/body weight ratio was recorded.

Analyses of enzymatic activity of these two groups of rats are presented in Table 6. As expected, there was no significant difference in alkaline phosphatase and maltase activity between the groups. Surprisingly, however, both "acidic" and "neutral" lactase's were significantly decreased in response to pentagastrin treatment.

Study 2c. The Effect of Pentagastrin on Intestinal Properties of 14-Day-Old Rats

In this experiment, 9-day-old littermate rats were divided into two groups, one of which received injections of pentagastrin while the other (controls) received injections of saline. The suckling rats were

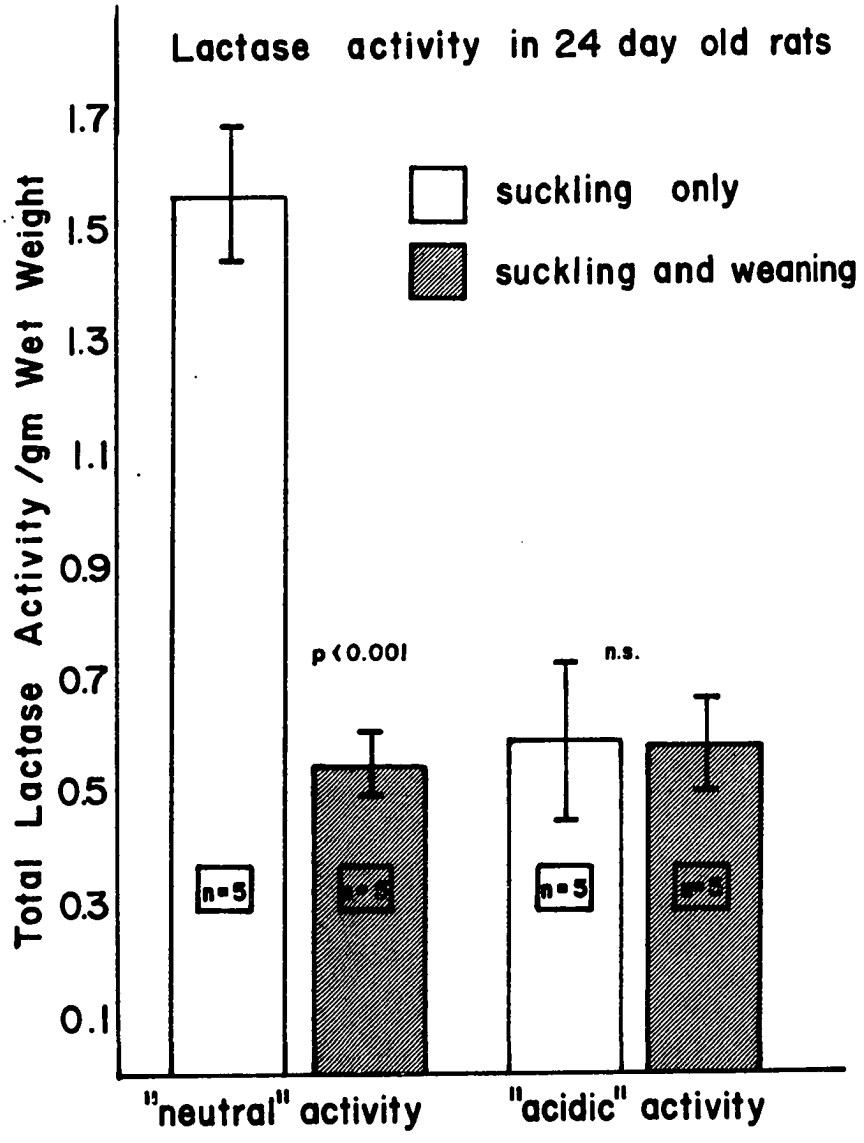

Fig. 10.--Lactase activity expressed as mean ± S.E. in uM of lactose hydrolyzed/min. at 37°C. at optimum pH.

TABLE 5

INTESTINAL STRUCTURAL PROPERTIES OF 22-DAY-OLD RATS IN RESPONSE TO PENTAGASTRIN TREATMENT (250 ug/kg, 3 X DAY)

Group	mg. protein[a] / 100 gm. body wt.	gm. wet wt.[a] / 100 gm. body wt.	mg. DNA[a] / 100 gm. body wt.	mg. RNA[a] / 100 gm. body wt.
Control n=6	488.13 ±12.68	4.075 ±0.101	20.886 ±2.746	42.100 ±0.932
Pentagastrin n=6	568.28 ±42.24	4.826 ±0.193	22.607 ±2.926	52.794 ±3.360
p	<0.05	<0.005	n.s.	<0.01

[a]Mean Value ± S.E.

TABLE 6

INTESTINAL ENZYMATIC ACTIVITY OF 22-DAY-OLD RATS IN RESPONSE TO PENTAGASTRIN (250 ug/Kg, 3 X DAY)

Group	Lactase		Maltase Activity[a] gm. wet wt.	Alkaline Phosphatase Activity[b] gm. wet wt.
	Acidic Activity[a] gm. wet wt.	Neutral Activity[a] gm. wet wt.		
Control n=6	4.194 ±0.185	7.364 ±0.329	11.657 ±0.768	51.052 ±2.692
Pentagastrin n=6	2.214 ±0.436	4.186 ±1.234	13.224 ±0.528	46.484 ±3.843
p	<0.005	<0.05	n.s.	n.s.

[a]Activity expressed as mean ± S.E. in uM of disaccaride hydrolyzed/ min. at 37°C. at optimum pH.

[b]Activity expressed as mean ± S.E. in mg. of phenolphthalein released/ 30 min. at 37°C. at optimum pH.

sacrificed on the fourteenth day of life.

The pentagastrin-treated rats showed the characteristic pattern of an approximate 10% increase in the structural parameters relative to the body weight. Significant differences were only found in the total intestinal protein/100 gm body weight ratio (458.87 ± 20.1 and 397.39 ± 12.42 for pentagastrin and saline injected rats, respectively).

Alkaline phosphatase activity, "acidic lactase," and maltase activity remained at their normal suckling levels in both groups and appeared to be insensitive to the hormonal treatment. "Neutral" lactase, however, did respond to pentagastrin, significantly decreasing towards adult values. This can be seen graphically in Figure 11.

Study 3. The Effects of Low Endogenous Gastrin Levels on Intestinal Properties

This problem was studied on antrectomized rats. Gastrointestinal continuity was then reestablished by two techniques (Billroth I and Billroth II). Sham-operated rats served as controls.

The first experiment in this study was performed on rats with an antrectomy followed by a gastroduodenostomy (Billroth I). Jejunal sections were removed for histological examination. No apparent difference in intestinal architecture was found between the antrectomized and sham-operated groups. Analytical studies indicated that intestinal structural parameters were somewhat reduced in the antrectomized group, with small decreases being recorded in intestinal protein, RNA, and DNA content when expressed in relation to body weight. A significant difference was found only between the intestinal RNA content of the antrectomized and the sham-operated rats (see Table 7).

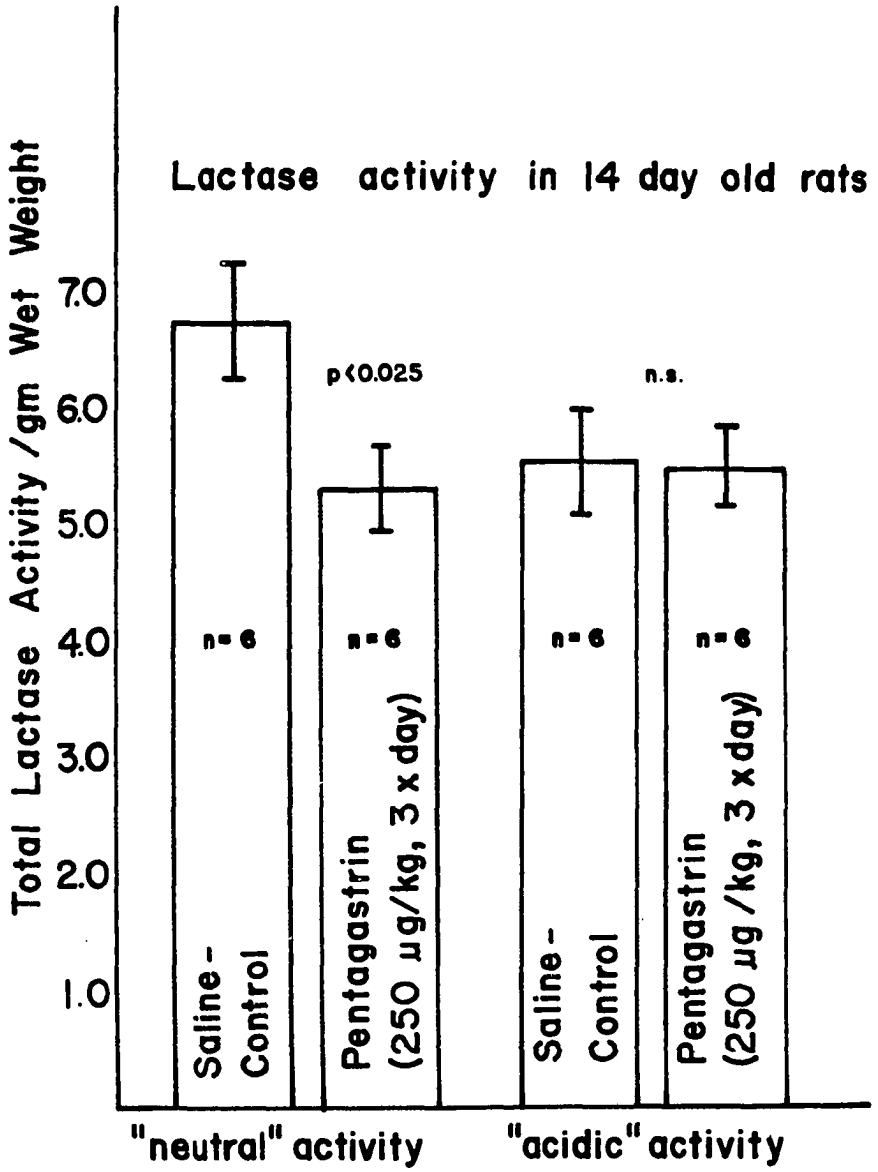

Fig. 11.--Lactase activity expressed as mean ± S. E. in uM of lactose hydrolyzed/min. at 37°C. at optimum pH.

TABLE 7

EFFECT OF LOW AND HIGH ENDOGENOUS GASTRIN LEVELS
ON INTESTINAL RNA AND DNA CONTENT

Group	mg. RNA[a] / 100 gm. body wt.	mg. DNA[a] / 100 gm. body wt.
Control	12.593 ±0.500	9.348 ±1.245
Antrectomy	11.163 ±0.165	7.848 ±0.648
p	<0.025	n.s.
Control	15.126 ±0.436	12.727 ±0.386
Distension	15.758 ±0.419	14.051 ±0.385
p	n.s.	<0.025

[a]Mean Value ± S.E.

Enzymatic analyses were then performed, and no significant differences in the levels of any of the four enzymes measured could be detected between the groups.

A Billroth II (gastrojejunostomy) procedure was then performed on a group of rats following antral resection. Another group was sham-operated. Unfortunately, there was a high mortality rate among the antrectomized animals, so that only several Billroth II-antrectomized rats survived the one month recuperation period. Upon sacrifice, another problem was revealed. The intestinal region of reanastomosis varied from distal duodenum in some animals to distal jejunum in others and a significant amount of adhesion of intestinal tissue to other organs occurred, so that the total length of intestine was not recovered in all cases. For these reasons, it was decided to use these rats for a pilot morphological study on the effects of this procedure on intestinal structure. Two sections for histology were removed from each animal, one from the proximal duodenum and one from the distal jejunum. In this way, it was possible to compare the effects of this surgical procedure on a duodenal region (which was deprived of contact with luminal contents) to a normal duodenal segment which was part of the normal gastrointestinal continuity, and at the same time, to compare the jejunal structure of the two groups of rats.

The averaged results of the quantitation of crypt and villus height of the duodenum and jejunum from the two groups are presented in Table 8. (Because of the few sections examined, no statistical analysis of the results was attempted.) It can be seen from the table that duodenal villus height appears to be markedly reduced and crypt height

TABLE 8

VILLUS AND CRYPT HEIGHT[a] IN ANTRECTOMIZED-BILLROTH II AND SHAM-OPERATED RATS

	Duodenum		Jejunum	
Group	Crypt Height	Villus Height	Crypt Height	Villus Height
Control	0.1333	0.3833	0.1130	0.2655
Antrectomized	0.1628	0.2614	0.1322	0.3126

[a]Expressed in millimeters

apparently is either unaffected or increased in the antrectomized-Billroth II group (see Figures 12a and 12b). On the other hand, both jejunal crypt and villus heights were slightly greater in the antrectomized-Billroth II animals than in their sham-operated counterparts. Other interesting observations include the marked decrease in the villus height/crypt height ratio in blind-ended duodenal loops of the antrectomized animal when compared to the ratio found in normal duodenal segments. There was no such difference recorded in the morphology of jejunal tissue. Also, it appeared that the villus height gradient along the intestinal length, which was present in the sham-operated rats, was absent in the antrectomized group, whereas a crypt height gradient could be observed in both groups.

Study 4. The Effects of High Endogenous Gastrin Levels on Intestinal Properties

This experiment was performed by surgically ligating the pyloric region in a group of rats while sham-operated rats served as controls. It was found that intestinal structural parameters were slightly increased by pyloric obstruction (antral distension). Small increases were also found in the relative amounts of RNA, DNA, and wet weight of intestine. As can be seen in Table 7, the DNA content was the only property that was significantly increased by this procedure which presumably caused hypersecretion of gastrin.

The results obtained with the enzymatic studies demonstrated that lactase activity was significantly lower in the distension group in comparison to the controls (see Figure 13). No differences in maltase or alkaline phosphatase was detected between the two groups.

Fig. 12a.--Duodenal structure of sham-operated rat. (X 113)

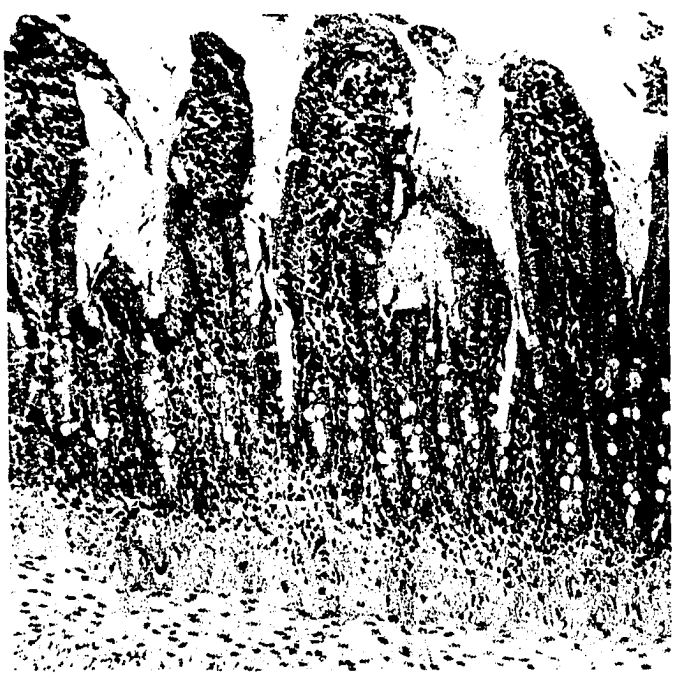

Fig. 12b.--Duodenal structure of antrectomized-Billroth II operated rat. (X 113)

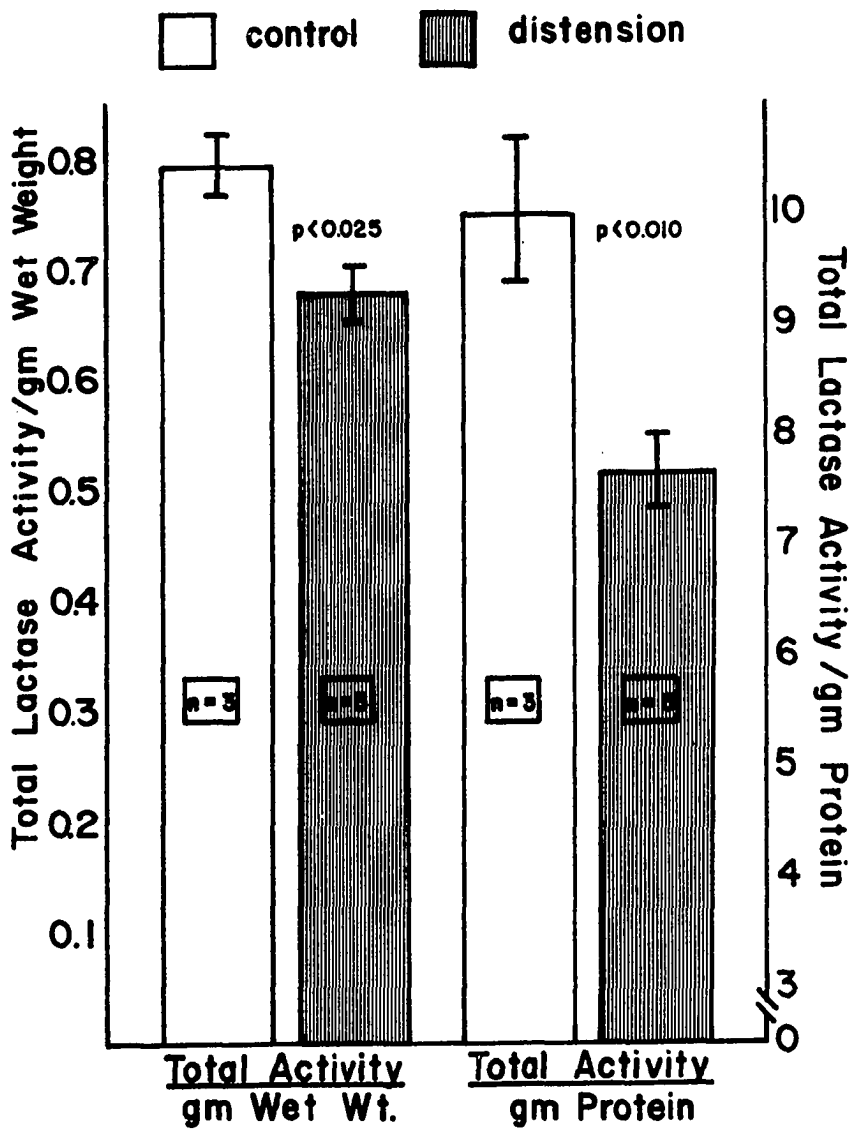

Fig. 13.--Effects of antral distension on lactase activity. Activity expressed as mean ± S.E. in uM of lactose hydrolyzed/min. at 37°C. at a pH of 5.6.

Study 5. The Effects of Pentagastrin on Duodenal Culture Cells

An adult rat duodenal culture line was established as described in the methods section. A week after the original explants were set down, one set of T flasks was started on daily doses of pentagastrin (0.5 ug/ml medium), and the controls were treated with an equal volume of saline daily. Approximately three months later, cellular growth kinetics were studied on the matching culture lines.

Clone counts were made on the pentagastrin and saline-treated cultures to get an estimate of the cellular doubling times (or the period it takes for the number of the cells/clone to double). The results can be seen in Figure 14. The pentagastrin-treated cultures were found to have a doubling time of 19.5 hours, whereas the saline-treated duodenal cells double their number every 31.5 hours. This difference was highly significant.

What was the cause of this marked increase in growth rate? Pentagastrin may be accelerating the cell cycle time; it may be increasing the percentage of proliferative cells in the culture; or it may be affecting both parameters simultaneously. In order to investigate this problem, a determination of the percentage of cells in the proliferative pool of each culture was made. The detailed techniques involved in this kinetic autoradiographic study are listed in the Appendix (page 151). The results are presented in Figure 15. The percentage of cells in the proliferative pool is markedly different in the two groups. Approximately 73% of the pentagastrin-treated cells incorporate ^{3}H-thymidine into their nucleus (and hence are proliferative), whereas, only 36% of

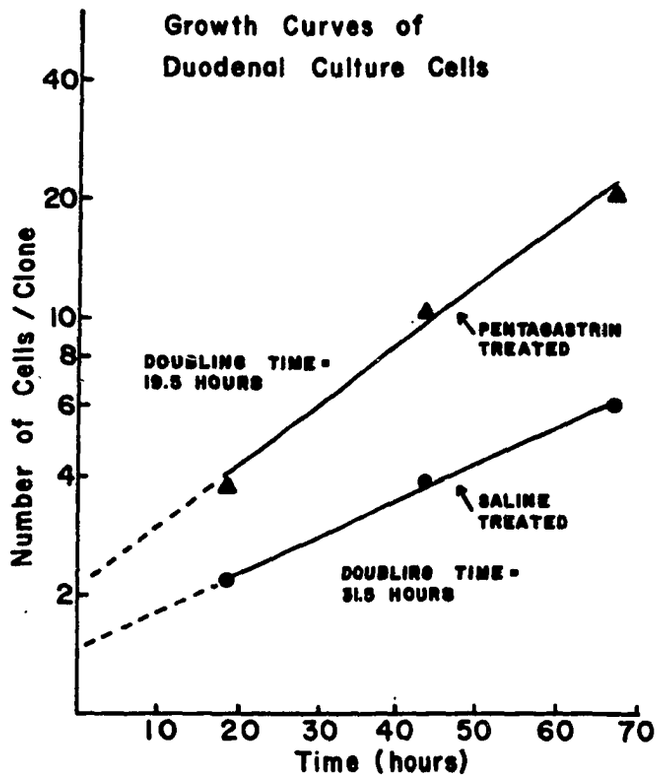

Fig. 14.--Each point represents a mean value of 15 Clone counts.

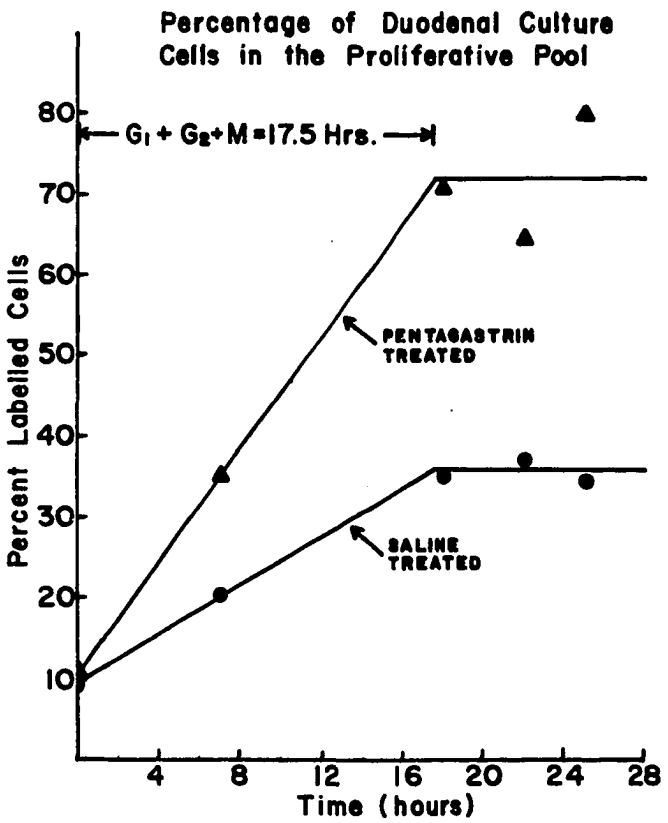

Fig. 15.--Each point represents the percentage of labelled cells in a field of 1,000 cells.

the saline-treated cells are proliferative. The rise time of both curves is 17.5 hours. As described in the Appendix (page 152), the duration of this phase is equivalent to $G_2 + M + G_1$.

Typical fields seen in both pentagastrin and saline-treated cultures (on the plateau region) are presented in Figures 16 and 17, respectively. The silver grains located in the cellular nucleus reflect the presence of ^3H-thymidine in the nuclear DNA, and are indicative of active DNA synthesis. As can be seen, the frequency of labelled cells in the control population is far less.

Using information obtained from the above two kinetic studies, a crude approximation of the cellular generation time (G_T) and the duration of the S phase can be calculated (see page 153 of the Appendix). The approximate values are listed in Table 9. As can be seen, there is roughly a two-fold difference in cell cycle time, and this is solely attributable to a much shorter S (DNA synthesis) phase in the pentagastrin-treated duodenal cultures.

The shortened doubling time of the pentagastrin-treated duodenal cultures, in comparison to the controls, therefore appears to be related to both an increase in the percentage of proliferative cells in the population, as well as to a greatly shortened generation time.

The cell populations of the two duodenal cultures were then examined with high and low-powered light microscopy. The pentagastrin-treated cultures appeared more epithelioid in both growth pattern and cell shape. As can be seen in Figure 18, the cells are cuboidal in shape, and have a prominent circular nucleus. The cells are oriented in a side by side manner and grow in a sheet-like fashion. These features

Fig. 16.--Autoradiograph of saline-treated duodenal culture cells, demonstrating percentage of cells labelled (grains in nucleus). (X 590)

Fig. 17.--Autoradiograph of pentagastrin-treated duodenal culture cells, demonstrating percentage of cells labelled (grains in nucleus). (X 366)

TABLE 9

EFFECT OF PENTAGASTRIN ON CELL CYCLE PHASES OF DUODENAL CULTURE CELLS

Group	$G_2 + M + G_1$[a]	S[a]	G_T[a]
Control	17.5	27	44.5
Pentagastrin	17.5	5	22.5

[a]Expressed in hours

where

S = DNA Synthesis

G_T = Generation Time

$G_2 + M + G_1 = G_T - S$

Fig. 18.--Autoradiograph of pentagastrin-treated duodenal culture cells (epithelial cells). (X 720)

are found predominantly in epithelial populations (45). Although an occasional fibroblast is found in the pentagastrin-treated cultures, they are much more frequently found in the control cultures. These cells are elongate and spindle-like in shape and have a relatively smaller nucleus (in comparison to epithelial cells), which has an oval configuration. Their growth pattern is much less organized than one finds in epithelial populations, for fibroblasts are oriented in a haphazard fashion in relation to neighboring cells. Epithelial clusters are also seen in the control cultures and represent approximately 50% of the population whereas, the pentagastrin-treated cultures consist of approximately 90% epithelial cells. A typical field seen in a control culture is shown in Figure 19.

Ultrastructural studies were then performed on the duodenal culture cells. An electron micrograph of an epithelial cell from the pentagastrin-treated culture is shown in Figure 20. Microvillus-like structures are sparsely distributed along the periphery of the cell. They are small and aligned in a disoriented fashion. A prominent trilaminar surface membrane covers these projections, and a thin, translucent fuzzy coat is seen overlaying the membrane. No terminal web was seen in association with the microvillus-type structures; however, small vesicular structures were frequently seen adjacent to the membranous surface. Another characteristic of these cells is depicted in Figure 21. In this cell, a large extruding cytoplasmic mass (several microns in diameter) appears. Cytoplasmic structures such as a Golgi network and endoplasmic reticulum are frequently seen enclosed in these cytoplasmic blebs. In general, the Golgi network is well developed in these

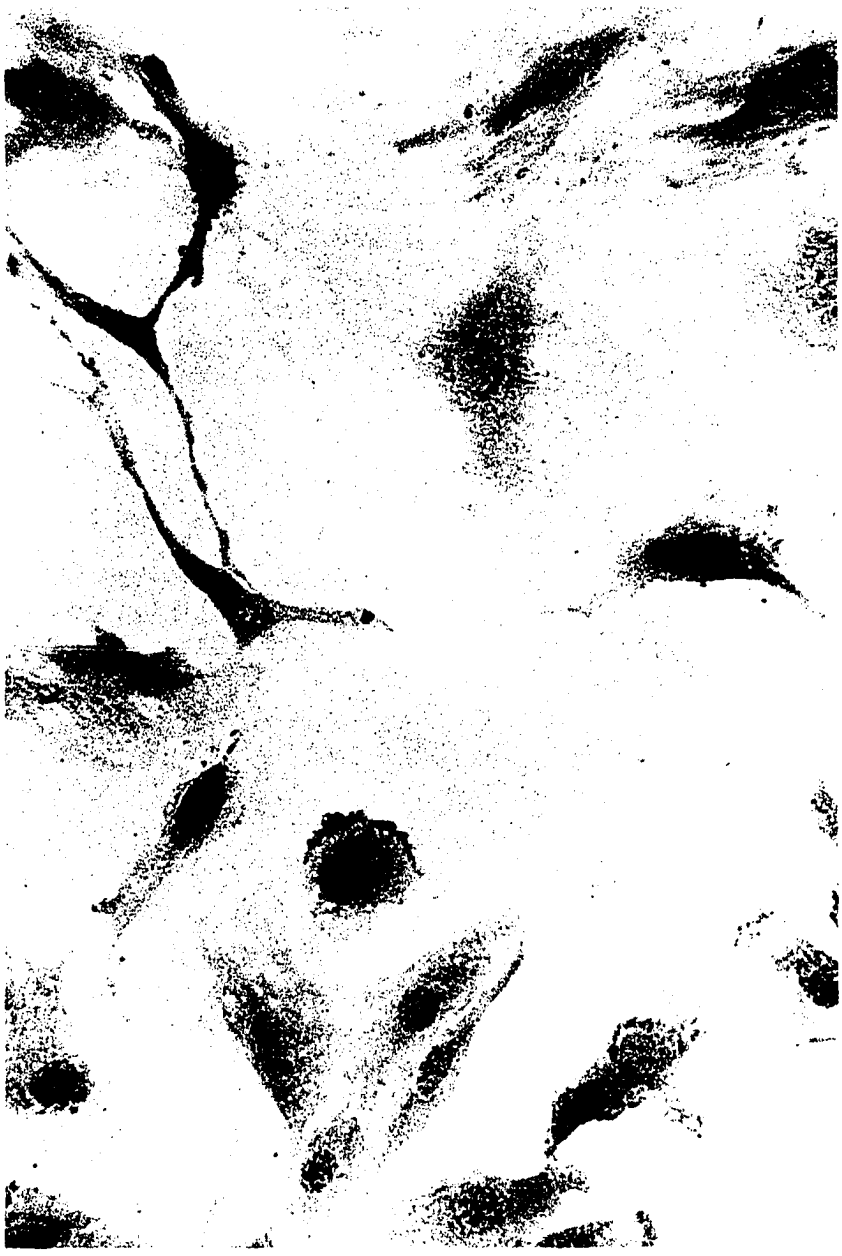

Fig. 19.--Autoradiograph of saline-treated duodenal culture cells (fibroblastic-epitheliod admixture). (X 235)

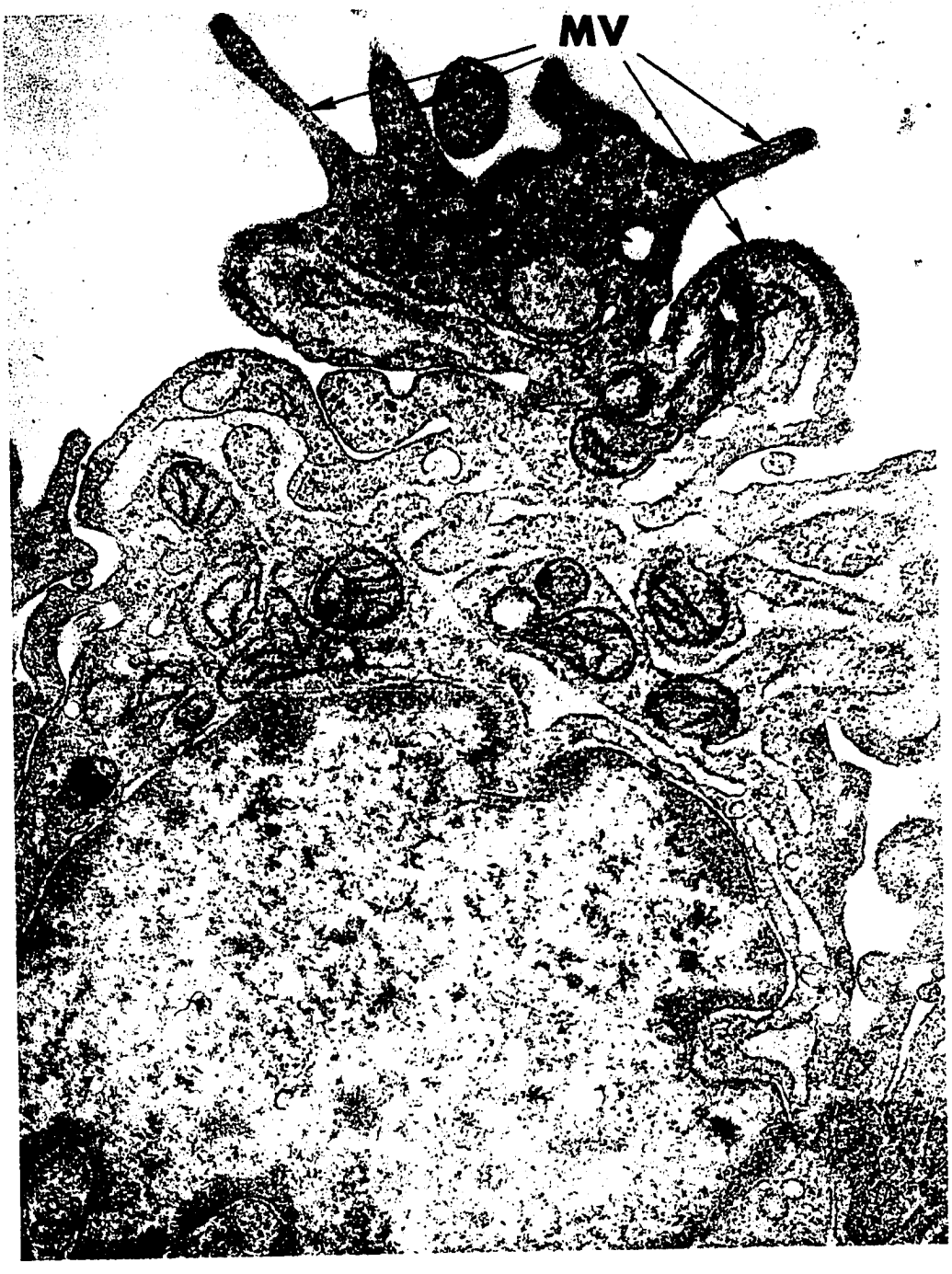

Fig. 20.--Electron micrograph of a pentagastrin-treated duodenal culture cell. Short microvillus-type structures (MV) and vesicles (V) can be seen lining the periphery of the cell. (X 33,000)

Fig. 21.--Electron micrograph of pentagastrin-treated duodenal culture cell, demonstrating cytoplasmic extrusions. Several cytoplasmic blebs (B) can be seen being extruded. (X 50,000)

cells.

If one now refers back to the discussion of the ultrastructure of immature crypt epithelial cells (found on page 8) of the Introduction), the marked similarities between these two types of epithelial cells becomes apparent. All the above ultrastructural properties are common to both cell types, and, indeed, it seems that the duodenal epithelial cells grown in tissue culture may be identical to crypt epithelial cells. It must be emphasized that this cell type is present in both the pentagastrin and control cultures; however, they are found much more frequently in the former.

The ultrastructure of a fibroblast, which represents a significant proportion of the control culture population, is shown in Figure 22. The fusiform shape of the cell is in marked contrast to the cuboidal configuration of an epithelial cell. These cells are characterized by a predominant endoplasmic reticulum, which can also be seen in the electron micrograph.

The rapidly dividing pentagastrin-treated cells seem to have lost the "normal" property of contact inhibition (when cell division and migration is inhibited upon contact with a neighboring cell). This is particularly evident when, after a confluent monolayer is formed, piling up of cells into a mound-like structure occurs (see Figure 23a). Contact inhibition is present in the control population, for a cellular monolayer persists after confluency is reached (see Figure 23b).

Fig. 22.--Electron micrograph of a saline-treated duodenal culture cell. Extensive endoplasmic reticulum (ER) can be seen in the cytoplasm. (X 22,000)

Fig. 23a.--Pentagastrin-treated culture at confluency. (X 102)

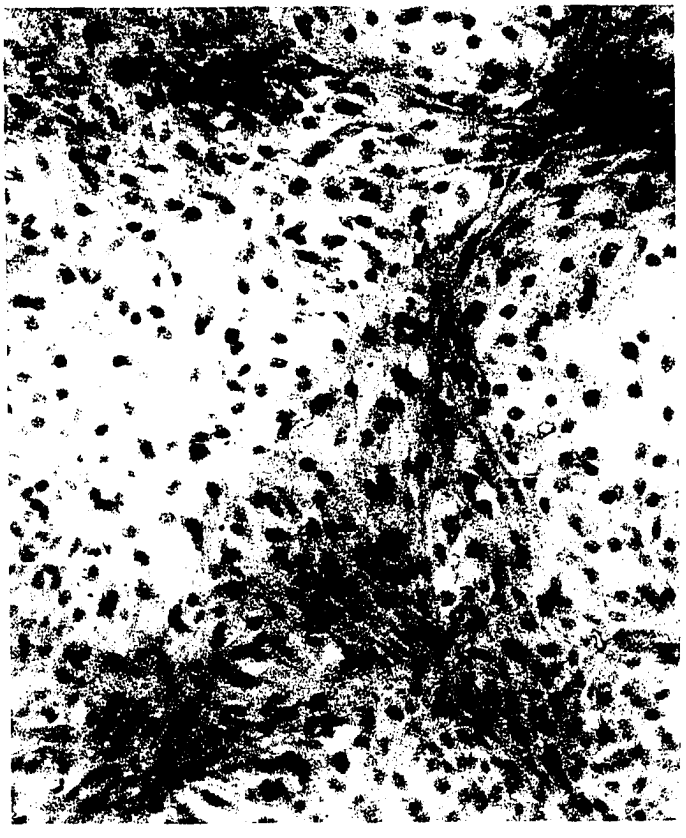

Fig. 23b.--Saline-treated culture at confluency. (X 102)

CHAPTER IV

DISCUSSION

In this chapter, the experimental results will be discussed and various interpretations of their significance will be considered. The results will be discussed in the order of their presentation in the preceding chapter, and an attempt will be made to interrelate the findings in all five studies. From the above information, hypotheses will be made about a possible role of gastrin in the regulation of the changeable intestinal properties, which were analyzed in detail in the introductory chapter.

Discussion of Experimental Results

Starvation Study

The effects of starvation on the small intestine. The preliminary results on the effects of starvation on the structural parameters of the small intestine basically verify previous reports on the subject made in other laboratories (88, 116). We found that starvation in the rat causes marked atrophy of villus and crypt height. Further quantitative analyses revealed disproportionate decreases in intestinal wet weight, RNA, DNA, and protein content when considered in relation to the effects of starvation on body weight as a whole.

Our results concerning enzymatic activity during starvation

were in general agreement with Troglia, et al. and McNeil, et al. who reported enhancement of enzymatic activity in undernutrition and starvation, respectively (89, 123). We found that disaccharidase activity (notably lactase) was dramatically increased during periods of starvation and that a linear type relationship exists between the number of days a rat is deprived of food and the specific activity of lactase. Maltase activity also increased during starvation, but it was not as affected by the lack of food as lactase, as it increased in activity at a slower rate. In contrast to the above enzymes, alkaline phosphatase activity was not changed during the starvation period. This is quite an interesting finding, since all three enzymes are associated with the brush border membrane of the intestinal epithelial cell.

The reason for the increase in disaccharidase activity during starvation is unclear at present. One possible explanation is that the serosal and submucosal regions of the small intestine may undergo greater atrophy during starvation than the mucosa, hence increasing the brush border enzymatic activity (activity/gm wet weight). Since it has been demonstrated that the mucosa is the most severely affected layer of the small intestine during starvation, this interpretation is probably not correct (88, 116). Another more acceptable interpretation is that the intestinal epithelial cells attained a more mature stage of differentiation during starvation. This theory is consistent with the findings of Hopper, et al. (which were discussed in the introduction) that the maturation phase of the crypt cell is significantly lengthened during a starvation period (51). This determination is based upon the increased generation time and decreased crypt migration rate of

intestinal epithelial cells from starved rats. It is believed that the cell is programmed for differentiation during this phase (i.e., the mRNA for disaccharidase production is synthesized). It is quite conceivable, then, that the duration of the maturation phase is directly related to the stage of differentiation which the cell will attain. If this is so, it may account for the increase in the number of disaccharidase molecules per cell and, hence, the greater enzyme activity. The differences in the effects of starvation on lactase, maltase, and alkaline phosphatase are more difficult to explain. It is possible that a balance exists between two antagonistic factors, one stimulating differentiation, the other dedifferentiation, and each brush border enzyme inherently has a different sensitivity to these two regulatory agents. During starvation, one of these factors, i.e., the dedifferentiation factor may be deficient, resulting in a non-uniform change in brush border enzymatic activity.

The extreme sensitivity of the small intestine, both structurally and functionally to food deprivation is unexplained. The severe atrophy of the structure of the small intestine during starvation has been noted many times, but the mechanism behind this dramatic change has never been investigated. The assumption underlying all these reports is that the intestine, unlike other tissues in the body, is dependent on sequestering its nutritional supply from two sources, namely the blood and the lumen, and, therefore, is expected to be most severely affected during starvation. While this theory may be true, it has not to date been tested. It was our desire to investigate another possible mechanism to account for the intestinal changes during

starvation.

The relationship between starvation and a gastrin deficiency. As mentioned in the introduction, gastrin release is believed to be triggered in part by the presence of food in the antrum, and thus is ideally suited to monitor the presence or absence of food in the gastrointestinal tract. Gastrin blood levels have been shown to increase approximately five-fold after the ingestion of a meal. Since gastrin has been shown to have a trophic influence on the gastric and duodenal mucosae, we formulated the hypothesis that effects of starvation on the small intestine are mediated by a gastrin deficiency. We tested this theory by injecting starved rats with pentagastrin. If the effects of starvation on intestinal structure and function are indeed due to a gastrin deficiency, replacement therapy of this hormone should reverse these changes. The pentagastrin-treated starved rats had a significantly higher intestinal RNA, protein, and possibly greater DNA content than the starved controls. In addition, lactase and maltase activities were significantly lower in the treatment group, whether they were expressed per wet weight, protein, or per DNA. There was no noticeable pentagastrin effect on alkaline phosphatase activity. Thus, many of the changes in intestinal properties associated with starvation were partially reversed by pentagastrin treatment, and it, therefore, can be concluded that the starvation-induced intestinal changes may be the result of a gastrin deficiency.

The inhibitory influence of pentagastrin on disaccharidase activity could be due to many factors. In light of the knowledge that starvation increases the maturation phase of cells essentially by

retarding both crypt cell generation time and migration rate, it is quite possible that pentagastrin has the reverse effect. In this case, pentagastrin would decrease the maturation phase by stimulating proliferative and migratory activity in the crypt and, in turn, reduce cellular development of those enzymes whose synthesis is dependent on the length of the maturation phase. It is, therefore, possible that gastrin may be the dedifferentiation factor, which is deficient during starvation. This deficiency would stimulate the development of the enzymes which are particularly sensitive to gastrin. From the data obtained in this study one would assume that the order of sensitivity of the three enzymes is: lactase > maltase >> alkaline phosphatase.

Our results support our preliminary hypothesis, in that the structural degenerative changes and increase in enzymatic capacity of the small intestine occurring during starvation may be attributed to a general state of gastrin deficiency. From these findings, one also could conclude that gastrin is a stimulant of intestinal growth, but not differentiation, and, therefore, has characteristics of an "intestinal trophic hormone."

Ontogenic Development

With these findings in mind, we investigated the role that gastrin may play in the ontogenic development of the small intestine. We earlier advanced the theory that gastrin plays a major role in the development of the intestine at the onset of weaning.

As discussed in detail earlier and demonstrated in our results, there is a dramatic increase in intestinal structural parameters the third week of life. At this time, enzymatic activities begin to

develop towards adult values (acidic and neutral lactases and alkaline phosphatase decreasing in total activity and maltase increasing in activity). The adrenal corticosteroids have been implicated as possible agents in the induction of some (not all) of the above developmental changes. These findings are supported by the fact that the initiation of the release of the steroids occurs in the third week of life. However, as described previously, the physiological importance of the steroids in this role is presently not clear cut. One of the major points of confusion is that early weaning can induce precocious development of adult intestinal properties, yet no known relationship exists between the ingestion of solid food and the initiation of the release of the steroid hormones.

The effects of prolonged suckling on intestinal development. We decided to look at the relationship between weaning and intestinal development further by studying the effects of a prolonged suckling period. We found that the "prolonged suckling" animals had lower intestinal RNA, DNA, protein, and wet weight relative to body weight, in comparison to their weaned littermates. However, it should be mentioned that this prolonged suckling into the fourth week did not prevent a significant developmental rise in these values from those of two-week-old animals. Prolonged suckling did not appear to have any effect on the normal development of alkaline phosphatase activity, "acidic lactase," or maltase activity, whereas development of brush border lactase was significantly retarded. It, therefore, appeared that the development of certain intestinal properties was unimpeded by the delay of weaning, whereas others were very much affected. A striking observation was that

the three enzymes uninfluenced by the process are those whose development are believed to be under the control of the adrenal cortex, while no evidence has been found to implicate the steroids in the development of brush border lactase. Consequently, it was concluded that, from the information gained in this experiment and the "early weaning" study, it seems highly likely that the steroids, along with other unknown factors, play an important role in the development of intestinal properties. The other agent(s) are extremely sensitive to the transition from suckling to weaning. As described previously, there is evidence that these intestinal changes are not due to the changes in the dietary composition of the food at the onset of weaning and, therefore, are most probably related to the change in the form of the food (from liquid to solid) (113).

The agent or agents which induce the intestinal changes at the onset of eating solid food are unknown, and gastrin seemed a likely candidate. It was reasoned that liquid diets would cause minimal antral distension, whereas significant distension and, hence, gastrin release would be initiated upon the ingestion of solid foods. This assumption was recently supported by Zelenkova, et al. who demonstrated that gastrin activity is negligible during the first few weeks of life, then increases dramatically in the third week (129). We, therefore, postulated that the developmental changes that are initiated at the onset of weaning are mediated through the release of gastrin.

The ability of pentagastrin to evoke precocious intestinal developmental changes. We then proceeded to test the above hypothesis by injecting pentagastrin into rats from the age of nine days until age

fourteen days in an attempt to evoke precocious developmental changes. The effect of hormonal treatment on structural parameters was highly suggestive of a positive response, with small increases in RNA, DNA, and protein content when expressed per body weight. The only significant increase was in the intestinal protein content. "Neutral lactase" was the only enzyme affected by the treatment, significantly decreasing from its high suckling level.

The results from this experiment, although demonstrating the overall trend expected, was not as clear cut as hoped for. This may have been due to a lack of sensitivity of the intestinal tissue to the hormone at the particular stage of development studied. Thus, another developmental period was chosen for further study of the problem. Since the intestine develops adult-like properties during the third week of life, it was thought that this may be the optimum period in which to test the role of pentagastrin in development. Therefore, in the last experiment of this study, pentagastrin treatment was administered between days 12-22 of life. Both groups were deprived of solid food during this period. This eliminated (or reduced) the possibility of endogenous gastrin being released in both the treatment and control groups (assuming its release is stimulated by weaning), which would cause serious difficulties in the analysis of the results.

The 22-day-old rats treated with pentagastrin had significant increases in many structural parameters (including protein, RNA, and wet weight) over the respective control values. Significant decreases in both lactases were also recorded in the treatment group, whereas no difference was found in alkaline phosphatase or maltase activity between

the groups. The reason for this discrepancy in the results concerning the response of "acidic lactase" to the presence and absence of pentagastrin at different ages is not apparent at this time, and will have to be investigated further in the future.

It appears that pentagastrin injections did succeed in evoking certain adult developmental changes in the "immature" small intestine. The small intestine of suckling rats is significantly different in structural properties and enzymatic capacity from the adult (70-day-old) small intestine (Figure 8). The parallelism in properties between the "immature" small intestine of suckling rats and the atrophied small intestine of starved rats (both of which are deprived of solid food) is impressive. The similarities and dissimilarities of intestinal properties of these two groups is presented in Table 10. Both of these groups of animals may be considered to be in a gastrin deficient state due to the absence of stimuli needed for gastrin release (solid food). It has been demonstrated that hormonal supplementation by exogenous administration of pentagastrin to both groups can change many of these properties towards normal adult values (see Table 11).

In light of the results just reported, it can be stated that pentagastrin has a significant effect on many intestinal properties, changing them from their immature suckling levels to adult values. This information supports the theory that gastrin may be a mediator of adult developmental changes, which are initiated at the onset of weaning.

Certain intestinal developmental changes, however, were insensitive to pentagastrin treatment, or for that matter, to the onset

TABLE 10

COMPARISON OF INTESTINAL PROPERTIES
OF SUCKLING AND STARVED RATS
RELATIVE TO ADULT VALUES

Property	Suckling	Starved
presumed blood gastrin level	-	-
villus height	-, [a], [b]	-, [a]
crypt height	-, [a], [b]	-, [a]
villus/crypt	+, [a], [b]	+, [a]
RNA/body wt.	-, [a]	-, [a]
DNA/body wt.	-	-, [a]
wet wt./body wt.	-	-, [a]
protein/body wt.	-	-, [a]
"neutral" lactase activity/ gm. wet wt.	+, [a]	+, [a]
"acidic" lactase activity/ gm. wet wt.	+, [a]	0
maltase activity/gm. wet wt.	-, [a]	+, [a]
alkaline phosphatase activity/ gm. wet wt.	+, [a]	0

+ Indicates greater than adult values.

- Indicates less than adult values.

0 Indicates no apparent difference from adult values.

[a] Represents a significant difference at the 0.05 level.

[b] Data obtained from the paper of Herbst, et al. (47).

TABLE 11

COMPARISON OF INTESTINAL PROPERTIES OF PENTAGASTRIN-TREATED SUCKLING AND STARVED RATS RELATIVE TO VALUES OF UNTREATED CONTROLS

Property	Pentagastrin-Suckling	Pentagastrin-Starved
presumed blood gastrin levels	+	+
villus height		0
crypt height		+
villus/crypt		-
RNA/body wt.	+, [a]	+, [a]
DNA/body wt.	+	+
wet wt./body wt.	+, [a]	+
protein/body wt.	+, [a]	+, [a]
"neutral" lactase activity/ gm. wet wt.	-, [a]	-, [a]
"acidic" lactase activity/ gm. wet wt.	0, -, [a]	0
maltase activity/gm. wet wt.	+	-, [a]
alkaline phosphatase activity/ gm. wet wt.	0	0

\+ Indicates greater than values of untreated controls.

\- Indicates less than values of untreated controls

0 Indicates no apparent difference from values of untreated controls.

[a] Represents a significant difference at the 0.05 level.

of weaning. These include the enzymes alkaline phosphatase, maltase, and possibly, also "acidic" lactase. The development of all three enzymes can be precociously evoked by the adrenal corticosteroids (24, 68, 94). If these three enzymes have a higher sensitivity to the steroids than they do to pentagastrin, one would expect little response in activity to an increase in gastrin levels, when the steroids are found in high concentrations in the blood. It is conceivable then, that the steroids represent the gastrin antagonists. If the sensitivity of the brush border enzymes to the steroids follows the relationship, alkaline phosphatase > maltase >> lactase which is the reverse of their order of sensitivity to gastrin, then the enzymatic changes recorded in Studies 1 and 2 may be explained. During starvation, the steroid levels in the blood are unchanged, while the gastrin level is decreased. This would result in a highly significant change in lactase activity, a smaller change in maltase activity, and essentially no change in alkaline phosphatase activity. Pentagastrin injected into starved rats would then tend to return these activities back to the normal levels found in fed rats. Prolonged suckling animals would have a low gastrin level and normal steroid levels. One, therefore, would expect changes in both brush border lactase and maltase activities, with no change in alkaline phosphatase activity in comparison to values from their weaned littermates. The brush border lactase and alkaline phosphatase acted according to these expectations. However, in this case, maltase activity is not significantly different between the two groups. This may be due to the characteristic maltase overshoot that occurs at this time (most probably due to the initiation of steroid release), which would

overwhelm any effect due to differences in blood gastrin levels. In the last experiment, pentagastrin injections into 22-day-old suckling rats stimulated activity changes in brush border lactase, but not maltase or alkaline phosphatase. Both the treatment and control rats would have normal steroid concentrations in the blood; consequently, due to the overshoot phenomenon, no difference in maltase or alkaline phosphatase activity would be expected between the two groups.

Another observation made during this study was that marked increases in intestinal structural parameters occurred in the third week of life, regardless of whether weaning took place or not (although highly significant differences existed between the prolonged suckling and weaning groups). These results would tend to indicate that another agent exists which trophically affects the small intestine in the third week of life, along with gastrin. The release of this agent would not be as dependent on the onset of weaning, as is gastrin. Growth hormone would be a likely candidate for being this agent. Leblond and Carriere have demonstrated that growth hormone acts as a mitogen on the intestinal epithelium (74). It is also known that the corticosteroids are "permissive agents" for the action of growth hormone on target cells (107). For this reason, it is quite conceivable that the intestinal epithelium is receptive to the trophic action of growth hormone upon the initiation of endocrine secretions from the adrenal cortex the third week of life. Consequently, both growth hormone (in the presence of steroids) and gastrin would play a role in the structural development of the small intestine, and gastrin and the adrenal corticosteroids would influence the development of adult enzymatic activity.

The Effects of High and Low Endogenous Gastrin Levels on Intestinal Properties

In the introduction we stated the hypothesis that high and low levels of endogenous gastrin would evoke hyperplastic and atrophic changes in intestinal structure, respectively. We also predicted diverse functional changes in these two conditions. This problem was studied by performing antrectomy and pyloric obstruction surgical procedures.

Resection of the antrum followed by a Billroth I gastroduodenostomy had very little effect on the structural and functional parameters of the small intestine. This was a very disappointing result, since removal of the site of the hormonal secretion, followed by changes in the properties of the target cells is one criterion used to prove an influence (107). A possible explanation for this lack of response is that a significant amount of gastrin may be released from intestinal sites, which obviously will not be affected by ablation of the antrum. Indeed, it is also conceivable that gastrin secreted by antral cells is normally ineffective in acting on the intestinal epithelium and that it is exclusively the gastrin released from duodenal endocrine cells which affect the intestinal mucosa.

We were encouraged by a recent communication, which reported that blood gastrin levels in dogs are significantly lowered by a Billroth II (gastrojejunostomy) reanastomosis, in contrast to a Billroth I procedure following antrectomy (115). These investigators hypothesized that their results indicated that food entering the duodenum (which would cause distension of the duodenal walls) may be a critical factor in gastrin release from these duodenal sites, as it is in the antrum. Based on these results, we performed a Billroth II procedure on a group

of rats in order to optimize our chances of signficantly inhibiting gastrin release. For technical reasons, we had to restrict this experiment to a pilot morphological study. While a marked decrease in duodenal villus height occurred, duodenal crypt height was either unaffected or actually increased by the antrectomy-Billroth II procedure. Jejunal structure appeared unaffected by this procedure.

An interpretation of these findings in relation to gastrin levels is premature at this point for several reasons: first, the results were based on very few observations, and secondly, the changes seen may not be solely attributed to a state of gastrin depletion, but may also be the result of the deprivation of the epithelium of the duodenal blind loop to luminal nutrition. However, if one does attempt to relate the changes in intestinal structure to a gastrin deficiency, two conclusions can be reached: gastrin (1) is needed for the maintenance of normal, long, finger-like duodenal villi and (2) is essentially responsible for the villus height gradient which occurs as one moves from the duodenum to the ileum. Another interesting point is that the presence or absence of gastrin does not appear to affect crypt height (and, indeed, a gastrin lack may even increase the height of the crypt). This result was surprising, when one considers the shallow crypts found in intestines of starved and suckled rats (which are believed to have a low blood gastrin concentration), and this finding will have to be pursued in more extensive studies before further discussion on this subject will be attempted.

In the next series of experiments, an attempt was made to increase endogenous gastrin levels, by surgically ligating the pyloric

region in rats. This is believed to markedly reduce gastric emptying, causing food to accumulate in the pylorus, resulting in distension of its walls and chronic release of gastrin. Suggestive increases in intestinal structural parameters occurred in the "distension" rats; however, few significant differences were recorded. The "distension" group had a significantly lower lactase activity than the sham-operated rats, whereas little difference was found in the other enzymatic levels between the distension and control rats.

These results, although not dramatic, are generally in agreement with the trend seen in other experiments, notably, that gastrin stimulates the formation of structural properties of the intestinal epithelium, as it inhibits the development of certain brush border enzymes.

The Effect of Pentagastrin on Duodenal Cells in Tissue Culture

If one makes the assumption that gastrin, or pentagastrin, has a direct effect on the intestinal epithelium, there are many reasons why the effect could be minimized when studied in an in vivo system. For one, the endogenous or exogenous hormone may be readily metabolized by various tissues of the body. Indeed, it has been demonstrated that gastrin's half life in the body is only about ten minutes and that its pentagastrin derivative may be metabolized many times more rapidly (55, 121). Another possibility to consider when studying an endocrine action in vivo is that the antagonist hormones (i.e., secretin) would be released and may essentially nullify any gastrin effect. Other hormones (i.e., the adrenal corticosteroids) may also be acting on the

epithelium, initiating other changes in intestinal properties. All these factors would tend to minimize and cloud the changes seen after gastrin levels are altered in the blood. For this reason, we decided to study the effects of pentagastrin on the intestinal epithelium in an *in vitro* system, namely, tissue culture. A tissue culture system provides the investigator with a great deal more control over the experimental conditions. If one does see a response to hormonal treatment, the possibility of a secondary or indirect action of the hormone on the target cells can virtually be dismissed.

This *in vitro* study was undertaken to test the hypothesis that pentagastrin directly stimulates duodenal epithelial cell growth. In this study, flasks containing duodenal culture cells were arbitrarily divided into two groups a short time after the original explants were set down. One group of flasks received daily injections of pentagastrin; the other flasks served as controls and received daily injections of saline. This procedure, along with the necessary subculturing, was then performed for a three-month period. At the end of this period, cellular growth kinetics and ultrastructure were analyzed in the two populations. The doubling time of the cells was markedly shorter in the pentagastrin-treated culture as compared with the controls. In order to gain better insight into the reason for this, the cellular proliferative potential was analyzed by a kinetic autoradiographic study. This study demonstrated that approximately 73% of the pentagastrin-treated culture cells were proliferative in nature, whereas only 36% of the controls were actively synthesizing DNA. A crude cell cycle analysis was then performed, and it was determined

that the generation time of the pentagastrin-treated cells was much shorter than that calculated for the control population and that this increase in cell cycle time was solely attributable to an accelerated S phase.

Another finding of interest was that the "normal" cellular property of contact inhibition appeared to be lost in cells treated with pentagastrin, for the cells readily piled up on top of one another when confluency was reached. This did not occur in the control population.

The cell-types in both the treatment and control cultures were then characterized. It was concluded that epithelial cells constituted a greater percentage of the cellular population in the pentagastrin-treated culture ($\sim$ 90%), while the control flasks contained an epithelioid-fibroblastic admixture ($\sim$ 50% epithelial cells). The ultrastructure of the epithelial cells was then analyzed and it was found to have many of the characteristics of an immature (undifferentiated) epithelial cell, which would normally be associated with the crypt.

All these results seem to indicate that pentagastrin evokes dramatic changes in the properties of duodenal cells grown in tissue culture. Hormonal treatment seems to select for epithelial cells in the culture population. These cells have many of the properties of undifferentiated cells, and indeed, like crypt cells, may be programmed for proliferation. Consistent with this conclusion is the high proliferative potential and rapid generation time of the pentagastrin-treated cells. In contrast the lower proliferative potential and longer generation time of the control population represents an average

of the values of the epithelial-fibroblast constituents. The averaged proliferative potential is much less and the generation time much longer in the control cell line than in cells treated with pentagastrin. Consequently, pentagastrin appears to have a direct trophic influence on duodenal epithelial cells, resulting in a selection for this rapidly proliferating cell type. It is well documented in the literature that malignant transformation readily occurs when a target cell is exposed to a trophic factor in high concentrations for long periods of time (107). The apparent loss of contact inhibition in the pentagastrin-treated cultures, therefore, may be representative of this type of phenomenon and indeed may be additional proof that pentagastrin trophically affects the intestinal epithelium.

One would suspect from the results of this dissertation work that the site of action of gastrin in vivo would be at the region of proliferation of intestinal epithelial cells, the crypts of Lieberkuhn. Gastrin would act on these cells by stimulating their proliferative activity. From the tissue culture studies it appears that gastrin can stimulate or initiate DNA synthesis (S phase) in the undifferentiated cells in the crypt. Increasing gastrin levels in the blood, either endogenously or exogenously, may effectively move the boundary separating proliferative (P) from nonproliferative cells (Q) further towards the mouth of the crypt. This would accelerate the movement of undifferentiated cells onto the villi. Employing Hopper, et al.'s method for the calculation of the duration of the maturation phase, one would find a marked shortening of this period. This may be the explanation for the decrease in disaccharidase development after injection of the hormone.

The result of increasing gastrin levels may, therefore, be an increased number of undifferentiated cells in the intestine, resulting in an increase in DNA, RNA, protein, and wet weight and a decrease in the activity of certain enzymes. A decrease in gastrin levels would cause an increased generation time and a narrower and more basally-located proliferative-nonproliferative boundary. This would effectively increase the maturation phase of the cell, resulting in increased disaccharidase activity.

Interpretation and Speculation

Gastrin's Role in the Maintenance of Normal Intestinal Properties

It is fairly well documented that gastrin is released from endocrine cells in the duodenum. Ultrastructural studies have revealed that endocrine-like cells are located at the basal one-third of the crypt. These cells, which constitute less than 1% of the crypt cell population, are known as argentaffin cells. It is conceivable that the gastrin molecule is released from these cells. It could then be transported to neighboring crypt epithelial cells by simple diffusion or via the microcirculatory system at the crypt, the cryptic capillary plexus. It is likely that both routes of transport are utilized in the duodenum, for the greatest amount of proliferative activity of the intestine occurs in the crypts of the duodenum. The proliferative activity in the more distal intestinal sites, the jejunum and ileum (which do not have gastrin releasing sites), would be solely dependent on duodenal gastrin reaching them via the blood supply. It has been shown that the cryptic capillary plexus interconnects adjacent villi, so that gastrin

could conceivably be transported along the length of the intestine in this fashion (111). If this would occur, one would expect a dilution of the hormone and its effect as it moves from a proximal to distal direction down the small intestine. Such a gradient of crypt proliferative activity does occur from proximal to distal small intestine (83). This gastrin dilution effect may also account for the gradation of crypt and villus height which occurs down the length of the small intestine (1). I would like to emphasize that this latter argument is purely speculative, and a "gastrin dilution effect" may be one factor out of many which contributes to the differences in structure along the length of the small intestine. Indeed, it is quite possible that gastrin released from duodenal sites into the microcirculatory system will be so diluted after traveling a short distance that it will have negligible activity in the mid and distal small intestine. Other factors which may prove to be important in causing this intestinal structural transition are the changing form and composition of the food bolus and the varied species and number of bacteria in the different regions of the gut.

When the proper concentration of gastrin reaches the target cell, whether it would be instantaneously, in the case of the duodenal crypt or after a finite period of time in the ileal crypt, DNA synthesis would be markedly accelerated. Only the cells located in a sharply delineated region in the basal one-third of the crypt are active in DNA synthesis, and, therefore, according to our theory, are susceptible to the hormone. This may be due to the availability of gastrin receptor sites in these cells only, or it may be due to the specific geometry of

the microcirculatory system. It has been shown by Carnie, et al. that between 60-70% of the cells in this region actively incorporate ^{3}H-thymidine, i.e., are proliferative (13). This figure agrees very well with our 73% proliferative pool value of the pentagastrin-treated duodenal culture cells.

When the crypt epithelial cells move across the boundary, proliferative activity ceases, and differentiation occurs. One possible explanation for this is that a balance exists between gastrin and a maturation agent (which could be a steroid or, possibly, secretin) and that the cells in this region of the crypt are more receptive to the maturation agent. Another equally plausible theory is that the immature epithelial cells will spontantaneously undergo differentiation unless gastrin is present. In the upper regions of the crypt gastrin is not found in sufficient concentration to block this process. After the cells leave the crypt, the gastrin influence is completely lost and other agents (i.e., steroids or secretin) will regulate the properties of the differentiating epithelial cell.

Implication of Gastrin in the Changes in Intestinal Properties Seen after Surgical Procedures

The three surgical studies referred to in the introduction all elicited changes in intestinal growth. In reviewing the procedures involved, it is possible that the changes may all be attributed to altered gastrin release.

Ileal resection was demonstrated to stimulate crypt proliferative activity and the migration of labelled (^{3}H-thymidine) crypt cells onto the villi (83). Generation time was also speeded up with marked

shortening of the S and possibly G_1 phase. The authors concluded that "an intestinal epithelial growth hormone" of unknown origin is released upon ileal resection. An equally plausible alternative is that sites of synthesis of a gastrin antagonist, possibly secretin, is removed during the operation. There is evidence that secretin may be released along the entire length of the small intestine (101). It is possible that the main route of transport of secretin is via the microcirculatory vessels. If proliferative activity along the height of the crypt is dependent upon the balance between the actions of gastrin and secretin (where the basally located cells have a higher affinity for gastrin, and the cells at the mouth of the crypt have a higher affinity for secretin), the removal of major secretin-releasing sites may move the proliferative-non-proliferative boundary towards the mouth of the crypt. This would effectively account for the greater proliferative activity and accelerated crypt migration rate which occurs after ileal resection. The shorter S period may also be due to an increased gastrin influence on the crypt cells.

Altman and Leblond's interpretation of the transposition experment was that a "villus enlarging factor" is released by the proximal duodenum and antrum, and a "villus reducing factor" is released in the ileum (1). They reasoned that these unknown factors are released in the chyme and act on the epithelial cells from the luminal side. Their results may be explained by an hypothesis, that the "villus enlarging factor" is actually gastrin. This possibility is strengthened when one considers the location of gastrin releasing sites in the G.I. tract (the antrum and the duodenum). This alternative explanation is also

supported by our results concerning the actions of the hormone. A transposed ileal segment in the duodenum would be interconnected with the surrounding gastrin-producing duodenal tissue via newly formed microcirculatory vessels. This tissue would then receive gastrin in higher concentrations than are usually found in the ileum, and consequently, crypt and villus size would be enlarged. A similar situation would occur when duodenal tissue is transplanted to the ileum, for the surrounding ileal tissue would be trophically affected by being exposed to the higher concentration of gastrin. In as much as the villus height of a jejunal segment in the ileum decreased, this may not involve the presence of a "villus reducing factor" at all, but may be due to the unusually low gastrin concentration in the ileal surroundings. If a "villus decreasing factor" does exist, it is quite possible that it may be identical with secretin.

In the last surgical procedure discussed, it was found that total abdominal vagotomy effectively stimulated proliferative activity in the crypt (112). Generation time of crypt epithelial cells was markedly shortened by vagal denervation, as were the S and G_1 phases of the cell cycle. The authors cited the ileal resection experiment and hypothesized that an "intestinal epithelial growth hormone" was released by this procedure. Recent experiments in the dog (the same species being studied in the above experiment) have demonstrated that a slightly lower amount of gastrin is released after truncal vagotomy, but that the parietal cells become hypersensitive to the hormone, resulting in a dramatic increase in gastric secretion in the presence of gastrin (127). If the crypt epithelial cells undergo a similar

"denervation phenomenon," it is quite possible that the increase in crypt proliferative activity is associated with a hypersensitivity to the hormone. The increase in proliferative activity and decrease in generation time (due to a shortened S phase) are entirely consistent with our results and is highly suggestive that these changes are mediated by gastrin.

From the above relationships, it appears that the "villus enlarging factor," the "intestinal epithelial growth hormone," and gastrin may all be identical.

In summary, we feel that the hypotheses advanced earlier have been supported by our experimental results. In light of our data and the reported findings in the literature, we contend that gastrin plays a major role in the maintenance of normal adult intestinal structural and functional properties. Gastrin may, indeed, be the "intestinal epithelial growth hormone." We also feel that the variability of these intestinal properties in response to changing conditions in the local environment (i.e., the presence or absence of food in the lumen) is in part a result of altered levels of gastrin in the blood.

CHAPTER V

SUMMARY AND CONCLUSIONS

At the start of this dissertation research, it was decided to investigate the role that gastrin may play in the regulation of intestinal properties. The properties of intestinal epithelial cells are quite variable and quickly respond to changes in the local environment. Gastrin was thought to be an ideal candidate for mediating many of these changes, since its release is dependent on the presence of stimuli located in the gastrointestinal lumen. Evidence from other laboratories indicates that gastrin trophically affects other gastrointestinal tissues and has the ability to significantly influence their enzymatic activity profiles. We, therefore, theorized that a major second action of gastrin (in addition to being a gastric secretagogue) is the fine regulation of intestinal structural and functional properties to meet the requirements dictated by the local environment.

The small intestine undergoes dramatic changes in properties during starvation. Our results demonstrated that severe degeneration of mucosal architecture, as well as enhancement of disaccharidase activity, occur with starvation. Food is thought to be the main stimulant for gastrin release (due to mechanical distension of the antrum). It was reasoned that during starvation there would be an absence of stimuli for gastrin release and a subsequent decrease in blood gastrin

levels. We, therefore, hypothesized that the intestinal structural and functional changes occurring during starvation could in part be due to a gastrin deficiency. In order to test this theory, we injected rats during a starvation period with pentagastrin. If the theory is correct, hormonal supplementation should partially reverse the intestinal changes which occur during starvation. Our results supported the above hypothesis, because both structural and enzymatic activity levels of the starved intestine changed towards values associated with the fed state upon injection of pentagastrin. It should be noted that hormonal treatment stimulated only partial reversal of the effects of starvation, but optimal conditions of obtaining a hormone-target cell interaction may not have been met in the in vivo system.

Marked intestinal developmental changes take place the third week of life. These changes are closely associated with the transition from suckling to weaning. We demonstrated that prolongation of the suckling period results in a significant retardation in the development of some intestinal properties. It was noted that the suckling and starved rat intestine are very similar in many structural and functional properties. Using the same reasoning as expressed in the previous study, we concluded that very little gastrin would be released when the young animal is on a liquid diet of milk (suckling) and that dramatic changes in gastrin levels would most probably occur at the onset of eating solid food (weaning). We therefore hypothesized that the initiation of gastrin release at the onset of weaning plays a major role in the stimulation of adult developmental changes of the small intestine. This theory was then supported when it was demonstrated that administration of pentagas-

trin to suckling rats stimulated changes in intestinal structural and functional properties towards normal adult values. A model was then designed to describe the possible endocrine interactions necessary for normal intestinal development. We concluded that both growth hormone (in the presence of steroids) and gastrin may play major roles in the development of adult intestinal structure, while the interplay of gastrin and the adrenal corticosteroids influence the development of adult intestinal enzymatic levels.

An attempt was then made to study the effects of increased and decreased endogenous gastrin levels on intestinal properties. This was accomplished by performing pyloric obstruction and antrectomy surgical procedures, respectively. The result of these experiments were only suggestive of the trend expected.

If gastrin's effect on the intestinal epithelium was to be definitively proven, an <u>in vitro</u> action of the hormone on the target cell would have to be shown. A duodenal culture line was established for this express purpose. It was our hypothesis at the onset of the experiment that the proliferative activity of the duodenal cells would be stimulated by the hormone. This prediction was confirmed by the demonstration that the pentagastrin-treated cells had a significantly shorter doubling time than the saline controls. Kinetic autoradiographic studies demonstrated that there was approximately a two-fold increase in the percentage of proliferative cells in the cultures treated with pentagastrin. Crude approximation of generation time (G_T) revealed that G_T is markedly reduced in pentagastrin-treated cultures in comparison to the controls; this was solely attributed to a

shortened S phase (DNA synthesis). The pentagastrin-treated cultures were primarily epitheloid in nature, whereas the controls consisted of an epithelial-fibroblastic admixture. Ultrastructurally, the epithelial cells appear very similar to undifferentiated crypt cells.

From the experiments performed, we conclude that gastrin does play an important role in the regulation of both intestinal structural and functional properties, notably, nucleic acid and protein content, wet weight, possibly villus and crypt height, and disaccharidase activity. According to our theory the major site of action is on the immature crypt epithelial cells, where gastrin acts to accelerate DNA synthesis. If a gastrin deficiency occurs, proliferative activity in the crypts is reduced and a greater than normal percentage of cells undergo maturation (resulting in a higher disaccharidase activity); in the case of an excess of gastrin (or hypersentivity of the target cells to the hormone), proliferation in the crypts is increased and the cells undergo a shortened maturation process (resulting in a decreased disaccharidase activity).

In conclusion, several studies were cited which, through surgical manipulation, changed the growth rate of the intestinal epithelium. The authors of these separate studies hypothesized the presence of an unknown growth factor which they labelled as either an "intestinal epithelial growth hormone" or a "villus enlarging factor." It is our contention that the actions and properties of this growth factor are consistent with those of gastrin.

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APPENDIX

Detailed Description of Methods

Detailed Description of Methods

Lowry Protein Determination (84)

Working Solutions:

1) Reagent A_I consists of 2% Na_2CO_2 plus 0.02% KNa tartrate made up in 0.1N NaOH. This solution is stored at 4°C.

2) Reagent A_{II} consists of 0.5% $CuSO_4$ solution made up in deionized distilled water. This solution is stored at 4°C.

3) Reagent A is made up of the following ratios of the above two solutions:

$$A_I : A_{II} : : 50 : 1$$

Due to its unstable nature, it is made immediately before use.

4) Reagent B is made by mixing 200 ml of F-Ciocalteau phenol reagent with 220 ml of deionized distilled water. It is stored at room temperature in a brown bottle, because of its light sensitivity.

5) A protein standard stock solution is made up to a concentration of 5.0 mg of crystalline serum albumin/ml. The solution also contains 1.8 mg of benzoic acid/ml as a preservative. This stock solution is stored at 4°C. At the time of assay, an aliquot is removed and a 1:10 dilution is made, making a final concentration of 0.5 mg/ml. A protein standard is made by adding 0.00, 0.05, 0.1, 0.2, and 0.4 ml of the above solution to a series of test tubes.

6) 0.1N NaOH

Working Procedure:

1) 0.1 ml of 0.1N NaOH is added to 0.1 ml of homogenate, or protein standard, in a test tube. This mixture is allowed to stand for one hour or longer.

2) At the end of this period, water is added to make a total volume of 2.0 ml. Once this is completed, 5.0 ml of Reagent A is added. This solution is vortexed and allowed to stand exactly 20 minutes.

3) At this time, 0.5 ml of Reagent B is added. The test tube is inverted and allowed to stand for exactly 45 minutes, at which time its O.D. is read at a wavelength of 660 mu.

4) The standard curve is plotted on log-log graph paper, and the protein values of the sample are read off the graph.

Theoretical Basis of the Technique:

It is known that under alkaline conditions, phosphomolybdic and phosphotungstic acids (both contained in Folin's Reagent) are readily reduced, yielding a bluish color. Tissue proteins contain a high concentration of aromatic amino acids which readily reduce these acidic compounds in Folin's Reagent. The Lowry protein determination is based on the above biochemical properties. It was found that an increased accuracy of the test can be achieved if $CuSO_4$ and $NaCO_3$ are used in the reaction mixture, for they increase the color intensity and stability, respectively. The $CuSO_4$ interacts at the site of peptide bonds.

RNA-DNA Extraction Procedure as Modified from Schmidt-Thannhauser (31)

Working Solutions:

1) 0.4N PCA, stored at room temperature.

2) 0.2N PCA, stored at room temperature.

3) 10% PCA, stored at room temperature.

4) 0.3M KOH, stored at room temperature.

Working Procedure:

1) 5.0 ml of 0.4N PCA is added to 5.0 ml of diluted homogenate in a 15 ml glass conical centrifuge tube and left in ice for 10 minutes. After this period, the centrifuge tubes are placed in a refrigerated swinging bucket centrifuge and spun at 600 g for 10 minutes. The centrifuge tubes are removed after this period, and the supernatant is poured off.

2) The pellet is then resuspended in 5.0 ml of 0.2N PCA and centrifuged as described above. This washing procedure is repeated two more times.

3) 4.0 ml of 0.3M KOH is then added to the pellet, and the mixture is incubated in a shaking water bath at 37°C for one hour.

4) At the end of this period, 2.0 ml of cold 10% PCA is added to the suspension, and the tubes are placed in ice for 10 minutes. The tubes are then centrifuged as described previously.

5) Upon removal of the tubes from the centrifuge, the supernatant is poured into 15 ml test tubes.

6) The pellet is then resuspended in 4.0 ml of 0.2N PCA and centrifuged once again. The supernatant is poured into the same 15 ml test tube (resulting in a total volume of 10 ml). This sample is then used for RNA determination.

7) The pellet is then resuspended in 4.0 ml of 10% PCA and placed in a boiling water bath (96°C) for 10 minutes.

8) The samples are then centrifuged, as described above.

9) 2.0 ml of the supernatant is then removed and pipetted into a clean test tube for DNA determination.

Theoretical Basis of the Technique:

The extraction procedure is based on the biochemical properties of RNA and DNA (95). The initial precipitation and subsequent washings in 0.2N PCA is based on the property that RNA and DNA both react with the acid, forming an insoluble salt, which can be isolated easily by low speed centrifugation. This initial washing procedure has been shown to successfully remove free nucleotides, sugars, and inorganic phosphates which would interfere with the quantitation of RNA and DNA (95). The RNA salt is then specifically solubilized in the KOH incubation procedure. This is based on the fact that the ribose moiety of RNA contains two hydroxyl groups (one being part of the phosphodiester bonds which link the adjacent sugars). In the presence of alkali, the phosphodiester bond is broken, and RNA is hydrolyzed into its component bases and sugars. This is not the case with the deoxyribose moiety of DNA, which has only one hydroxyl group. Consequently, upon the addition of PCA to the incubation mixture and the subsequent centrifigation, the RNA is released into the supernatant, whereas the DNA salt is precipitated. This RNA and DNA separation is important, because DNA will significantly interfere with the subsequent spectrophotometric analysis of RNA. The DNA precipitant is readily solubilized by incubating it with hot (96°C) PCA. This is due to the fact that the sugar-purine bonds are broken (with only few scissions of the phosphodiester backbone) under these conditions, forming apurinic acids (38). This solution is then allowed to cool and then is centrifuged, as described previously. The pellet which forms is primarily protein. This final separation of protein is necessary for an accurate determination of tissue DNA content.

Modified DNA Determination (11, 34)

Working Solutions:

1) 4% solution of diphenylamine made up in glacial acetic acid (made up fresh immediately before performing the assay).

2) 1.6 mg/ml acetaldehyde, stored at 4°C.

3) DNA standard of 200 ug DNA/ml, stored at 4°C.

4) 12.5% PCA, stored at room temperature.

Working Procedure:

1) Add 0.00, 0.05, 0.10, 0.20, 0.30, and 0.40 ml of DNA standard to a series of test tubes, add water to each to give a volume of 0.4 ml in each tube. Add 1.6 ml 12.5% PCA to each. Boil the DNA standard tubes for 10 minutes, as performed on the samples.

2) Add 2.0 ml of diphenylamine solution to all the tubes (both samples and standards). This should be followed by pipetting in 0.1 ml of acetaldehyde solution. The tubes are then placed in an incubator at 30°C for a period of 18-24 hours. After this time, they are removed and the O.D.'s are read at wavelengths of 595 and 700 mu. The O.D. at 700 mu should then be subtracted from the reading at 595 mu.

3) The standard curve is drawn on linear graph paper, and the DNA values of the samples are read off the curve.

Theoretical Basis of the Technique:

This technique is based on the fact that diphenylamine specifically reacts with the deoxyribose sugars released from the purine bases. This reaction is catalyzed by small quantities of acetaldehyde. The blue color appears shortly after the incubation is initiated and becomes stable after 18 hours. Protein has been shown to slightly interfere with the spectrophotometric reading. Because its contribution to the O.D. is approximately the same at wavelengths of 595 and 700 mu (whereas the blue color produced by the diphenylamine-deoxysugar reaction does not absorb light at 700 mu), the double wavelength measurement is employed (34).

RNA Determination of Fleck and Berg (30)

Working Procedure:

1) The absorbency of the RNA solution is measured by reading the O.D. at 260 and 232 mu. The following formula is employed in the determination of the quantity of RNA:

$$\frac{\text{ug RNA} - \text{P}}{\text{ml}} = 3.40\ (\text{O.D.}_{260\ \text{mu}}) - 1.44\ (\text{O.D.}_{232\ \text{mu}})$$

2) This difference is then multiplied by 10.2 to give the total ug RNA/ml of the sample.

Theoretical Basis of the Technique:

This technique is based on the fact that RNA absorbs maximally and minimally at wavelengths of 260 and 232 mu, respectively, in the ultraviolet spectrum. In contrast, contaminating peptides have a greater O.D. at 232 mu than at 260 mu. Consequently, the RNA $O.D._{260}$/protein $O.D._{260}$ ratio yields a maximal value, and the RNA $O.D._{232}$/protein $O.D._{232}$ ratio yields a minimal value. Therefore, it was found that by taking the difference between the O.D.'s at the two wavelengths, one can best correct for interference by peptide contaminants. The coefficients (3.4 and 1.44) were determined by considering the contribution and changes of the extinction coefficients of RNA and proteins at the two wavelengths (30).

Dry Weight Determination as Modified from Schedl, et al. (110)

In this determination, the intestine is removed from the body, slit open, and washed as described previously. It is then placed in a tared glass scintillation vial and weighed. The scintillation vial is placed in a dessicator bottle, which is subsequently evacuated. The dessicator is then placed in an oven set at 65°C. The vial is removed at different time periods and weighed. When there is no further decrease in weight with time, it is assumed that the tissue is completely dehydrated, and a final dry weight measurement can be recorded. The weight of tissue water is the difference between the wet weight and dry weight.

Disaccharidase Assay by Dahlqvist (19)

Working Solutions:

1) Tris Glucose Oxidase Reagent (TGO), stored at 4°C. 100 ml of this reagent has the following composition:

   a. 97.0 ml of 0.5M Tris at a pH of 7.0

   b. 0.5 ml of type 1 horseradish peroxidase solution (1 mg/ml)

   c. 0.5 ml of O-Dianisidine suspension (10 mg/ml 95% ethanol)

   d. 1.0 ml Triton X-100 solution (0.25 gm Triton X-100/ml of 95% ethanol

   e. 1.0 ml of glucose oxidase reagent (C-5816, Miles Co.)

2) Glucose Standard (200 ug/ml). Benzoic acid at a concentration of 2.5 mg/ml is used as a preservative. The standard is stored at 4°C. At the time of the assay, 0.00, 0.025, 0.05, 0.10, and 0.20 ml of the standard is pipetted into a series of test tubes, and each tube is made up to a volume of 0.2 ml with water.

3) Lactose substrate solution, pH 3.5, stored at 4°C. This solution is made up to a concentration of 0.056M in a 0.1M sodium acetate buffer solution. A drop of toluene is used as a preservative.

4) Lactose substrate solution, pH 5.6, stored at 4°C. This solution is made up to a concentration of 0.056M in a 0.1M maleate buffer. A drop of toluene is added as a preservative.

5) Maltose substrate solution, pH 6.0, stored at 4°C. This solution is made up to a concentration of 0.056M in 0.1M maleate buffer. A drop of toluene is added as a preservative.

Working Procedure:

1) 0.1 ml of substrate solution is added to 0.1 ml of homogenate (or diluted homogenate) and is incubated at 37°C in a shaking water bath for one hour. A substrate blank is made, which contains 0.1 ml substrate and 0.1 ml water.

2) At the end of this period, the tubes are placed in ice, and 3.0 ml of TCO reagent is immediately added to the sample and standard tubes.

3) All the test tubes are then placed into a shaking water bath at 37°C for one hour.

4) At the end of this period, the O.D. of the orange-colored samples and standards are read against a substrate blank at a wavelength of 420 mu. (An enzyme blank, consisting of 0.1 ml of homogenate plus 0.1 ml of water was only used when the enzyme activity of recently fed animals was being determined.)

5) The standard glucose curve is then drawn on linear graph paper, and the glucose concentrations of the samples are read off the graph.

6) The enzymic activity of the disaccharidases are determined as follows:

$$\frac{\text{uMoles of disaccharide hydrolyzed}}{\text{hour}} = \frac{\text{ug glucose released}}{\text{hour}} \times \frac{1 \text{ hour}}{60 \text{ min.}} \times \frac{\text{uMoles of disaccharide}}{n \text{ (180 ug of disaccharide)}}$$

where n = 1 for lactose and n = 2 for maltose (depending upon the number of glucose molecules in one molecule of the disaccharide).

7) Calculation of brush border ("neutral") lactase is determined by the following formula (121):

brush border activity = lactase activity, pH 5.6 - 0.4 (lactase activity, pH 3.5)

Theoretical Basis of the Technique:

These enzymes are known to hydrolyze the disaccharides, cleaving the bond between the two monosaccharide components and, thus, releasing free glucose. The varying substrate pH's correspond with the pH optima of the three enzymes being studies (67, 105). Koldovsky, et al. have demonstrated that, due to the broad pH activity range of "acidic lactase," it still has 40% of its maximal activity at a pH of 5.6 (65). In contrast, "neutral lactase" has a narrow pH optimum curve and has negligible activity at pH 3.5. For this reason, the above formula (#7 of working procedure) is used in the calculation of "neutral lactase" activity. At the end of the incubation period, the TGO is added. In addition to reacting with the free glucose, as described below, the 0.5M Tris effectively stops the disaccharidase reaction (19). The glucose oxidase activity is based on the following reactions:

$$\text{glucose} \xrightarrow{\text{glucose oxidase}} \text{gluconic acid and } H_2O_2$$

$$H_2O_2 + \text{reduced chromogen (O-Dianisidine)} \xrightarrow{\text{peroxidase}} H_2O + \text{oxidized chromogen}$$

It is the oxidized chromogen which gives the solution its orange color.

Alkaline Phosphatase Assay Using the Phosphatabs-
Alkaline Method of Klein, et al. (63)

Working Solutions:

1) Alkaline phosphatase substrate, pH 9.9, made fresh, consisting of one Phosphatab tablet (Warner-Chillcot) (containing 0.3 mg sodium phenolphthalein phosphate) per 0.6 ml of water.

2) 10 mM NaOH, stored at 4°C.

3) 0.50 M NaOH, stored at 4°C.

4) Phenolphthalein standard, made up at a concentration of 20 ug/ml. At the time of the assay, serially pipette volumes of 0.5, 1.0, 2.0, and 3.0 ml of standard into test tubes, add 0.1 ml of 0.50M NaOH and dilute with water to a volume of 5.7 ml.

Working Procedure:

1) 0.6 ml of the substrate is added to 0.1 ml of the diluted homogenate, and the test tubes are placed in a shaking water bath at 37°C for 30 minutes.

2) At this time, the test tubes are removed, and 5.0 ml of 10.0 mM NaOH is added.

3) 1.0 ml of this purple-colored solution is then removed and pipetted into a test tube containing 2.0 ml of water (making a 1:3 dilution).

4) The O.D. of this final solution is immediately read at a wavelength of 560 mu against a water blank.

5) The alkaline phosphatase activity is expressed as the ug of phenolphthalein released/30 minutes at 37°C.

Theoretical Basis of the Technique:

This assay is based on the following reaction sequence: phenolphthalein phosphate $\xrightarrow{\text{alkaline phosphatase}}$ phenolphthalein + phosphate The NaOH solution is added to terminate the reaction. It is the quinonoid structure of the phenolphthalein in alkaline conditions that has the purplish hue that is measured colorimetrically (53).

Measurement of Villus and Crypt Height by the Method of Altmann, et al. (1)

Intestinal ring sections are cut from the intact intestine, using a sharp scalpel. The intestinal rings are immediately placed in a cold 10% formalin solution. The fixed tissue is then embedded in paraffin. Longitudinal sections (5u thick) are then cut, using a hand-driven microtome. These sections are placed on slides and stained with PA-Schiff hematoxylin.

Villus and crypt height are measured in portions of sections that contain almost exclusively villi and crypts cut along their length. The villi that are counted should have a finger-like appearance, and there should be a single layer of epithelial cells lining the villi. If all these conditions are met, it indicates that the villi and crypts have been cut perpendicularly (in contrast to tangential or oblique sectioning) and that they are suitable for quantitation. The villus height is measured by an occular scale and is expressed in millimeters.

Tissue Culture Growth Experiment as Modified from Kollmorgen, et al. (70)

Working Solutions:

1) Fetal calf medium, consisting of the following ingredients:

   a. 80% Minimum Essential Medium with glutamine and non-essential amino acids (Eagle) (25)

   b. 10% NCTC-135

   c. 10% fetal calf serum

d. 1 mM sodium pyruvate

e. 100 U/ml penicillin

f. Fungizone (2.5 ug/ml) (Gibco). This was used only when the original explant was set down.

2) Pentagastrin (25 ug/ml).

3) Isotonic saline.

Working Procedure:

At subculture, an extremely dilute inoculum of cells from pentagastrin and control stock cultures is injected into two sets of T_{25}'s for each group. After two hours, the trypsin-containing medium is removed and is replaced by fresh medium. At this point, representative fields (containing at least ten evenly dispersed single cells) are selected in each flask. The increase in the number of cells/clone is then measured with time in the control and pentagastrin cultures. This relationship is then drawn on a semilog graph. If the cultures are growing in an exponential fashion, a straight line should connect the points. The time it takes for the average clone population to double is referred to as the doubling time.

Tissue Culture Proliferative Pool Determination
as Modified from Kollmorgen, et al. (70)

Working Solutions:

1) Fetal calf medium, containing 0.1 uCi of ^{3}H-thymidine/ml.

2) Pentagastrin (25 ug/ml).

3) Isotonic saline.

4) Fixative (3 parts methanol and 1 part glacial acetic acid).

5) Kodak Nuclear Track Emulsion (NTB-2), made up 1:1 with water.

6) Giemsa stain (1 part Giemsa to 1 part phosphate buffer).

7) Kodak D-19 Developer.

8) Kodak Stop Bath Mixture.

9) Kodak Photographic Fixer.

Working Procedure:

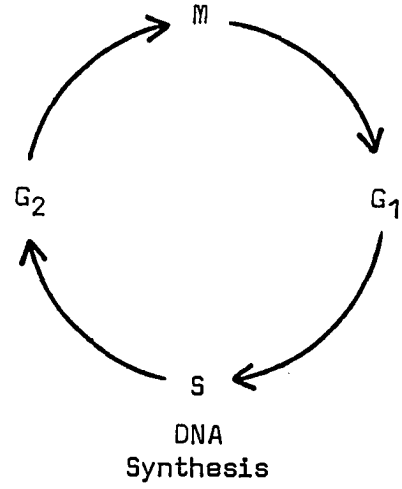
A dilute cellular suspension of both pentagastrin and control stock cultures (as described above) is innoculated into a series of Leighton tubes containing coverslips. Upon removal of the trypsin-containing medium, it is replaced with fetal calf medium containing ^{3}H-thymidine at a concentration of 0.1 uCi/ml. The medium is subsequently changed daily. The cultures are then serially sacrificed at different time periods (from 2-40 hours). At the time of sacrifice, the medium is discarded, and fixative is added to the Leighton tubes and is left in the tubes for approximately 15 minutes. The coverslips are then removed and mounted on slides. After the sacrifice of the cells of the last set of Leighton tubes, autoradiography is performed. This is accomplished initially in a dark room by dipping each slide into diluted NTB-2, having them air dry in a vertical position, and placing the emulsion-covered slides in a black plastic slide box. The slide box is then sealed with tape and stored at 4^{o}C for 4 days. At the end of this time, the slides are developed and fixed, just as prints are made from photographic negatives. The slides are then removed from the dark and stained with Giemsa. Upon viewing these cells under light microscopy, labelled nuclei (containing silver grains) are easily visible and can be distinguished from unlabelled cells. The percentage of labelled cells in a field of 1000 cells is then determined. It can be seen that the percentage of labelled cells increases with time and levels off at a certain period when the percentage of labelled cells remains constant (the plateau region of the curve). The time it takes for the curve to plateau is considered to be representative of the duration of the G_2 + M + G_1 period.

Theoretical Basis of the Technique:

The S phase (DNA synthesis), by definition, is the period during which the nucleus will incorporate ^{3}H-thymidine. Those cells that are not active in DNA synthesis, and hence are not proliferative, do not incorporate the thymidine molecules into their nuclear chromatin. In order to monitor the frequency and kinetics of this process in different cell populations, autoradiography is performed. Autoradiography is a process in which a light-sensitive, finely-grained emulsion is laid over a sample which is actively emitting radioactive particles. The radioactive element of choice is ^{3}H. This is because ^{3}H emits low energy particles, which have an extremely high ionization potential. The particles released from ^{3}H have a relatively straight path (unlike the scatter of other radioactive particles), and hence ionize substances directly over them. Consequently, ^{3}H is ideal for autoradiography, for it delineates the location of its incorporation in the cell on the overlaying emulsion. The e^-'s released by the emission serve to reduce the silver ions of the emulsion in the following way, forming metallic silver: $Ag^+ + e^- \longrightarrow Ag$. Upon developing the emulsion, an increased number of metallic silver particles are formed in the vicinity of the reduced silver grains that are produced by β emission, so that nuclei containing incorporated thymidine are easily visualized under light

microscopy.

The time it takes for the curve to plateau is considered to be equal to the G_2 + M + G_1 phase, because it is representative of the period it takes the proliferative cells which just completed DNA synthesis (when the H-thymidine was initially added) to proceed completely around the cell cycle and to enter DNA synthesis once again, as graphically shown below:



Crude Approximation of Generation Time (69)

This can be performed using the information from the results obtained from the above two determinations.

Given: doubling time = X hours

% of cells in the proliferative pool = Y%

% of nonproliferative cells = (100-Y)%

The technique below is then employed.

	No. of cells in each group in population of 100, at 0 time	No. of cells in each group in 2nd generation	No. of cells in each group in 3rd generation
Proliferative	Y	2Y	$[Y \times 10^{-2}(100 + Y)]2 = L$
Nonproliferative	100 - Y	100 - Y	$4Y-[Y \times 10^{-2}(100 + Y)]\ 2 + [100 - Y = M]$
Total	100	100 + Y	

Since Y percentage of the total population will be proliferative, in order to calculate the number of proliferative cells present in the third generation, the following calculation should be performed:

$$[Y \times 10^{-2}(100 + Y)]2$$

This number is designated "L". The number of cells in the nonproliferative pool is represented as

$$4Y - [Y \times 10^{-2}(100 + Y)]2 + [100 - Y]$$

This term is designated "M". Now, one can state that the time it takes for Y number of cells to grow to L number of cells is equal to the duration of two doubling times, or 2X hours. If these two points (Y and L, at times 0 and 2X) are then plotted on semilog graph paper, the doubling time for the proliferative cells can be ascertained. The doubling time of proliferative cells is synonymous with the generation time, or cell cycle time (G_T).

If G_T is determined, and $G_2 + M + G_1$ is known from the proliferative pool graph, S can be determined by deduction: $G_T - (G_2 + M + G_1) = S$.

Preparation of Duodenal Culture Cells for Ultrastructure as Described by Miller, et al. (92)

The cell suspension obtained by tryptic digestion is fixed in cold isotonic 2% glutaraldehyde in 0.08% cacodylate buffer for 15 minutes, as described by Gordon, et al. (36). The cells are then spun down, fresh fixative is added, and the mixture is allowed to stand for several hours. The glutaraldehyde solution is then removed, and the cells are post-fixed with osmium tetroxide. They are then dehydrated and embedded in Maraglas. Thin sections are then cut with a diamond knife ultramicrotome. The sections are stained with uranyl acetate and lead citrate. The sections are then examined with an RCA electron microscope.