1981

Circadian Changes in Cells of the Rat Gastric Mucosa

Danielle M. Jacobs
Loyola University Chicago

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CIRCADIAN CHANGES IN CELLS OF
THE RAT GASTRIC MUCOSA

by

Danielle M. Jacobs

A Dissertation Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

September
1981
ACKNOWLEDGMENTS

The author wishes to express her appreciation to Dr. Ruthann P. Sturtevant for her guidance and encouragement.

Sincere appreciation is also extended to Drs. Charles C.C. O'Morchoe, John McNulty and Charles Ehret for their excellent assistance and Drs. Mary Manteuffel and Rajinder Nayyar for their advice.

The author also wishes to thank Mr. Gregg Sunday of the Loyola University Academic Computer Services for the many hours of assistance that he provided.
VITA

The author, Danielle M. Jacobs is the daughter of Raymond F. and Jacqueline Jacobs. She was born on March 25, 1952, in Chicago, Illinois.

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CHAPTER I

INTRODUCTION

The correlation between the gross anatomical structure of the body's organs and their functions is usually apparent. Recently, it has become evident that this correlation also exists at the cellular level; there is a relationship between subcellular structure and the activities of a cell. Histological studies continue to yield information about the function of tissues, the mechanisms of cellular activities and their pathological deviations.

In many tissues, the physiological state is reflected by subcellular structure. The gastrointestinal system, in particular, displays many examples of this relationship. Salivary glands (Albegger and Muller, 1973), pancreas (Bolender, 1979), liver (Muller et al. (1966), intestines (Schafer et al., 1974) and stomach (Helander, 1964) all display variations in organelles or enzyme levels that can be correlated with function. Within the stomach itself, several cell types are known to vary morphologically as their level of activity changes. Weber (1958) observed that the RNA of chief cells varies in relation to their physiological state. Helander (1964) also noted that zymogen granules in stimulated chief cells are a different size than those in resting cells. Parietal cells, which produce gastric acid, were also seen to vary with function; secretory canaliculi were observed to be
"thicker" in secreting cells (Hoerr, 1936). It was noted at the ultrastructural level that a stimulus also caused a decrease in the number of microvesicles (Rosa, 1963; Tarnowski et al., 1980). A third organelle reputed to vary is the multivesicular body, which has been reported to increase in number with certain stimuli (Hally, 1959; Rosa, 1963). It is not known, however, whether this is a part of the secretory mechanism or an end product thereof.

Rough endoplasmic reticulum, involved in protein production, is found in the parietal cell, often perinuclearly or peripherally in the cytoplasm. While fluctuations in the amount and configuration of this organelle have not been correlated with secretory or other specific functions in the parietal cell, such observations have been made in hepatocytes (Muller, 1971) and pinealocytes (Welsh et al., 1979).

Mucus is produced by surface epithelial and mucous neck cells of the gastric epithelium. Little was known about their relationship to each other or their derivation until very recently, and the literature still contains conflicting views on this subject. Although the mucus is necessary for the protection of the stomach lining (Davenport, 1972), the respective roles of the two mucus-producing cells are not clear. The subject has rarely been studied by histological methods. Rhythmic variations have never been demonstrated in the cells, although their morphology does change after a secretory stimulus (Gerard et al., 1968).
For many years, the theory of homeostasis, attributed to Claude Bernard, was accepted as the manner by which the body survives environmental changes. The systems of the body were thought to respond to external stimuli and stress by returning to a "milieu constant" or optimal state. It is now apparent, however, that the body's systems do not maintain a steady state equilibrium; instead, the body's physiological functions and susceptibility to insult fluctuate in a rhythmic manner. Oscillations characterized by a predictable time span of about 24 hours are known as circadian rhythms.

In nature the length or period of rhythmic fluctuations is often synchronized by environmental cues such as the light-dark cycle or social interactions, which in turn affect feeding phases and sleep-activity phases (Ashoff, 1976). If the persistence of an oscillation depends on such an external time cue, it is said to have an exogenous cause. If a rhythm persists in the absence of external synchronizers, it has an endogenous source.

The existence of such rhythms is presumed to be advantageous in dealing with predictable changes in the environment. Many vertebrate species have daily peaks in plasma corticosteroid levels which may serve to prepare the animal for the stresses of its activity phase (Dunn, 1974).

Dissociations of phase-relationships among physiological rhythms can have a detrimental effect on health and the sense of well-being. A
well-known example is the phenomenon of "jet-lag". In long distance flights to the east or west, passengers are subjected to a rapid time-zone shift. During the reentrainment to the new time zone, there is a temporary dissociation of physiological rhythms, as rhythm readjustment rates vary. Psychological effects as well as an impairment of ability to perform discrete motor tasks may be noted. This has been of particular concern in the aviation industry. Precautions are taken to minimize schedules that subject flight crews to unusual stresses when they are least able to deal with them (Siegel et al., 1969).

A variety of theories has been proposed to explain such pathological conditions as gastritis and gastric ulcers. They are commonly encountered disorders frequently associated with stress and with work-shift changes. While diet, genetic predisposition, sex, blood type, viruses and other sources have been named as contributing factors (Borg and Andren, 1980), almost nothing is known about the cellular mechanisms that promote lesions. It is not known whether the condition is related solely to overproduction of acid, underproduction of mucus, a combination of both or neither. It is the premise of this dissertation that further study of the cellular mechanisms of mucus and acid production is necessary to understand the disease states to which they may be related.

The purpose of this study was to determine whether there are histologically demonstrable cycles in gastric cellular morphology and to
examine the premise that changes in structure may reflect functional changes of the cells.
CHAPTER II

REVIEW OF LITERATURE

Chronobiology

General concepts

Chronobiology is the study of the time structure of organisms (Halberg, 1977). All organisms undergo changes related to time. Perhaps the most obvious of these changes are growth, development and aging. Throughout life, however, many cycles of a shorter duration occur in most organisms. These include repeated rhythmic fluctuations in cellular physiological and morphological states, and susceptibility to stress. In the past, such changes were assumed to be responses to changes in the organism's environment, and followers of the 19th century physiologist, Claude Bernard, coined the term "homeostasis" to describe a process by which the body was able to maintain equilibrium of the internal milieu. More recently, however, investigators have ascertained that changes in the body's physiological processes are not always a response to a change in the environment. Instead, some of these variables fluctuate in the absence of environmental challenge. In studying these oscillations, chronobiologists have designated the word "rhythm" to describe a regularly repeating fluctuation that has a set length or period.
In the past, biological rhythms were sometimes considered to be manifestations of geophysical or environmental changes. Some aspects of fertility and timing of birth, for example, may in some way be affected by lunar cycles (Menaker and Menaker, 1959). Brown (1960) observed that in a wide range of living forms including seaweed, carrots, potato plants, quahogs and fiddler crabs, variations in oxygen consumption are related to fluctuations in geomagnetism, cosmic ray cycles and atmospheric changes. Von Mayersbach (1965) also noted seasonal changes in liver morphology and histochemical staining properties in laboratory animals kept under conditions of constant temperature and humidity and standardized light-dark cycles. Such fluctuations (challenged by other investigators) suggest a pervasive geophysical source and are said to be exogenously caused.

Recent studies, however, have demonstrated biological rhythms with endogenous causes, i.e. sources within the organism itself. Wever's (1975) experiments with human beings living under constant conditions without environmental time-cues or "Zeitgebers", indicate that not only do rhythms persist in the absence of external time cues, but also that different physiological systems within a body may become "uncoupled". Thus different rhythms in a single organism may have different free-running periods. Many rhythms free-run with a period longer than that usually seen under natural conditions. For example, Ashoff (1965) demonstrated human rhythms which, in the absence of environmental cues, have periods of 24.7 and 32.6 hrs but in the presence of time cues such
as social interaction and the light-dark cycle, have periods of 24.0 hours.

Circannual rhythms, characterized by a period of about 12 months, occur frequently in nature. Hibernating animals generally have a dormancy-wakefulness cycle of this length. A relationship has been shown among many physiological and anatomical states such as mean body temperature (Folk, 1974) and cellular structure of such organs as the stomach (Ryabchikova and Vinogradova, 1979) and pineal gland (McNulty and Dombrowski, 1980). The degree of susceptibility of the body to certain stresses also displays a circannual rhythm (v. Mayersbach, 1976).

A rhythm with a period of about 30 days is defined as a circatrigintan rhythm. The female menstrual cycle is an example of such a rhythm. Rhythms with weekly and biweekly periods as well as periods of shorter lengths have all been described (Halberg, 1977).

One of the most widely studied biological oscillations is the circadian rhythm with a period of about 24 hours. Body temperature and activity (Wever, 1975) as well as a large number of physiological and histological variables are known to fluctuate rhythmically with a period of approximately 24 hrs. Mitotic indices of the cornea (Scheving, 1976) and parts of the digestive tract (Scheving et al., 1979) vary daily and in a predictable manner. Rates of glandular secretions such as parotid gland saliva (Dawes, 1975) and gastric acid secretion (Moore and Englert, 1970) also vary predictably with a circadian period.
Furthermore, susceptibility of the organism to pharmacological challenge may vary in a circadian fashion (Sturtevant et al., 1978). Possible factors mediating such changes may include circadian fluctuations of biogenic substances such as corticosterone, serotonin, histamine, norepinephrine and alkaline phosphatase, cell counts of neutrophils, eosinophils, lymphocytes (Ehret et al., 1978) and mast cells (Polat, 1980) and a wide range of enzymes (Ehret et al., 1978). In short, many of the body's indices for determining and mechanisms for dealing with stress also vary with a circadian periodicity.

Histological manifestations

If a relationship exists between structure and function at the microscopic level, then a change in subcellular morphology reflecting the above mentioned physiological fluctuations is indicated. Indeed, within the past 20 years, many examples of organelle population, cellular inclusion and variations in enzyme staining relating to physiological changes have been reported. Within the gastrointestinal tract these include mitotic figure frequencies throughout the alimentary canal (Scheving et al., 1979), salivary gland ultrastructure (Albegger and Muller, 1973), cellular structure of stomach cells (Ryabchikova and Vinogradova, 1979; Zavicic et al., 1980), enzyme staining characteristics in the small intestine (Schafer et al., 1976), and lysosomal content (Bhattacharya and v. Mayersbach, 1976), glycogen deposits (Muller et al., 1966) and ultrastructure of the liver (Uchiyama, 1980).
Gastric mucosa

The mucosa of the gastric corpus is lined by surface epithelial cells, mucous neck cells, parietal cells, chief cells, and by relatively few enterochromaffin cells. The stomach surface is lined exclusively by surface epithelial cells, which also line the foveola, or pits. Emptying into each foveola are about three to five gastric glands. Each gland has an isthmus, lined by surface epithelial cells interspersed with parietal cells, a neck, lined by parietal cells and mucous neck cells and a base, comprised by chief cells and parietal cells (Fig. 1).

Parietal cells

The gastric parietal cell is responsible for hydrochloric acid (HCl) production, which aids in the digestive process by softening fibrous foods and by catalyzing the conversion of pepsinogen to pepsin, a digestive enzyme. The acid also lyses ingested bacteria (Davenport, 1972). These beneficial effects are occasionally accompanied by detrimental effects; i.e. HCl secretion may be related to formation of some peptic ulcers (Weiner, 1977). In addition to HCl production, parietal cells in some species produce gastric intrinsic factor, necessary for the absorption of Vitamin B. However, while this is the case for the parietal cells of man, the Rhesus monkey, cat, rabbit, guinea pig and cow, there are indications that the rat and mouse produce this protein in the chief cells; and the hog produces it in the pylorus and Brunner's glands of the duodenum (Hoedemaeker, 1965).
Fig. 1. Schematic drawing of gastric glands and their component parts. SE = surface epithelial cell; MN = mucous neck cell; C = chief cell; P = parietal cell.
gastric lumen

foveola

isthmus

neck

base
Histology

In general, parietal cells are described as being somewhat pyramidal in shape, with the apex pointed toward the glandular lumen. The centrally located nucleus is round or oval and relatively pale. Unstained intracellular spaces, which are seen at the light microscopic level in semithin (1 micron) sections, are revealed to be secretory canaliculi at the ultrastructural level (Bloom and Fawcett, 1975). The canalicular system appears to branch through the cytoplasm; but all canaliculi are believed to be continuous with the glandular lumen, at least in the active state (Forte et al., 1977; Ito and Schofield, 1978). Within the cytoplasm, microvesicles, also called tubulovesicles, are seen. Although these were once postulated to be either smooth endoplasmic reticulum or a network of channels connecting directly with the outer cell (Ito, 1961; Rubin, 1969), current indications are that they are probably discrete vesicles independent from, although possibly structurally related to, the plasmalemma (Black, 1980). Rough endoplasmic reticulum is seen sparsely distributed throughout the cytoplasm (Bloom and Fawcett, 1975). Dense bodies (Helander, 1976) and multivesicular bodies (Hally, 1959) are also present, although not in great numbers.

Parietal cells are located along the entire gastric gland, from isthmus to base. There are indications that there are some differences between parietal cells of the neck of glands (that area from the deepest
mucous neck cell to the stomach lumen) and those of the base of glands (below the deepest mucous neck cell to the glandular base) (Wattel and Geuze, 1977). Descriptions of these differences have not always concurred. As early as 1960, Lawn reported that at the ultrastructural level rat parietal cells of the necks of glands had more rough endoplasmic reticulum but fewer microvesicles and mitochondria than cells in the base. He also indicated that in neck cells, secretory canaliculi are "closed and may be less extensive". Rosa (1963) also reported that in human parietal cells of glandular necks, secretory canaliculi are less extensive, although he reported increased numbers of microvesicles. Helander (1976) observed that neck parietal cells contained some mucous granules (perhaps reflecting their recent differentiation from cells also giving rise to mucous neck cells). He noted, in contrast to Lawn (1960) and Rosa (1963), that these cells had an increased secretory surface. Ryabchikova and Vinogradova (1979) also reported that as cells migrate basally (away from the neck) that the "level of the development of secretory membranes of the parietal cells gradually falls". This study also noted more ribosomes and lysosomes in basally located cells.

Structure and function

As noted above, physiological state is often reflected by the microanatomical structure of a tissue. Although most of these changes in the parietal cell are best seen at the ultrastructural level, some
differences had already been seen with the light microscope before electron microscopes were commonly used. As early as 1936, Hoerr noted that "the more active the stomach is, the thicker are the intracellular canaliculi."

By far, however, the largest number of studies on this topic is based on transmission electron microscopic studies. One of the more striking changes noted between the active and resting parietal cells is in the conformation of the secretory canaliculi. Upon stimulation by such factors as food, drugs or the vagus, secretory canaliculi undergo a change that has been described as a proliferation of the surface membrane (plasmalemma facing the glandular lumen) (Rubin, 1969; Sedar 1969; Forte, et al., 1977; Zalewsky and Moody, 1977; Tarnowski et al., 1980). Others have observed a dilation of the canaliculi (Rosa, 1963; Frexinos, 1971; Zaviacic et al., 1980) or a proliferation and branching of the canalicular network (Rohrer, et al., 1965; Helander and Hirchowitz, 1972). This reaction appears to be widespread; the species represented in these studies are the rat, human, bullfrog, pig, dog and rabbit.

A second striking ultrastructural change occurring in stimulated parietal cells is the rapid depletion of microvesicles. This has been noted in man, the rabbit, pig, mouse, rat, dog and frog (Rosa, 1963; Roher et al., 1965; Frexinos et al., 1971; Tarnowski et al., 1980; Berglindh et al., 1979; Forte et al., 1977; Black, 1980; Ito and

From these studies it becomes apparent that a stimulus to the parietal cell causes a disappearance of membrane-bound vesicles from the cytoplasm and a concomitant increase in the surface area and/or size of secretory canaliculi. These observations have led to the hypothesis that upon stimulation, the microvesicles, which presumably contain HCl or precursors, approach the luminal surface of the cell and fuse with the plasmalemma, exocytosing the contents into the secretory canaliculi. The secretory surface of the cell thereby becomes more extensive. Either by changes in the conformation of the membrane caused by increased surface area or by the pressure of the newly-secreted HCl in the canaliculi, the latter become dilated (Schettino et al., 1980). During the non-stimulated state, parts of the newly expanded surface membrane presumably are taken into the cytoplasm again to form new microvesicles, replenishing the depleted supply (Forte et al., 1977).

Several other studies support this hypothesis. In a morphometric study Helander and Hirchowitz (1974) demonstrated that "the nutrient membrane (capillary side of cell) remains practically constant regardless of the functional state of the cells. A reciprocal relationship is . . . found between the secretory (canalicular) and the tubulovesicular membranes; an increase of the former is paralleled by a decrease of the latter, and vice versa." This postulated interchange of
membranes is supported by freeze etch studies (Leeson, 1972; Black, 1980), showing that the membrane face with the most particles points outward (extracellularly) in the cell membrane, while this same surface faces inward (towards the contents) in the microvesicles. This would be the case if there were an inversion and eversion of the microvesicles with the plasmalemma. Finally, Berglindh et al. (1980) have shown with Nomarski and fluorescent microscopy that the site of acid production is in the secretory canaliculus. This is supported by the demonstration that carbonic anhydrase lines the surface membrane of parietal cells (Cross, 1970; Sato et al., 1980; Sugai et al., 1980).

Another cellular structure, the multivesicular body, has been observed to change under some secretory stimuli. Injections of pilocarpine nitrate (Hally, 1959) and Histolog (Rosa, 1963) have been reported to increase numbers of multivesicular bodies, although Ito and Schofield (1974) reported that neither insulin nor pentagastrin, both potent stimuli of gastric HCl secretion, caused any observable change in this cellular organelle.

The role of rough endoplasmic reticulum in parietal cells is not well understood. It may function solely in normal cell maintenance. It may produce the peptide gastric intrinsic factor that is known to be formed in parietal cells of some species. The rat, however, is not one of those species, although its parietal cells contain the organelle. The general function of rough endoplasmic reticulum is to produce
protein. It may undergo circadian structural fluctuations, as in other tissues of the body, notably the salivary glands (Albegger and Muller, 1973), liver (Muller, 1971) and pineal gland (Welsh et al., 1979).

A wide variety of environmental, neural, pharmacological, pathological and physiological factors are known to influence parietal cell secretion. The resting state, characterized by narrow canaliculi and large numbers of microvesicles, is seen during a fast (Rosa, 1963; Roher et al., 1965; Ito and Schofield, 1974). Hibernating animals may display a majority of parietal cells with these characteristics (Ryabchikova and Vingradova, Figs. 1a and 1b, 1979). In addition, some pharmacological inhibitors of gastric secretion such as atropine (Roher et al., 1965; Helander and Hischowitz, 1974), cimetidine, isoproterenol, thiocyanate (Zalewsky and Moody, 1977), metiamide (Black, 1980) and high doses of reserpine (Ito and Schofield, 1974) have been observed to induce quiescent cells.

The morphologically active or secretory state with wider canaliculi or increased secretory plasmalemma area and fewer microvesicles is induced by many factors, some of which may be dependent on each other (Bohman et al., 1980; Gottrup et al., 1980). The nervous system appears to have profound effects on the parietal cell. Most of these effects are probably mediated through the vagus nerve, experimental stimulation of which induces a morphologically active state in the parietal cell population (Sedar and Friedman, 1961). It should
be noted that although the morphological effects have not as yet been tested, physiological studies have shown that other neurally mediated factors such as stress (Hoelzel, 1942) sham feeding (Stendquist and Olbe, 1980) and anticipation of food (Weiner, 1977) are associated with increased gastric acid output. Certain brain lesions (Grijalva et al., 1980) may also cause this effect. In addition, humoral factors affect acid secretion. Gastrin (Hirchrowitz, 1967) and histamine (Knight et al., 1980), are known to be endogenous secretogogues. Morphological studies have used pharmacological doses of such secretogogues as Histalog (Rosa, 1963), histamine (Sedar, 1961; Roher et al., 1965; Helander, 1972; Forte, 1977; Ito and Hirchowitz, 1972; Forte et al., 1977; Ito and Schofield, 1977; Black, 1980), insulin (Sedar, and Friedman, 1961; Ito and Schofield, 1974) pentagastrin (Frexinos, 1971; Ito, 1974; Black, 1980; Tarnowski et al., 1980) and low doses of reserpine (Ito and Schofield, 1974). While the majority of cells do respond to these chemical agents, it should be noted that not all cells appear to be active simultaneously (Frexinos, 1971; Ito and Schofield, 1978).

**Mucus-producing cells of the stomach**

Although mucus-producing glands are located throughout the esophagus, gastric cardia and fundus, the stomach mucosa itself contains two cell types that produce mucus. Their product is clearly important in the protection of the stomach wall (Davenport, 1972). Aside from
lubricating the chyme, the mucous layer lining the stomach lumen is considered by some investigators (Allen and Garner 1980) to make an "unstirred layer" for the prevention of back-diffusion of acid. That is, acid produced by intraglandular parietal cells is delivered to the stomach lumen where it reacts with chyme. While some of the acid diffuses back toward toward the mucosa, these potentially harmful hydrogen ions are met by a viscous layer of mucus adhering to the cells. This gel-like coating, which forms a water- and ion-permeable lattice-work, allows the diffusing hydrogen ions to interact with bicarbonate ions moving out from the surface epithelial cells (Allen and Garner, 1980; Williams and Turnberg, 1980). Supporting this hypothesis is a study by Williams and Turnberg (1981) demonstrating a pH gradient across the mucous layer coating the stomach.

Surface epithelial cells

Surface epithelial cells line the entire gastric lumen and extend into the foveolae. They are tall columnar cells with a basally located nucleus and pale-staining cytoplasm. Apically in the cytoplasm is a "packet" of small mucous granules which stain a dark red-purple with periodic acid Schiff and dark blue with toluidine blue. The former reaction indicates a highly sulfated glycoprotein and the latter, an acid pH of the mucus (Gerard et al., 1967). While histochemical staining has given some insight into the biochemical nature of the stored mucus (Gerard et al., 1967; Wattel and Geuze, 1977; Sato and
Spicer, 1980), the physiological significance of these substances is as yet unknown.

The staining properties of these cells at different glandular locations has been noted to vary; the mucus is less sulfated in cells lining the stomach lumen than in cells in the isthmuses (Gerard et al., 1967; Wattel and Geuze, 1977). This may be related to the age of the cell. The size and number of mucous granules also increases as cells migrate to the gastric lumen (Wattel and Geuze, 1977).

Surface epithelial cells appear to release mucus by at least three methods: exocytosis, by which only a few granules are released at a time; apical expulsion, during which granules coalesce and the apical plasmalemma "pinches off", sending a stream of mucus into the stomach; and cell exfoliation, during which the entire cell is extruded from the stomach surface (Zalewsky and Moody, 1979). The functional significance of each of these methods is not known, although total cell extrusion is the response to mucosal tissue damage as well as the fate of all degenerating surface epithelial cells (Harding and Morris, 1977).

Mucus secretion in the stomach can be stimulated by carbachol, pentagastrin, secretin, histamine and cholecystokinin (Vagne and Perret, 1976). Unfortunately, in physiological studies of gastric mucus output, it is presently not possible to distinguish mucus produced by antral and cardiac glands from that produced by surface epithelial cells. To avoid this problem, investigators have used Heidenhain pouches to collect
secretions from the gastric corpus, alone. In these studies, however, it is still not possible to separate the secretions of surface epithelial and mucous neck cells.

Morphological analysis is one method of detecting physiological changes in the cells. In general, surface epithelial cells change very little in different functional states. Food, urecholine (Gerard et al., 1968) and glucagon (Ivy et al., 1981) have been observed to cause an extrusion of mucus from the cells. Histamine and gastrin, on the other hand, appear to cause an increase in production but no visible extrusion of the mucus (Gerard et al., 1968).

Mucous neck cells

Mucous neck cells lie between the isthmus and base of gastric glands. They are irregular in shape, with a basal nucleus and apical mucus-packed cytoplasm which appears to be deformed by surrounding parietal cells. Mucus granules are larger than those in surface epithelial cells (Bloom and Fawcett, 1975) and stain lightly with periodic acid Schiff, indicating a low sulfate content in the mucus (Gerard et al., 1967). In a perfusion study of precursors of mucus production, these cells incorporated the labeled substances at a low rate, leading Wattel et al., (1979) to conclude that they are not important in gastric mucus synthesis. The method of mucus secretion from these cells has not yet been described; they are by far the least studied mature cell type of the gastric mucosa. Urecholine, a
vagomimetic agent, may cause a release of mucus from the cells (Gerard, et al., 1968). Helander (1964) reported a slight increase in the size of mucous granules of reed mice when compared to the fasting state. Unfortunately, since he referred to the cells as mucoid and did not distinguish the neck cells from the surface cells, the significance of these data is unclear.

Gastric cell renewal

Leblond (1981), in a review of early studies, has shown that some cell types of the gastrointestinal tract belong to a rapidly renewing cellular population. When autoradiographic techniques were used cells of the small intestine were observed to develop in the base of isthmuses. They then migrate superficially along the villus and are sloughed off the villar tip. Later studies of mitotic frequency in the stomach mucosa (Stevens and Leblond, 1953; Bertalanffy, 1960; Lipkin et al., 1963) showed cells dividing only in the neck of gastric glands. At least four different timepoints of the 24-hr cycle were sampled in these experiments. The results indicate that cells of the stomach migrate in two separate directions and that not all cell types have the same renewal rate.

In a variety of histological studies using routine techniques as well as more sophisticated histochemical and autoradiographic techniques, parietal cells have rarely if ever shown evidence of DNA synthesis or mitosis (Bertalanffy, 1960; Lipkin et al., 1963; Willems,
et al., 1972; Wattel and Geuze, 1977). Since parietal cells do reappear after injury to the mucosa (Wattel et al., 1977; Blom and Helander, 1981), they are presumed to develop from undifferentiated cells in the neck of glands and to migrate to the base of the gland (Willems et al., 1972). The renewal of surface epithelial cells is well studied. Of all gastric cell types it appears to follow the renewal pattern of small intestinal cells most closely. The cells arise from dividing, undifferentiated cells of glandular necks and migrate toward the glandular lumen, into which they are sloughed off (Stevens and Leblond, 1953; Bertalanffy, 1960; Lipkin et al., 1963; Wattel et al., 1977). The process is quite rapid in these cells as compared to that in other tissue types; the entire surface epithelial cell population is believed to be replaced within three (Stevens and Leblond, 1953) to four (Lipkin et al., 1963) days. The development and fate of mucous neck cells is less clear. They do appear to arise from undifferentiated cells (Stevens and Leblond, 1953) or dividing mucous neck cells (Bertalanffy, 1960); and since these mitoses are more numerous than would be necessary to replace the very small number of degenerated cells seen, mucous neck cells may slough off into the lumen without having migrated (Stevens and Leblond, 1953). Their mitotic rate is lower than surface epithelial cells (Bertalanffy, 1960), and complete renewal is believed to occur every six and one half days (Stevens and Leblond, 1953). In short, "the proliferative activity of undifferentiated cells and differentiating mucous cells, and the late reappearance of parietal...cells (after
fast neutron irradiation) indicate that the progenitors of all fundic epithelial cell types must be sought among the undifferentiated cells and the differentiating mucous cells" (Wattel et al., 1977).

Feeding habits of rats

The rat is a nocturnal animal, with most motor activity and feeding behavior occurring during the dark phase of the 24-hr light-dark cycle (Gibbs, 1979). Siegel and Stukey (1947) noted that when the stomachs of ad libitum-fed rats were weighed at four equidistant timepoints of the 24-hr cycle, maximal weights, and thus the greatest amounts of food, were found during the dark phase. More frequent measurements of feeding behavior over the circadian period revealed a definite food intake pattern in which a decline to the least stomach weight was found during the first half of the light phase. In the second half of this phase, feeding gradually increased (Siegel, 1961). Subsequent studies have shown that in the dark phase there is a bimodal peak in feeding/stomach weight, the first peak occurring in the mid-dark span and the second peak in the late dark to early light phase (Armstrong and Taylor, 1978; Peraino et al., 1980).

While many rhythmic fluctuations of function and structure have been described for the gastrointestinal tract, their causes are often difficult to determine because of complicating effects of periodic food intake. One possible solution is to fast the animal to determine
whether the variation persists in the absence of food. There are at least two problems inherent in this procedure, however. First, a rhythmic fluctuation may persist during the beginning of a fast (Furuya and Yugari, 1974; Dawes, 1975) because of a psychological or physiological "anticipation" of feeding (Siegel, 1961; Bolles and Stokes, 1965) but may diminish in a longer fast (Saito et al., 1976). Second, while one may starve a small mammal such as the rat for as long as five consecutive days (Saito et al., 1976), this practice is ethically unsound. It also introduces the possibility of health/stress hazards which may affect experimental results.

A second method sometimes used to determine whether a gastrointestinal rhythm is dependent on food intake is to phase-shift the feeding pattern of the animal (Scott and Potter, 1970; Furuya and Yugari, 1974; Sitren and Stevenson, 1978; Kersten, et al., 1980; Saito et al., 1980). While such food restriction or "meal-feeding" (Scott and Potter, 1970) may cause an initial weight loss, the long-term effects are not considered serious enough to affect the experimental results (Kaul and Bernadier, 1975; Saito and Noma, 1980).

Rodents such as the rat indulge in coprophagia (Weber, 1958) to retrieve nutrients from ingested food. To avoid this complication during food restriction studies, experimental animals often are housed in cages with wire mesh floors that allow fecal material to fall below (Helander, 1964; Ito and Schofield, 1974; Saito et al., 1980).
Morphometry

Morphometry is the measurement of structures. When these measurements are used to estimate three dimensional structures, the study is called stereology (Elias and Hyde, 1980). When applied to biological structures the assumption is made that "quantitative relationships exist between the average dimensions of a large number of organelles and those of their profiles on sections" (Weibel, 1973). It is also understood that an average of the data of a large population will be made. Morphometric studies may be made to confirm previous visual observations (Helander et al., 1972), to compare results of different experiments (Helander, 1978) and occasionally will reveal subtle differences that may not be apparent otherwise.

While computer "image analyser" systems are sometimes used, Mathieu et al., (1981) has shown that with sufficient sample size, point counting is just as accurate and sometimes more rapidly performed. Weibel et al., (1966) described an efficient "multipurpose test system" that may be used simultaneously for point-counting and line intersections. The method is described in Appendix II.
CHAPTER III

PURPOSE OF STUDY

1. To determine whether there are circadian fluctuations in characteristics of gastric parietal cells as revealed by
   a) secretory canaliculi
   b) microvesicles
   c) rough endoplasmic reticulum
   d) multivesicular bodies

   and surface epithelial and mucous neck cells as revealed by
   a) cell volume
   b) nucleoli
   c) mucous content
   d) mucus staining characteristics.

2. To determine the results of the following feeding regimens on the above characteristics:
   a) ad libitum feeding
   b) fasting
   c) restricted food availability.

3. To assess circadian variation in gastric acid and mucus production as effected by feeding regimens.
This paper was published in Cell and Tissue Research 211:175-177 (D. M. Jacobs and R. P. Sturtevant, 1980).
CHAPTER IV

CIRCADIAN MORPHOMETRIC VARIATION IN GASTRIC PARIENTAL CELL POPULATIONS OF THE RAT

Summary

Gastric parietal cells of rats maintained under standardized conditions and fed ad libitum were examined by electron microscopy at 7 timepoints of the 24-hr day. Morphometric determinations were made on 4 cell characteristics. The volume density of secretory canaliculi was maximal at the mid-dark sampling point and decreased during the light phase; a secondary peak was seen 1 hr before the onset of darkness. The surface density of microvesicles and RER fluctuated inversely with the pattern displayed by secretory canaliculi. The number of multivesicular bodies per cytoplasmic area exhibited a single peak, 1 hr after the onset of darkness. It was further noted that parietal cells in the necks and bases of glands differed morphologically and that their organelle populations varied at individual circadian rates.
Introduction

Rosa (1963) and Helander and Hirschowitz (1972) reported that when stimulated by secretagogues or by fasting-refeeding, the parietal cells of the stomach responded with an increase of the secretory canalicular area and a decrease of the microvesicles that appear to contain HCl or its precursor(s). In addition, Hally (1959) reported an increase in multivesicular bodies upon repeated stimulation of the cells by pilocarpine nitrate.

Qualitative circadian studies have indicated that 24-hr changes occur in the morphology and enzyme-staining characteristics of these cells in animals maintained under standardized conditions and fed ad libitum (Zaviacic and Brozman 1978; Zaviacic et al., 1979). Although differences in the parietal cells of the necks and bases of gastric glands have been noted at the light microscopic and ultrastructural levels (Lawn, 1960; Helander, 1972), it is not known to what extent the two populations vary from each other or if they do so at all phases of the circadian cycle. The purpose of the present study was to quantify these differences and to relate them to the rats' circadian physiological pattern.
Materials and methods

Thirty male Charles River rats weighing 150-200 g were caged in groups of 5 in environmental chambers in which a 12 hr:12 hr light-dark cycle was maintained. Food and water were supplied ad libitum. Animals were handled frequently during the initial 1-month acclimatization period. In the winter of 1979, animals were decapitated in groups of 5 at timepoints corresponding to mid-dark, mid-light, and 1 hr before and after the onset of light and dark phases. The stomach was rapidly removed, and a 2mm x 2mm block of tissue was excised from the anterior corpus, 1mm inferior to the limiting ridge. The tissue was placed immediately in ice-cold Karnovsky's fixative. Each block was further trimmed into smaller rectangles to promote rapid fixation and to allow subsequent orientation in the embedding molds. The tissue was post-fixed in 2% osmium tetroxide in cacodylate buffer, processed for electron microscopy by standard techniques, and embedded in epon in flat molds.

For each animal, electron micrographs were taken on 20 cells from the neck and 20 cells of the base of glands. The cells were sampled systematically and micrographs were taken on an Hitachi electron microscope (Model HU-11B) at x2,000. The negatives were projected onto a Nikon Profile Projector screen (Model 6C) over which a system of test lines was laid, thus providing a final magnification of x20,000. The test lines were used as a multipurpose test system, i.e., line
intersections and point counts. Four cell characteristics were estimated according to Weibel and Bolender (1973) as follows: (1) volume density (Vv) of secretory canaliculi, (2) surface density (Sv) of microvesicles, (3) Sv of RER and (4) number of multivesicular bodies (Nv) per non-nuclear cytoplasmic area.
Results and discussion

The Vv of secretory canaliculi was maximal at the mid-dark sampling time, when the Sv of microvesicles and RER reached minimal levels. A secondary peak and trough, respectively, were seen 1 hr before the onset of darkness. Multivesicular bodies were most numerous 1 hr after the onset of darkness and decreased thereafter (Fig. 1).

The rat, a nocturnal animal, usually consumes most of its food during the dark phase of a cycling illumination regimen. It is, therefore, not surprising that parietal cells presented a classical picture of secretion at 2300 (Fig. 1) when microvesicles were depleted and secretory canaliculi expanded. Conversely, during the animals' resting phase, secretory canaliculi were less distended and the microvesicular population appeared to have been replenished. The observation that RER increased with the latter may indicate increased membrane formation and/or production of related proteins. The secondary indication of activity noted in the cells before the onset of darkness may indicate either that the animals begin feeding at this time or that there is an anticipatory release of acid in the stomach prior to initiation of feeding. Since the function of multivesicular bodies in the parietal cell has not been established, the reason for their increase early in the dark phase remains obscure.

Parietal cells in the necks of glands appeared to present a more "active" picture at almost all circadian phases than the cells in the
Fig. 1. Circadian variation in 4 characteristics of parietal cells from ad libitum-fed rats. Dashed line shows mean value ± SE of cells from necks of glands; solid line shows mean value ± SE of cells from bases of glands. For each point n=100 except in the case of multivesicular bodies where n=200. White and black bars below abscissa indicate light and dark hours of illumination regimen.
basal portion of the glands. Secretory canaliculi were more prominent, more RER was present, and fewer microvesicles were seen. However, it is of note that while fluctuations in morphometric values of the 2 cell populations were generally in parallel, cells in the bases of glands appeared to return to the resting state earlier than those in the necks of glands.

The results of this study suggest that a complete understanding of the morphological and functional properties of the parietal cell population requires a systematic sampling from all glandular levels at several phases of the animal's circadian physiological system. The common practice of observation made at a single timepoint may be misleading.
This paper was accepted for publication in The Anatomical Record in August, 1981 (Danielle M. Jacobs and Ruthann P. Sturtevant).
CHAPTER V

CIRCADIAN ULTRASTRUCTURAL CHANGES IN RAT GASTRIC
PARIETAL CELLS UNDER ALTERED FEEDING
FEEDING REGIMENS: A MORPHOMETRIC STUDY

Abstract

The ultrastructure of rat gastric parietal cells was studied at six timepoints of the 24-hr day. The rats, maintained on an LD 12hr:12hr regimen, had been subjected to either a 40-hr fast or to a four-hr mid-light restricted feeding period. At each timepoint, the volume density (Vv) of secretory canaliculi, surface density (Sv) of microvesicles and RER, and the numerical density (Nv) of multivesicular bodies were determined in cells of the neck and base of glands. Circadian variation of the four variables was suggested in both experiments. Canalicular and microvesicular measurements suggested that a rhythm in gastric acid secretion may persist during fasting; a peak and trough, respectively, occurred in the late dark phase, as in our previous report on ad libitum-fed rats. Restriction of feeding to that which is normally the rat's resting phase caused an apparent 180 degrees phase-shift in the rhythm. The data suggested, however, that additional factors may have influenced the cellular activity pattern. At all timepoints in both experiments, cells of the neck of glands had higher
RER and canalicular values than did cells of the base of glands. This suggests that parietal cells in glandular necks may be more active than those farther removed from the stomach lumen. There was no correlation between the Nv of multivesicular bodies and glandular location of the cells.
Introduction

Parietal or oxyntic cells are the source of gastric HCl. With the exception of the foveola (Wattel et al., 1977), they are found throughout the entire gastric gland from upper isthmus to base. When stimulated to secrete, the parietal cell acquires the morphology characteristic of its active state: secretory canaliculi become dilated, and the number of microvesicles decreases (Frexinos et al., 1971). The resting state, characterized by narrow canaliculi and a cytoplasm rich in microvesicles, is simulated by administering an inhibitor of HCl secretion such as cimetidine (Zalewsky and Moody, 1977).

Another cellular characteristic reported to vary during altered physiological conditions is the multivesicular body. Hally (1959) and Rosa (1963) both described changes in this organelle after administration of pilocarpine nitrate and Histolog. The organelle itself is rather ubiquitous in the body's tissues, but its function is not clear. Friend (1969) indicated that the matrix of multivesicular bodies of rat epididymal cells contains acid phosphatase, a lysosomal enzyme. Novikoff et al. (1964), Holzman (1976) and others suggested that they may be lysosomal in nature.

Circadian (circa 24-hr) rhythms of physiological function have been described for many of the body's tissues (v. Mayersbach, 1965). Since physiological changes in a tissue are often reflected
morphologically, many cells display circadian variations in enzyme staining, cell inclusions and organelle populations. In the gastrointestinal tract such changes have been noted in the salivary glands (Albegger and Muller, 1973), liver (Uchiyama, 1980) and stomach (Zaviacic and Brozman, 1978). While some physiological rhythms may be generated by the periodic intake of food, others persist during starvation (Saito et al., 1976) and continuous intravenous infusion of nutrients (Stevenson et al., 1980).

It has been well documented that the parietal cells in the neck and base of gastric glands differ morphologically. Lawn (1960) suggested that cells of the neck may have narrow, less extensive secretory canaliculi. Other investigators (Helander, 1976; Ryabchikova and Vinogradova, 1979), however, have reported that parietal cells of glandular necks have fewer microvesicles and more elaborate secretory canaliculi than those in the glandular base.

In an earlier study (Jacobs and Sturtevant, 1980), we found statistically significant changes in the morphometry of four variables in ad libitum-fed rats maintained under controlled light and dark conditions (12hr:12hr). Secretory canaliculi, microvesicles, rough endoplasmic reticulum and multivesicular bodies all varied over the 24-hr period. For the present report our observations were extended to rats maintained under similar lighting conditions but altered feeding regimens. In both food restricted (phase-shifted) and fasted animals
secretory canaliculi and microvesicles were studied as indicators of secretory activity. Rough endoplasmic reticulum and multivesicular bodies were studied in an effort to elucidate their respective roles in the parietal cell. In all studies the data from cells in the neck and base of gastric glands have been compared in order to identify further the differences between cells in these two glandular locations.
Materials and methods

Sixty adult male outbred albino rats obtained from Charles River Breeding Laboratories were housed in groups of five in cages with wire mesh bottoms to prevent coprophagia. Cages were kept in environmental chambers in which lights were on from 0500 to 1700 and off from 1700 to 0500. Temperature and humidity were monitored continuously. Water was available ad libitum and diet (Purina Rat Chow) as described below. All animals were acclimatized to these conditions for at least one month and weighed 250-350 g at the time of the experiments.

Manipulation of feeding

Food restricted experiment: thirty rats were allowed to feed from 0900 until 1300 (mid-light) daily for eight weeks.

Fasting experiment: thirty rats were subjected to four periods of feeding: starvation (8hr:40hr) during which food was available from 1800 to 0200 on alternate days. They were sacrificed after the last starvation phase.

Experimental procedure

Groups of five rats each were sacrificed at six timepoints: mid-dark, mid-light, one hr before and one hr after the onset of both light and darkness. Animals were removed individually from the environmental chambers and decapitated within 20 seconds. The stomach
was removed and a piece of tissue 2mm x 2mm excised from the anterior wall of the corpus, approximately 2mm inferior to the limiting ridge. This was placed immediately into a modified Karnovsky's fixative of 2% formaldehyde and 2.5% glutaraldehyde in sodium cacodylate buffer (0.2M, pH 7.2) to which 0.0025% calcium chloride was added. The size and contents of the remaining part of the stomach were noted. The excised tissue was cut into smaller rectangular blocks for later orientation in the embedding mold and fixed for four hrs at 4 degrees C. After two hrs post-fixation in 1% osmium tetroxide in sucrose cacodylate buffer, the tissue was dehydrated in graded ethanol solutions and propylene oxide and flat-embedded in Epon.

Electron microscopy and morphometry

For each animal, 20 different cells from the neck and 20 cells from the base of glands were selected for morphometric analysis by the following procedure. One micron sections from tissue blocks were cut through the entire stomach wall and stained with toluidine blue to allow orientation. Blocks were then trimmed to show either the neck (from the mucous neck cells to the lumen) or base (deep to the mucous neck cells) of glands. At least five grids were prepared per block. Between each grid, several thick sections were cut to prevent repeating any cell on the next grid. Thin sections were stained routinely with lead citrate and uranyl acetate. Grids were scanned under a Hitachi (Model HU 11B) electron microscope. One section was chosen per grid and all complete
parietal cell profiles were photographed at X2,000 except those showing poor fixation (disrupted plasma, vesicular or mitochondrial membranes). Also, any cell profile in which the nucleus later proved to be less than 3% of the total cell area was deleted. While scanning, it was possible to confirm the location of the parietal cells (neck vs. base of glands) by their orientation to surrounding cell types (mucous neck, chief, etc.).

The negatives were projected (final magnification X20,000) onto a screen overlaid with a morphometric "multipurpose test system" as described by Weibel et al. (1966). Test lines were 1.5 cm in length (representing 750 nm on the projected negatives); there were 32 test lines in the field. Morphometric determinations were made on four variables: (1) volume density (Vv) of secretory canaliculi, (2) surface density (Sv) of microvesicles, (3) Sv of rough endoplasmic reticulum (RER) and (4) numerical density (Nv) of multivesicular bodies. Vv was obtained by counting the number of line end-points that lay on the secretory canaliculi versus those that fell on the non-nuclear cell (including secretory canaliculi) and represents an estimation of the volume of canaliculus per non-nuclear cell volume (see Appendix II). (When canaliculi were visibly connected with the glandular lumen, a line drawn between the two apical points of the canalicular sides was defined as the apical cell boundary.) Sv, obtained by line intersections with the variable, represents the surface area of the component per non-nuclear cell volume. Nv was obtained by counting the number of
multivesicular bodies per non-nuclear cell volume. All values were calculated according to the method of Weibel and Bolender (1973). A Student's t-test was used to establish probability values; the 0.05 level was considered significant.

Acid phosphatase study

Pairs of rats feeding ad libitum were decapitated at 1800 and 0700, timepoints at which parietal cells showed relatively fewer and more multivesicular bodies, respectively, in our preliminary studies. Stomach tissue fixed for 1 hr at 4 degrees C in isotonic glutaraldehyde/tris maleate buffer was incubated at room temperature in a solution containing beta-glycerophosphate after the method of Barka and Anderson (1963). Tissues incubated with buffer alone at room temperature or with substrate plus sodium fluoride, an enzyme inhibitor, served as controls.
Results

Phase-shifted feeding experiment. Morphometry.

The stomachs of rats sacrificed during the feeding phase (1100) were notably distended with food, measuring up to 5.5cm x 3.0cm. Stomachs of animals sacrificed at 1600, 1800 and 2300 also contained relatively large amounts of food. Noticeable decreases in stomach size (2.5cm x 1.5cm) were not observed until the 0400 and 0600 sacrifice timepoints.

From 1100 until 2300 the Vv of secretory canaliculi was elevated as compared to values at 0400 and 0600 (Fig. 1c and Table 1). Significantly lower values were seen only at the 0400 and 0600 sampling times. Microvesicular Sv, in contrast, was maximal at 0400 and 0600 and significantly less at 1100, 1600 and 2300 (Fig. 2c). A second, statistically significant increase in Sv was noted at 1800, although there was no concurrent decrease in canalicular Vv. RER displayed maximal values at 1600 (Fig. 3c). The Nv of multivesicular bodies displayed peak values at 2300 and 0600, after which values continued to decline through 1800 (Fig. 4c).

At all timepoints, there were consistent differences between cells of the neck and cells of the base of glands. The location of parietal cells with respect to other glandular cells varied (Fig. 5). Only cells in the base of glands were truly "parietal" or peripheral to other cells
Fig. 1. Volume density (Vv) of secretory canaliculi under three different feeding regimens. Data from cells of the neck (____) and base (---) of glands are plotted separately. Time (clock hr) is indicated on the abscissa; white and black bars below indicate light and dark hrs of the illumination regimen. Each point is the mean value of 100 cells ± S.E. Vertical stippled bars indicate hrs of food availability during phase-shifted feeding.

Fig. 2. Surface density (Sv) of microvesicles. Details as in Fig. 1.
Table 1. Means ± S.E. from 5 phase-shifted feeding animals. Twenty cells each from the glandular neck and base per animal (n=100 ± S.E.). Significant differences between timepoints are indicated below.

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>TIME OF DAY</th>
<th>2300</th>
<th>0400</th>
<th>0600</th>
<th>1100</th>
<th>1600</th>
<th>1800</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secretory canaliculi</td>
<td>neck</td>
<td>17.32 ± 1.68</td>
<td>10.40 ± 0.86</td>
<td>11.51 ± 1.35</td>
<td>19.30 ± 1.27</td>
<td>20.51 ± 1.12</td>
<td>19.46 ± 1.26</td>
</tr>
<tr>
<td></td>
<td>base</td>
<td>11.03 ± 0.53</td>
<td>5.37 ± 0.62</td>
<td>5.92 ± 0.88</td>
<td>14.21 ± 0.85</td>
<td>10.84 ± 0.35</td>
<td>11.14 ± 2.35</td>
</tr>
<tr>
<td>Microvesicles</td>
<td>neck</td>
<td>3.34 ± 0.89</td>
<td>11.15 ± 2.51</td>
<td>6.88 ± 1.17</td>
<td>1.88 ± 0.50</td>
<td>2.51 ± 0.66</td>
<td>5.80 ± 1.06</td>
</tr>
<tr>
<td></td>
<td>base</td>
<td>5.31 ± 1.35</td>
<td>16.50 ± 3.87</td>
<td>9.38 ± 1.20</td>
<td>4.22 ± 0.53</td>
<td>4.75 ± 0.90</td>
<td>10.51 ± 2.76</td>
</tr>
<tr>
<td>Rough endoplasmic reticulum</td>
<td>neck</td>
<td>1.95 ± 0.28</td>
<td>1.70 ± 0.37</td>
<td>1.82 ± 0.19</td>
<td>2.42 ± 0.57</td>
<td>2.98 ± 0.41</td>
<td>2.14 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>base</td>
<td>1.20 ± 0.03</td>
<td>0.73 ± 0.21</td>
<td>0.57 ± 0.14</td>
<td>1.28 ± 0.16</td>
<td>2.27 ± 0.26</td>
<td>1.07 ± 0.12</td>
</tr>
<tr>
<td>Multivesicular bodies *</td>
<td></td>
<td>0.15 ± 0.05</td>
<td>0.10 ± 0.02</td>
<td>0.20 ± 0.05</td>
<td>0.11 ± 0.03</td>
<td>0.05 ± 0.02</td>
<td>0.04 ± 0.01</td>
</tr>
</tbody>
</table>

Secretory canaliculi (neck) - 2300 vs. 0400, p<0.01; 0400 vs. 1100, p<0.01.
Secretory canaliculi (base) - 2300 vs. 0400, p<0.01; 0400 vs. 1100, p<0.01.
Microvesicles (neck) - 2300 vs. 0400, p<0.01; 1100 vs. 1800, p<0.01.
Microvesicles (base) - 2300 vs. 0400, p<0.05; 1100 vs. 1800, p<0.05.
Rough endoplasmic reticulum (neck) - 0600 vs. 1600, p<0.01.
Rough endoplasmic reticulum (base) - 0600 vs. 1600, p<0.01.
Multivesicular bodies - 2300 vs. 1800, p<0.01.
*Pooled values from neck and base cells.
Fig. 3. Surface density (Sv) of rough endoplasmic reticulum. Details as in Fig. 1.

Fig. 4. Numerical density (Nv) of multivesicular bodies. Data from cells of the neck and base of glands are plotted together; each point is the mean value of 200 cells ± S.E. Other details as in Fig. 1.
AD LIBITUM-FED

FASTED

PHASE SHIFTED-FEEDING
Fig. 5. Semithin toluidine blue-stained section of gastric glands. Parietal cells of the neck (NPC) directly line the glandular lumen; secretory canaliculi are seen as unstained intracellular spaces. Parietal cells of the base of glands (BPC) are peripheral to the chief cells (C) lining the glandular lumen. Homogeneous cytoplasmic appearance results from the small area occupied by secretory canaliculi. SE = surface epithelial cell; MN = mucous neck cell; LP = lamina propria. (X 330).
lining the glands, whereas parietal cells in the necks of glands bordered directly on the lumen. In addition, values of secretory canaliculi $V_v$ as well as microvesicular and $\text{RER} \; S_v$ varied among cells of the two locations. Secretory canalicular volume densities were consistently greater in parietal cells in the neck of glands than in cells of the base of glands. Another difference observed in cells of the two locations was the variability of the secretory canaliculi. The canalicular $V_v$ of cells in both locations increased between 0400 and 1100; i.e. when the stomachs were empty and full of food, respectively. However, the magnitude of change was greater in cells of the neck than in cells of the base of glands. The $S_v$ of RER, like the secretory canalicular $V_v$, was also consistently greater in cells of the neck than in cells of the base of glands. In contrast, the microvesicular $S_v$ was less in parietal cells of the neck than in those of glandular bases. Multivesicular bodies were the only cellular characteristic studied that did not vary in number with respect to cellular location. Therefore, these data were combined at each experimental timepoint.

Fasting experiment. Morphometry.

Although the animals had been starved for 40 hrs at the time the first group was sacrificed, food was still present in the stomachs, which ranged in size from 1.5cm x 1.0cm to 3.0cm x 1.5cm at 2300. However, at the last timepoint sampled (1800), the stomachs were empty except for a brown-colored fluid. During the dark phase the animals
displayed feeding behavior even in the absence of food; i.e. a metal cage-restraining wire was chewed in half, and some stomachs contained what appeared to be scales from tails.

As seen in Figures 1b through 4b and in Table 2, fasting effected a marked attenuation of the rhythms of the four variables. Among these variables, secretory canaliculi showed the largest fluctuation, which reached maximal Vv from 2300 through 0400 and decreased thereafter. A small but significant increase was seen at 1800, possibly indicating the beginning of the next secretory cycle. Conversely, the microvesicles showed their lowest values at 2300 and 0400 and increased in Sv thereafter, with maximal values attained at 1100 and 1600. RER appeared to fluctuate very little over the 24-hr period except for a small but significant peak from 1800 through 2300 in the cells of the glandular necks, only. Multivesicular bodies reached maximal values at 1800.

Parietal cells of the neck and base of glands displayed the same characteristic differences as in the previous experiment. Those in the neck had a greater canalicular Vv and RER Sv, and a lower microvesicular Sv. No correlation was noted between the location of the cells and the
Table 2. Means ± S.E. from 5 fasted animals.
Twenty cells each from the glandular neck and base per animal (n=100 ± S.E.). Significant differences between timepoints are indicated below.

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>TIME OF DAY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2300</td>
</tr>
<tr>
<td>Secretory canaliculi</td>
<td></td>
</tr>
<tr>
<td>neck</td>
<td>17.36 ± 1.24</td>
</tr>
<tr>
<td>base</td>
<td>8.96 ± 1.44</td>
</tr>
<tr>
<td>Microvesicles</td>
<td></td>
</tr>
<tr>
<td>neck</td>
<td>2.19 ± 0.28</td>
</tr>
<tr>
<td>base</td>
<td>4.05 ± 1.07</td>
</tr>
<tr>
<td>Rough endoplasmic reticulum</td>
<td></td>
</tr>
<tr>
<td>neck</td>
<td>1.68 ± 0.21</td>
</tr>
<tr>
<td>base</td>
<td>0.77 ± 0.20</td>
</tr>
<tr>
<td>Multivesicular bodies</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.14 ± 0.04</td>
</tr>
</tbody>
</table>

Secretory canaliculi (neck) - 2300 vs. 1100, p<0.01; 1100 vs. 1800, p<0.05.
Secretory canaliculi (base) - 2300 vs. 1100, p<0.01; 1100 vs. 1800, p<0.01.
Microvesicles (neck) - 2300 vs. 1600, p<0.01; 1600 vs. 1800, p<0.05.
Microvesicles (base) - 2300 vs. 1600, p<0.01.
Rough endoplasmic reticulum (neck) - 0400 vs. 1800, p<0.05.
Multivesicular bodies - 2300 vs. 1800, p<0.05.

* pooled values from neck and base cells
numerical density of the multivesicular bodies. As in the food-restricted experiment, the secretory canalicular Vv did not fluctuate so strongly in cells of the base as in cells of the neck of glands. The greatest difference in their values was again seen when the stomach contained food (2300 through 0400). As noted above, some fluctuations of microvesicular and RER Sv occurred in cells of the neck but not in cells of the base of glands.

Acid phosphatase study

Tissue incubated in solution with beta-glycerophosphate showed positive reaction product in a few parietal cell dense bodies. However, although more than 400 cells were scanned from both 0700 and 1800, in no case was reaction product associated with multivesicular bodies.
Discussion

The rat normally feeds primarily during the dark phase of the LD cycle when allowed free access to food and water. In a previous study (Jacobs and Sturtevant, 1980) we found that in ad libitum-fed rats the stomachs were quite full of dry food at 2300 and 0400, were less full at 0600 and almost empty, except for a brown fluid, by 1800. In the food-restricted experiment a feeding-phase shift of approximately 12 hrs was imposed on the rats, allowing them to eat for only four hours during the middle of the light phase. They apparently responded to this by gorging, as the stomachs were distended with food from 1100 until 2300. Nearly empty stomachs were not observed until the 0400 and 0600 sampling times. Gorging may also have been a response to periods of enforced fasting.

Food deprivation is a technique commonly used to determine whether certain gastrointestinal rhythms are dependent on food intake. A potential problem in such studies is that in animals not previously fasted rhythms may persist early in the fast, perhaps in anticipation of food presentation (Saito, et al., 1976). The rhythms may eventually disappear if the fast is continued; in addition, one must consider the potential effects of the stress of starvation. In an effort to minimize this problem investigators have attempted to habituate animals to starvation by imposing alternate feeding and starvation periods before beginning the experiment (Saito et al., 1980).
The animals in the current study were subjected to four consecutive feed:starve periods. Forty hours after the last feeding phase ended some food was still present in the stomachs. This was clearly left over from the previous feeding phase, as the cages were free of food during the fast; and the wire mesh cage bottoms prevented coprophagia. There were clear indications of feeding behavior even in the absence of food. This behavior was most pronounced during the dark phase, when ad libitum-fed animals are known to feed most actively.

Any stimulus to gastric HCl secretion causes two morphological modifications in the parietal cell: microvesicles, which have been postulated to contain the acid or its precursors (Schettino et al., 1972), decrease in number, and the secretory canaliculi appear to dilate while the surface membrane area increases. While the latter has been demonstrated morphometrically, the dilation has not yet, to our knowledge, been confirmed by measurements. Stimuli include food (Helander, 1964), increased vagal tone (Helander et al., 1972), hormones such as gastrin (Helander et al., 1972) and insulin (Sedar and Friedman, 1961), histamine (Forte et al., 1977) and such pharmacological secretogogues as Histolog (Rosa, 1963) and pentagastrin (Tarnowski et al., 1980). The histological changes are well documented in the rat (Helander, 1976), among other species. While it is agreed that an increase in canalicular area is accompanied by a decrease in the number of microvesicles and vice versa, Forte et al. (1977) noted that some conformational changes of the plasmalemma may preceed changes in the microvesicular compartment of the cell.
The results of our previous study (Jacobs and Sturtevant, 1980) with ad libitum-fed animals are shown in Figs. 1a to 4a and in Table 3. The peak secretory canalicular Vv was found at 2300 and minimal values at 1100. In contrast, maximal microvesicular Sv values were observed at 1100 and minimal values at 2300. A secondary peak and trough of canaliculi and microvesicles, respectively, occurred before the lights went off. Stomachs at this time contained some dry food. Rough endoplasmic reticulum Sv fluctuated in a manner similar to the Sv of microvesicles; i.e., lowest values were in the mid-dark phase and maximal values at the mid-light sampling point. The Nv of multivesicular bodies increased sharply at 1800 and was significantly lower at all other sampled timepoints.

Phase-shifted feeding

The results of the food-restricted experiment supported the findings of Helander et al. (1972), Sedar and Friedman (1961) and other investigators. Peak values at 2300 and 1100 in secretory canalicular Vv were accompanied by troughs of the Sv of microvesicles. Similarly, a trough in canalicular values at 0400 was accompanied by peak microvesicular values. However, while there were no differences in the canalicular values at 1100, 1600, 1800 and 2300, microvesicles did display a statistically significant increase in Sv at 1800. The morphological evidence of increased secretion from 1100 to 2300 may have been a physical response to the presence of food in the stomach. When the stomachs were less distended with food at 0400 and 0600, canalicular
Table 3. Means ± S.E. from 5 ad libitum-fed animals. Twenty cells each from the glandular neck and base per animal (n=100 ± S.E.). Significant differences between timepoints are indicated below.

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>TIME OF DAY</th>
<th>2300</th>
<th>0400</th>
<th>0600</th>
<th>1100</th>
<th>1600</th>
<th>1800</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secretory canaliculi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>neck</td>
<td></td>
<td>19.92 ± 2.50</td>
<td>20.48 ± 2.21</td>
<td>17.25 ± 1.55</td>
<td>8.55 ± 0.95</td>
<td>14.91 ± 2.14</td>
<td>10.94 ± 1.22</td>
</tr>
<tr>
<td>base</td>
<td></td>
<td>19.37 ± 2.14</td>
<td>13.06 ± 0.45</td>
<td>6.81 ± 1.01</td>
<td>5.28 ± 0.75</td>
<td>12.58 ± 1.04</td>
<td>7.34 ± 0.61</td>
</tr>
<tr>
<td>Microvesicles</td>
<td></td>
<td>3.69 ± 1.61</td>
<td>1.80 ± 0.47</td>
<td>4.10 ± 1.04</td>
<td>13.94 ± 1.57</td>
<td>7.27 ± 1.68</td>
<td>11.17 ± 2.32</td>
</tr>
<tr>
<td>neck</td>
<td></td>
<td>5.97 ± 2.03</td>
<td>6.11 ± 1.40</td>
<td>8.64 ± 1.71</td>
<td>15.88 ± 2.03</td>
<td>8.28 ± 0.96</td>
<td>17.26 ± 1.18</td>
</tr>
<tr>
<td>base</td>
<td></td>
<td>1.75 ± 0.25</td>
<td>0.95 ± 0.24</td>
<td>1.26 ± 0.20</td>
<td>4.00 ± 0.66</td>
<td>2.42 ± 0.41</td>
<td>2.99 ± 0.50</td>
</tr>
<tr>
<td>Rough endoplasmic reticulum</td>
<td></td>
<td>2.00 ± 0.13</td>
<td>2.30 ± 0.30</td>
<td>2.07 ± 0.37</td>
<td>4.83 ± 0.46</td>
<td>1.93 ± 0.31</td>
<td>3.38 ± 0.51</td>
</tr>
<tr>
<td>neck</td>
<td></td>
<td>1.75 ± 0.25</td>
<td>0.95 ± 0.24</td>
<td>1.26 ± 0.20</td>
<td>4.00 ± 0.66</td>
<td>2.42 ± 0.41</td>
<td>2.99 ± 0.50</td>
</tr>
<tr>
<td>base</td>
<td></td>
<td>0.13 ± 0.03</td>
<td>0.11 ± 0.02</td>
<td>0.11 ± 0.02</td>
<td>0.06 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.27 ± 0.05</td>
</tr>
</tbody>
</table>

Secretory canaliculi (neck) - 2300 vs. 1100, p<0.01; 1600 vs. 1800, p<0.05.
Secretory canaliculi (base) - 2300 vs. 1100, p<0.01; 1600 vs. 1800, p<0.01.
Microvesicles (neck) - 2300 vs. 1100, p<0.01.
Microvesicles (base) - 2300 vs. 1100, p<0.01; 1600 vs. 1800, p<0.01.
Rough endoplasmic reticulum (neck) - 2300 vs. 1100, p<0.01.
Rough endoplasmic reticulum (base) - 2300 vs. 1100, p<0.01.
Multivesicular bodies* - 2300 vs. 1800, p<0.01.

*pooled values from neck and base cells
and microvesicular values, respectively, decreased and increased. The reason for the anomalous increase in the microvesicular $S_v$ at 1800 is not clear. It is possible that either microvesicles were being produced at a greater rate at 1800 than at other timepoints, or that they were being released more slowly. The possibility that the conformation of the plasmalemma (therefore secretory canaliculi) and the microvesicles may respond to different stimuli might also be a factor.

The function of RER is protein production. It may possibly be involved in normal parietal cell maintenance; however, its other roles in this cell are not clear. While man, cats, guinea pigs and rabbits appear to produce the peptide gastric intrinsic factor in the parietal cell (Hoedemaeker, 1965), the work of Hoedemaeker et al. (1964) and Boass and Wilson (1963) indicates that in the rat the chief cell is the site of gastric intrinsic factor production. In other organs such as the liver (Muller, 1971) and pineal gland (Welsh et al., 1979) fluctuations in the RER have been associated with changes in cell inclusions and enzyme and carrier-protein levels. Whatever its function in the parietal cell may be, RER had a circadian rhythm in feeding-phase shifted rats. Maximal values occurred at the transition from light to dark and minimal values at the transition from dark to light. Fluctuations were not so pronounced as during ad libitum feeding (Jacobs and Sturtevant, 1980).
Multivesicular bodies are widely distributed throughout the body's tissues including the adrenal medulla, neurons and esophagus. Although they have been considered to be lysosomal in nature (Holzman, 1976), their function in the parietal cell is not understood. Hally, (1959) found an increase in the number of multivesicular bodies in mouse parietal cells when the animals were injected repeatedly with pilocarpine nitrate. He suggested that multivesicular bodies increase during increased secretory activity. Our food-restricted data do not support this theory for the rat. Increases in the number of multivesicular bodies accompanied a decreased food stimulus. Secretory canalicular and microvesicular data suggested that the cells were returning to the resting state.

Fasting experiment

As in the previous experiment, peak values in secretory canalicular Vv appeared concurrently with lowest values in microvesicular Sv. Conversely, lowest canalicular values (1100 and 1600) were accompanied by greatest values in microvesicles. The increase in canalicular Vv at 1800 which was accompanied by a decrease in multivesicular Sv only in cells of glandular necks, may indicate that these cells either respond more quickly or are more sensitive to a secretory stimulus.

The elevated canalicular values at 2300 may have been due to the presence of a small amount of food in the stomach. However, there are
indications that other factors may have been involved. First, there was virtually no food present in stomachs of animals sacrificed at 1800, when a second peak and trough of canaliculi and microvesicles occurred, respectively. Second, since sham feeding is known to cause gastric HCl secretion (Weiner, 1977), the persistence of feeding behavior by the fasted rats during the dark phase may have been a stimulus to the parietal cells. While insulin is also said to be a stimulus to HCl secretion when administered as a pharmacological agent (Brobeck, 1979), it is questionable whether normal endogenous levels of insulin have a direct, if any, effect on gastric acid secretion in the rat. Jolin and Montes (1973) and Bellinger et al. (1975) reported a rhythm of serum insulin in both ad libitum-fed and fasted rats that peaked during the last half of the light phase. This being approximately 12 hrs removed from the morphological evidence of peak parietal cell secretory activity suggests that normal physiological fluctuations in rat serum insulin do not directly effect gastric acid secretion. Additional studies of vagal tone, histamine and gastrin effects on the stomach over the 24-hr period are needed to clarify the apparent persistence of rhythms of gastric acid secretion during fasting.

Fasting appeared to dampen the rhythm of RER S\textsubscript{v} considerably, as compared to the rhythm in ad libitum-fed animals. The minor peak during the early and middle dark phase occurred later than values of food-restricted and ad libitum-fed rats, and this peak was 180 degrees out of phase with the peak in ad libitum-fed rats.
Once again, our results do not support those of Hally (1959) who suggested that increased numbers of multivesicular bodies may be associated with increased gastric acid secretion. In the present study these organelles were sometimes more numerous in fasted than in phase-shifted or ad libitum-fed animals (1100 and 1600). Also, at these timepoints canaliculi and microvesicles in the fasted animals indicated decreased acid secretion, but multivesicular bodies did not decrease. It is suggested that the lag time since the last feeding may influence this organelle.

Differences in parietal cells of the neck and base of gastric glands have been described previously by Lawn (1960), Rosa (1963), Helander, (1976) and Ryabchikova and Vinogradova (1979), although not all investigators have agreed on the nature of these differences. Morphometric analysis of cells from rats subjected to different feeding regimens and fasting have allowed us to discern certain consistent differences in parietal cells at the two glandular sites. Cells of the neck of glands had more dilated secretory canaliculi and fewer microvesicles (indicating a more active secretory state) and more RER (indicating potential for more protein production) than cells of the base of glands. These results suggest that parietal cells of the neck are more active than those in glandular bases. Parietal cells of the neck and base of glands in the resting and active phases are shown in Figures 6 and 7.
Figs. 6a (top) and 6b (bottom). Electron micrographs of parietal cells from glandular necks. (X 16,600).

Fig. 6a. Actively secreting parietal cell with very dilated intracellular secretory canaliculi (SC) into which microvilli project. A few microvesicles (MV) are situated near canaliculi. RER = rough endoplasmic reticulum; M = mitochondrion.

Fig. 6b. Resting parietal cell characterized by collapsed secretory canaliculi and abundant microvesicles distributed throughout the cytoplasm. Two multivesicular bodies (MVB) are seen. The glandular lumen (L) and a mucous cell (MC) are seen.
Figs. 7a (top) and 7b (bottom). Electron micrographs of parietal cells from glandular bases. (X 16,600).

Fig. 7a. Actively secreting parietal cell with dilated secretory canaliculi (SC) which are not as pronounced as in cells of the necks of glands (Fig. 6a). Microvesicles (MV) are few in number and approach the canaliculi. M = mitochondrion.

Fig. 7b. The greatest density of microvesicles is seen in resting parietal cells in the base of glands. Secretory canaliculi are collapsed.
It has been suggested that parietal cells become more quiescent as they age during migration from neck to base (Willems et al., 1972). It is our observation, however, in spite of the presence of a few parietal cells in the lower neck region, which appear to be transitional, that the change from "neck parietal cell" to "base parietal cell" occurs abruptly. Even at the light microscopic level, (Fig. 5) one can see wide, unstained secretory canaliculi in the cells of the neck, while those in the base of glands stain homogeneously. It should also be noted that the orientation of parietal cells with respect to other glandular cells also changes abruptly; that is, in the neck of glands parietal cells alternate with mucous neck cells in bordering the lumen, while parietal cells in glandular bases are almost always peripheral to the chief cells lining the lumen. These observations lead us to suggest that cell-to-cell interactions or possibly simple physical constraints may play a role along with aging in the differentiation of neck parietal cells to base parietal cells.

As previously mentioned, the function of the multivesicular body in the parietal cell is not clear. Friend (1979) found acid phosphatase, a lysosomal enzyme, in the matrix of multivesicular bodies of rat epididymal cells, thus suggesting a lysosomal function to the organelles. He cautioned, however, that this did not necessarily imply that the vesicles were lysosomes. Although we examined parietal cells at their circadian peak and trough phases of organelle density, we saw no evidence of acid phosphatase in multivesicular bodies. We did,
however, note reaction product in some dense bodies in the parietal cell. Thus, the function of this organelle remains obscure.

In conclusion, the results of a circadian, morphometric study of gastric parietal cells of rats maintained under conditions of LD 12hr:12hr and subjected to either a phase-shifted feeding regimen or fasting indicate that (1) measurements of secretory canaliculi, microvesicles, RER and multivesicular bodies fluctuate over the 24-hr period, (2) cells in the neck and base of glands display characteristic and consistent differences in canaliculi, microvesicles and RER but not of multivesicular bodies, (3) secretory canaliculi and microvesicles of cells of the neck of glands may be able to react more strongly to some secretory stimuli than cells of the glandular base and (4) in addition to aging, the orientation of parietal cells with respect to other glandular cells may play a role in producing the differences between cells of the neck and base of glands.
CHAPTER VI

CIRCADIAN STUDY OF GASTRIC MUCUS-PRODUCING CELLS

Abstract

The gastric surface epithelial and mucous neck cells of rats maintained on three different feeding regimens were studied at the light microscopic level over the 24-hr period. After reaction with PAS stain for glycosaminoglycans, surface epithelial cells of the deep isthmus stained more darkly than those lining the gastric lumen, which, in turn, stained more darkly than the mucous neck cells.

The amount and distribution of intracellular mucus and the cell and nuclear areas of the surface epithelial cells were temporally invariant. Staining intensity did vary in a circadian manner. The largest number of cells with dark red-staining mucus was seen in the late dark phase in ad libitum-fed rats and from mid-light until mid-dark in rats fed in the mid-light phase. This state may indicate an increase in the rate of migration of the cells from glandular isthmus to gastric lumen. At other timepoints pink-staining mucus was more obvious. In fasted animals, values fluctuated widely among timepoints. The percentage of surface epithelial cells with visible nucleoli also varied in all three experiments. Peak values always coincided temporally with
indications of increased gastric acid secretion noted in a previous study on parietal cells. This suggests that mucus production may increase when the release of potentially harmful gastric acid increases.

Mucous neck cells were found to contain granules of varying staining intensity at all timepoints. Cell size, however, did vary over time. In all experiments, the largest mucous necks cell profiles were found from mid-dark until the early light phase.
Introduction

It has been established that the cytological structure of a tissue often reflects its physiological state. Among the cell types of the stomach, the parietal cells manifest striking morphological differences between the quiescent and secretory states. These changes in organelle populations and cellular characteristics vary over the 24-hr period and suggest a circadian rhythm in HCl production. In the present experiment, mucous neck and surface epithelial cells were studied over a 24-hour span to determine if they display morphological fluctuations.

Both cell types contain mucus, which is presumed to protect the stomach against mechanical erosion by lubricating food in its passage through the alimentary tract (Davenport, 1972). The mucus may also protect the stomach from the lytic properties of other gastric products such as pepsin and acid (Williams and Turnberg, 1980; Allen and Garner, 1980).

Surprisingly little is known about the mucous neck and surface epithelial cells' respective functions, secretory mechanisms or their relationship to each other. Physiological studies of mucus production in the stomach are hampered by the presence of cardiac and antral mucous glands; it is presently not possible to distinguish their secretions from those of the mucous neck and surface epithelial cells. Histological studies that indicate variations in physiological state of cell types are a possible solution to this problem.
The purpose of this experiment was to study the histological changes in mucous neck and surface epithelial cells over the 24-hr period. The effects of modified feeding regimens were also studied in an effort to compare the function of these cell types and to determine their relationship to each other. Finally, it is envisioned that a comparison of these results with those of the earlier studies on gastric parietal cells may provide additional information on how the stomach protects itself from its own secretions.
Materials and methods

The tissue for these experiments was harvested during the previously described experiments on parietal cells ("Circadian Morphometric Variation in Gastric Parietal Cell Populations of the Rat"; "Circadian Ultrastructural Changes in Rat Gastric Parietal Cells under Altered Feeding Regimens: A Morphometric Study"). Adult male outbred albino rats from Charles River Breeding Laboratories were housed in cages with wire mesh floors in environmental chambers in which the lights were on from 0500 to 1700 and off from 1700 to 0500. They had been habituated to one of three feeding regimens: ad libitum-feeding, fasting (after four consecutive feed:starve habituation phases) or phase-shifted (restricted) feeding from 0900 to 1300. Animals were sacrificed in groups of five at 2300, 0400, 0600, 1100, 1600 and 1800. The stomachs were excised and processed as previously described.

Semithin sections (1 micron) were cut on a Reichert microtome (Model Om U2). Five sections each through the entire stomach wall were floated onto a drop of water on a microscope slide, and the slides dried on a hot plate (temperature 115 degrees C). It was found that leaving the slides on the hot plate for one to two minutes after they were dry prevented their loss during subsequent manipulation. Ten slides were prepared per animal. After drying, the slides were treated either with toluidine blue or Periodic Acid Schiff reagent (PAS) (details are in Appendix IV.)
Tissue from all three feeding regimen experiments was studied at the light microscopic level for 24-hr changes in mucous neck and surface epithelial cells. A blind study evaluating a variety of traits was conducted as discussed below. When applicable, temporal differences were examined by morphometric techniques. Unless otherwise specified, a Zeiss Videoplan image analyser was employed.

General observations

The relative PAS-staining intensities of gastric luminal surface epithelial, isthmus surface epithelial and mucous neck cells were noted at all timepoints in all experiments. The presence or absence of a mucous layer lining the gastric lumen was also noted at all timepoints in all experiments.

Surface epithelial cells

PAS-stained surface epithelial cells subjected to a blind study were evaluated for the following characteristics:

Staining intensity: mucus was judged to be either predominantly dark red or red and pink.

Amount of mucus in cells: for each slide, a "representative" cell was designated and the depth of its mucous packet was measured with the aid of an eyepiece micrometer. The cell selected was the largest cell that was sectioned through the nucleus.
Uniformity of mucous content: concurrent with the choice of this cell, the surface epithelial cell population was evaluated as being either uniform or non-uniform in mucous content.

Cell and nuclear area: a morphometric study was made on the cell and nuclear areas of toluidine blue-stained surface epithelial cells lining the gastric lumen. Two timepoints at which maximal differences in PAS staining intensity were detected in the blind study were examined: 0400 and 1100 in the phase-shifted feeding experiment. Data from 100 cells per animal were recorded.

Percentage of cells with visible nucleoli: the percentage of cells with visible nucleoli per animal at each timepoint was evaluated. For each animal, one hundred consecutive toluidine blue-stained nucleoli from cells lining the gastric lumen were counted, and the number containing nucleoli was noted. When more than one tissue section was required to obtain sufficient data, non-consecutive sections were used.

Mucous neck cells

PAS-stained mucous neck cells subjected to a blind study were evaluated for the following characteristics:

Shape of cell: the population was judged to be either predominantly cuboidal, narrow or mixed.
Homogeneity of staining intensity: cells were judged as having either predominantly homogeneously or non-homogeneously staining cytoplasm. They were distinguished from "intermediate" cells by their lack of a distinct packet of dark red mucus in the apical region.

Cell and nuclear areas: a morphometric study was conducted to determine the cell and nuclear areas at three timepoints in the ad libitum and phase-shifted feeding animals. The timepoints were 2300, 0400 and 0600 in the former and 2300, 0400 and 1100 in the latter. The results of this morphometric study were compared with those of the blind study at the specified timepoints to determine if the observed trends in cytoplasmic area were confirmed and to determine whether the nuclear area fluctuated with cellular activity.
Results

General observations

Karnovsky's fixative and osmium tetroxide provided excellent fixation of the mucus-producing cells as had been the case for the parietal cells. Each tissue section included approximately ten glands. PAS stained basal laminae and nuclear membranes of all cells. The cytoplasms of chief, parietal, endocrine and connective tissue cells remained for the most part unstained. Mucus producing cells, however, contained pink to dark red-staining mucus. In all sections observed, the darkest staining mucus was found consistently in surface epithelial cells of the isthmus of glands. Surface epithelial cells lining the gastric lumen contained mucus either as dark as that in the isthmus or lighter in color. Mucous neck cells were consistently a lighter pink than the mucus in surface epithelial cells (Fig. 1). Cells were observed that appeared by their location and staining intensity to be intermediate between the two cell types (Fig. 1). Toluidine blue stained all cells varying shades of blue, purple and green. Surface epithelial cells had a homogeneous pale blue cytoplasm. The apical mucous granules stained dark blue. Mucous neck cells were distinguished by location, a lighter-staining and more abundant cytoplasm than "intermediate" cells and by having purple cytoplasmic granules. (Chief cell granules were unstained).
Fig. 1. One micron section of epon-embedded gastric glands stained with PAS. Mucus in surface epithelial cells in glandular isthmuses (IS) stains more darkly than in cells of the surface (SS). Mucus neck cells more lightly stained than surface epithelial cells of either location. Intermediate cells (I) are located between mucous neck and surface epithelial cells. (X 830)
No consistent pattern was seen as to the presence or absence of mucus lining the stomach wall. This may have been due in part to mechanical stresses during tissue processing. It was noted that mucus on the surface was the same color as that in the underlying cells.

Surface epithelial cells

Staining intensity: in *ad libitum*-fed animals, indications of the most intensive PAS staining were in the late dark phase. The most lightly stained sections were observed in animals sacrificed at the end of the light phase. Transitional stages were seen at the other timepoints. The results from the fasting experiment showed no discernible trend of change in the variable. Results fluctuated strongly among timepoints. In the phase shifted experiment, most tissue harvested from mid-light until late dark was judged to be darkly staining. A majority of lighter staining cells was found in tissue harvested in the early light (Fig. 2). Examples of the two extremes in staining intensity are seen in Fig. 3.

Amount of mucus in cells: cells in the gastric isthmus contained a packet of mucus that was about 0.5 to one micron thick. This increased to roughly three micra in the upper foveola and decreased to about two micra on the stomach surface. No significant differences were detected among timepoints or experiments by this method.

Uniformity of mucous content: no trends of change were found in this variable among timepoints or experiments.
Fig. 2. Sections evaluated as having red-staining mucus (stippled bar) in surface epithelial cells or red mucus with a pink component (white bar). Asterisks indicate when pink and red staining slides are two significantly different populations by a chi-square test. Ordinate indicates percent of slides examined. Abscissa indicates hour of day and white and black bars, the light and dark phases of the illumination regimen.
Figs. 3a (top) and 3b (bottom). One micron sections of PAS-stained, epon-embedded tissue. (X 680)

Fig. 3a. Surface epithelial cells in which the mucus stains predominantly dark red.

Fig. 3b. Surface epithelial cells in which the mucus contains a pink-staining component.
Cell and nuclear area: a pilot morphometric study of the cells harvested at 0400 and 1100, timepoints at which maximal and minimal staining intensities were detected in phase-shifted feeding animals, showed no significant changes in cell or nuclear areas.

Percentage of cells with visible nucleoli: the number of visible nucleoli changed significantly in all three experiments when a Student's t-test was applied. Maximal values were seen at 0400 and 1600 in the ad libitum-feeding experiment. A peak was also seen at 0400 in the fasting experiment. Data from phase-shifted fed animals showed elevated values from 1100 until 2300 and lower counts at 0400 and 0600 (Fig. 4).

Mucous neck cells

Cell shape: in ad libitum-fed animals, the largest number of cuboidal cells were noted in the mid-dark and early light phases. In the fasted animals, the peak number of slides evaluated as having cuboidal mucous neck cells was found in the mid-dark phase. In phase-shifted feeding animals, cuboidal cells appeared to be most numerous in the late dark and early light phases. Cells from mid-light until early dark were notably narrow (Fig. 5). Examples of the two extremes in cell shape are seen in Fig. 6.

Homogeneity of staining: aside from the "intermediate" cells, mucous neck cells in all slides showed cytoplasmic areas of variable staining intensity.
Fig. 4. Surface epithelial cells with visible nucleoli (____) and parietal cell secretory canaliculi (- - -). Asterisks mark significant increases in the number of cells with nucleoli (Student's t-test; p < 0.05). Ordinate on left indicates per cent of surface epithelial cells with visible nucleoli; ordinate on right indicates canalicular volume density. Abscissa indicates hour of day and white and black bars, the light and dark phases of the illumination regimen.
Fig. 5. Mucous neck cell shape. Sections evaluated as having cuboidal cells are marked by a stippled bar, narrow cells by a black bar and both types a white bar. Ordinate indicates per cent of cells; abscissa indicates the hour of day and white and black bars, the light and dark hours of the illumination regimen.
Figs. 6a (top) and 6b (bottom). One micron sections of epon-embedded, PAS-stained tissue. (X 680)

Fig. 6a. Mucous neck cells (MNC) which are predominantly cuboidal in shape.

Fig. 6b. Mucous neck cells which are predominantly narrow in shape.
Cell and nuclear areas: a morphometric study of three timepoints each in two experiments confirmed the observations of the blind study on mucous neck cell shape. No significant changes in the nuclear size were seen at these timepoints.
Discussion

A series of histological studies was made on gastric mucus-producing cells to determine if there were morphological manifestations of activity change over the 24-hr period and if the feeding regimen affected the manifestations. These studies were performed at the light microscopic level to allow visualization of larger populations more easily than would be seen at the electron microscopic level. It also allowed the utilization of PAS staining to yield information on the comparative histochemistry of the cells' mucus.

General observations

Because of the apparent common origin of surface epithelial, mucous neck and possibly chief cells (Wattel et al., 1977), there was occasionally difficulty in classifying certain cells. Surface epithelial cells were distinguished by their location (upper glandular isthmus and gastric lumen) and by their unstained cytoplasm with apical dark granules. "Intermediate" cells were distinguished from the former by staining light pink with an apical dark red area with PAS reagent and dark blue with toluidine blue. Cells were classified as mucous neck cells if they were found in the area of the glandular neck and if the entire PAS-stained cytoplasm was light pink. Toluidine blue-stained mucous neck cells were lighter in color than the intermediate cells. The most difficult cells to distinguish were the deepest mucous neck and the most superficial chief cells, which were morphologically similar.
The observations made during these experiments would appear to support the hypothesis that chief cells differentiate from mucous neck cells. In a variety of autoradiographic studies, chief cells were only rarely seen to divide (Chen and Withers, 1975). There is, however, a turnover in these cells, albeit more slowly than those of parietal or mucous cells. It is postulated that the cells arise from the neck of glands (Lawson, 1970) where most mitoses and DNA synthesis are observed to occur (Pyatnitskii et al., 1980). One of the more likely sources of these cells is differentiation from mucous neck cells. The areas these two cell types occupy are topographically contiguous, and the cells are often very difficult to distinguish at the deeper boundary of the neck. In this area, cells exist that with both PAS and toluidine blue staining contain granules that are lighter than the mucus in mucous neck cells yet are darker than the unstained zymogen granules of chief cells. Also, deeper mucous neck cells appear to have more cytoplasm and more oval nuclei than those closer to the stomach lumen, making the deep cells resemble chief cells (Fig. 7).

It has been known for some time that the mucus in surface epithelial and mucous neck cells stains differently. In PAS staining, a darker red-staining mucus indicates a high sulfate content, while a pink-staining mucus may indicate a higher sialic acid content (Gerard et al., 1967). The present results confirm previous observations that surface epithelial cells of the glandular isthmus stain more intensely than those on the gastric surface; and these, in turn, stain more darkly.
Fig. 7. One micron toluidine blue-stained, epon-embedded section through stomach wall. C = chief cell; MNC = mucous neck cell; UC = unidentified cell with characteristics of both C and MNC. (X 830)
than the mucous neck cells. (Wattel and Geuze, 1977). In addition, the present study yielded the information that this relationship holds throughout the 24-hr cycle in *ad libitum*-feeding as well as in fasting and phase-shifted feeding animals.
Surface epithelial cells

The results of the three experiments indicate that at least two surface epithelial cellular characteristics vary over the 24-hr period. The first of these to be studied was the staining intensity of the interfoveolar cells with PAS (for glycosaminoglycans). While cells of the isthmuses consistently stained dark red, the cells lining the gastric lumen fluctuated between dark red and a state where lighter pink mucus mingled with the darker granules.

The significance of these data lies in the chemical composition of the mucus. Mucin found free in the stomach has three components: sialic acid and two sulfated substances. One is a sulfated glycoprotein and the other, chondroitin sulfate B, which is said to derive from connective tissue (Horowitz, 1963). Some of the stomach mucus comes from glands in the cardia and pylorus, while the rest is secreted from mucous neck and surface epithelial cells of the corpus.

A series of histochemical investigations has indicated that sulfated glycoprotein is the source of the darkly staining mucus in surface epithelial cells of the deep isthmus while the lighter staining granules in luminal cells represent sialic acid (Gerard et al., 1968; Wattel et al., 1977; Sato and Spicer, 1980). This gradual change in cellular mucous content has been explained in three general hypotheses. Zalewsky and Moody (1979) suggested that during migration, as the cells approach the gastric lumen, sulfate may diffuse away into the cytoplasm,
leaving only sialic acid granules. Gerard et al. (1968) and Zalewsky and Moody (1979) also suggested that there may be a "precocious discharge" or exocytosis of sulfated granules during migration leaving (or "unmasking") sialic acid granules in older cells. Finally, the work of Wattel et al. (1979) strongly suggested that surface epithelial cells of the isthmus and stomach surface actually produce different mucosubstances. Their autoradiographic study showed that the relative uptake of certain key substances changes as the cells migrate luminally. For example, isthmus cells incorporate relatively more amino acids, possibly for cell maturation, and more sulfate, which gives the mucus its dark red appearance with PAS reagent. On the other hand, cells closer to the stomach lumen take up greater amounts of sugar, presumably for production of the glycoprotein sialic acid which stains light pink.

It is possible that this indicates a difference in function of the cells. Sato and Spicer (1980) proposed that the sulfated mucus of younger cells reflects the process of cell differentiation and maturation. Dietrich et al. (1976) had shown that each tissue produces a distinct sulfated glycosaminoglycans, and the abundance of the sulfate in young surface epithelial cells led Sato and Spicer to suggest that the mucus may be used specifically in cell differentiation. An alternative (or parallel) hypothesis proposed by the same authors is that exocytosed sulfated mucus may be reincorporated by the cells to form the glycocalyx seen on their apical plasmalemma. This glycocalyx may be protection against the steady flow of acid from parietal cells.
Because of its different staining (thus chemical) properties, the glycoprotein sialic acid produced by the surface epithelial cells of upper pits and the gastric lumen may be presumed to serve another purpose.

Heatley (1959), Davenport (1972), Allen and Garner (1980) and Williams and Turnberg (1980) suggested two functions of the mucus that lines the walls of the stomach. First, by lubricating the walls, it protects the epithelial cells from abrasion of food passing through the alimentary tract. Second, it may protect the stomach from autodigestion in two ways. First, it appears that the viscous nature of the mucus is resistant to the proteolytic enzyme pepsin. The glycosaminoglycans molecules may also be chemically bonded in such a way as to form a lattice with a meshwork too small for pepsin to diffuse through to the cells (Edwards, 1978). In addition, a model has been proposed by Heatley (1959) to explain protection of the cells from acid in the gastric lumen. Presumably, the surface epithelial cells, besides secreting mucus also produce bicarbonate. This, flowing luminally through the latticework of mucus, meets hydrogen diffusing from the stomach lumen. The resulting reaction forms water, which is unharmful to the cells. Since the work of Gerard et al., (1967) suggests that the pink-staining mucus of interfoveolar surface epithelial cells contains sialic acid, and since the current study has shown that the mucus lining the stomach walls stains the same color as mucus in these cells, it seems quite possible that this mucus functions to protect the stomach walls from mechanical abrasion and autodigestion.
It has been shown repeatedly (Stevens and Leblond, 1953; Bertalanffy, 1960; Lipkin et al., 1963; Wattel et al., 1977) that surface epithelial cells migrate from isthmus to stomach surface from which they are presumably extruded (Harding and Morris, 1977; Zalewsky and Moody, 1979). This may, in effect, be another line of defense against damage from the digestive process. As rapid renewal of surface cells is a normal process, total replacement occurring approximately every three days (Stevens and Leblond, 1953), any damage can be quickly repaired. The results of the present studies indicate that the mucus in cells lining the gastric walls varies in staining intensity over the 24-hr period. If cells in the isthmus do indeed produce a different glycosaminoglycans than cells on the surface, then red-staining mucus should indicate a younger cell. Given the constant, or frequent migration of the cells, the periodic appearance of red mucus in cells of the gastric surface may indicate that during that phase, migration is more rapid than when pink-staining mucus is seen.

During the ad libitum-fed experiment, the greatest number of cells seen with red-staining mucus was at 0600 which is also the end of the major feeding phase of the rat (Armstrong et al., 1978; Peraino et al., 1980), and when the stomachs were seen to be distended with food. The question that presents itself, then, is if there is increased migration and presumably more cell division during this phase, does this reflect an inherent mitotic rhythm or does the presence of food cause mitosis and migration? It is of note that although a small amount of food was
apparently eaten at 1600, this did not increase red-staining cells on
the surface. It is possible that not enough food was ingested to
stimulate migration or that the latter only occurs at a "preset" time,
\textit{e.g.} late in the dark phase. Unfortunately, there are no studies
available on gastric mitotic rhythms in the rat. Scheving \textit{et al}. (1979)
did, however, observe a DNA synthesis rhythm in the mouse, another
nocturnal rodent. Peak values were found in the middle to late dark
phase.

Results from the fasting experiment were unclear and without
trends of change. Instead, values fluctuated widely from one sample
timepoint to the next and from one animal to the next. It is possible
that 40 hrs and more of food deprivation perturbed the internal milieu
sufficiently to desynchronize the rhythm.

The results were most striking in animals forced to feed only
during the mid-light phase. During the course of the experiment, it had
been noted that the rats' stomachs were very distended with food from
1100 until 2300, while at 0400 and 0600 they were less full.
Interestingly, the PAS study on surface epithelial cells of the gastric
lining demonstrated red-staining mucus from 1100 until 2300 and
significantly more pink cells at 0400 and 0600. Thus, the presence of
food in the stomach may be one factor controlling surface epithelial
cell migration.
The second morphological characteristic that fluctuated in surface epithelial cells in these experiments was the number of cells displaying nucleoli. At different timepoints the number of cells with visible nucleoli ranged from approximately 20 to 40%. There are two possible reasons for this. Either the number of cells with nucleoli changed or the size of nucleoli fluctuated, so that when they were smaller, they were less likely to be encountered upon sectioning, or were less visible at the light microscopic level. The latter seems more likely, as nucleoli are responsible for RNA synthesis which is necessary in normal cell maintenance and function. Also, Adamstone and Taylor (1972) showed that jejunal epithelial cells which are analogous to gastric surface epithelial cells do all contain nucleoli.

In general, two factors control the size of nucleoli: age and cellular activity. In Adamstone and Taylor's (1972) study of jejunal epithelial cells, it was found that like their gastric analogues, they arise from undifferentiated cells in isthmuses and migrate towards the lumen of the alimentary canal. In their younger stages their nucleoli are large; while in the terminal stages of life this organelle disperses, and the remaining components become very small and compact. If the same changes occur in gastric surface epithelial cells, then the presence of very large, visible nucleoli in cells lining the gastric lumen may indicate that migration has just occurred or is occurring; and younger cells are appearing on the surface. In this case, if the results of the previously discussed PAS-staining experiment also
indicate the relative age of cells on the gastric surface, then every peak in nucleolar count should be accompanied by increased numbers of cells with red-staining mucus. For the phase-shifted experiment this proved to be the case. Nucleolar values and those of cells with red-staining mucus were elevated from 1100 through 2300 and decreased at 0400 and 0600. The other two experiments did not support the hypothesis. While elevated values did occur in both variables at 0400 in the ad libitum-fed animals, the increased nucleolar count at 1600 was accompanied by peak values in pink-staining mucus. Also, in the fasting experiment, there were no consistent trends in the staining changes. Values fluctuated widely between consecutive sampling times. Nucleolar data, however, showed one peak at 0400 and lower values at other sample timepoints.

A second possibility proposed to explain increased nucleolar values was change in activity of the cells. Busch (1974) stated that increased cellular activity is reflected by an increase in nucleolar size. In order to compare this theory to the nucleolar results of the present study, it is necessary to review what is known about stimuli to surface epithelial cells' mucus production.

Unfortunately, the literature on this subject is sparse and sometimes contradictory. Perhaps one of the best candidates for gastric mucigogue is topical acid application. Although it has not been demonstrated in the surface epithelial cells per se, acid does cause an
increase in mucus output, especially from pyloric glands (Ivey and Oyama, 1921; Menguy, 1969). The effect of food on surface epithelial cells is not clear. While Gerard et al. (1968) reported that feeding caused a discharge of mucus from mucous neck and surface epithelial cells, Menguy (1969) reported no change in the cells after feeding. The effect of vagus nerve activity has also been studied. Jennings and Florey (1940) showed that increased vagal stimulation gave histological evidence of mucus discharge from cardiac and pyloric glands and mucous neck cells, although not surface epithelial cells. This is not, of course, conclusive, as the histological manifestations of mucus secretion may have been too short-lived to be seen with the sampling procedure used. Pearl et al. (1965) also reported increased mucus collected from Pavlov pouches after vagal stimulation.

A problem with these studies, as Menguy (1969) has stated, is that neither feeding nor vagal stimulation experiments were able to control for the effects of gastric acid secretion. Food and increased vagal tone are both known to stimulate acid secretion. Since the acid is produced by parietal cells which surround mucus-producing cells, perhaps the mere presence of acid is enough to stimulate mucus secretion.

The results of the parietal cell experiments described earlier (in this dissertation) afford one way of testing this hypothesis. It has been established in a number of studies that the histological appearance of parietal cells reflects their physiological state. It was shown that
known stimuli of gastric acid secretion such as the vagus and its pharmacological analogues, food and humoral stimuli all cause two changes in the parietal cells: the number of microvesicles decreases, and a configurational variation of the secretory canaliculi occurs. The latter has been described as a proliferation and branching or as a dilation of the canaliculi. The present study has shown, by morphometric analysis, that during a food stimulus the decrease in microvesicles was accompanied by an enlargement or dilation of the secretory canaliculi. The latter was measured as a volume density which reflects the percentage of cell volume occupied by the canaliculi.

This, then, offers the opportunity to compare gastric acid output with nucleolar counts. Fig. 4 compares the results of the two cells' characteristics. If one considers the hypothesis that an elevated surface epithelial cell nucleolar count reflects increased cellular activity and thus mucus production, and that elevated parietal cell canalicular values indicate increased acid secretion, then a peak in the values of one should be accompanied by a peak in the values of the other. The results of the two cell types have been plotted together. For the sake of visual clarity, only the volume density of parietal cell canaliculi in glandular necks is shown. (Although parietal cells of the necks and bases of glands displayed the same trends of variation, values of cells in the neck varied more distinctly and the cells were also topographically closer to the surface epithelium, possibly contributing a greater effect.)
The results of the comparison are notable in all three experiments. Considering first the *ad libitum*-feeding experiment, elevated values of both variables were seen at 0400 and 1600. Lowest values were noted at 1100 and 1800. There was a discrepancy between values at 2300, however, for which no explanation is offered at present. In the fasting experiment once again peak values were seen in both variables at 0400. A second increase in parietal cell canalicular volume density was accompanied by a slight, but non-significant increase in nucleolar number at 1800. Once again, as in the *ad libitum*-fed experiment, there was a discrepancy in values of the two variables, but only at 2300. In the phase-shifted feeding experiment, elevated values in both are seen from 1100 through 2300, and decreased values at 0400 and 0600.

While these results are inconclusive, they may suggest that either acid is a stimulus to surface epithelial cell activity or that parietal cells and surface epithelial cells respond to similar stimuli.

The results of the other studies of surface epithelial cells are inconclusive. There appeared to be no significant variation in the amount of mucus contained in cells among timepoints or experiments. There were also no indications that cell or nuclear volume varied between timepoints of peak and least activity and/or migration. Either these variables did not in fact change, or the changes were not of sufficient magnitude to be detected by the methods used.
Mucous neck cells

Probably less is known about the mucous neck cells than any other cell type of the adult gastric mucosa. They appear to derive from either undifferentiated cells or mitoses of other mucous neck cells (Bertalanffy, 1960; Wattel et al., 1977). As to their fate, hypotheses abound; but at the present time none has been proved. There are two groups of hypotheses concerning the function of the cells. One concerns protection of glandular cells from autodigestion of the stomach; the other concerns these cells as precursors of other cell types.

If one postulates that the cells participate in the protection of the stomach from autodigestion, then a biochemical analysis of their mucus would be meaningful. This has not been reported. While pink staining of mucus is said to be due to sialic acid in surface epithelial cells, Gerard et al. (1967) failed to mention whether the mucus of mucous neck cells is also susceptible to sialidase. As to the acidity, Sato and Spicer (1980) demonstrated an acidic mucousubstance with the dialysed iron method and Wattel et al. (1977), a neutral mucus with the alcian blue method. In the present experiment, it was found that while the mucus of neck cells always stained light pink, there were patches of cytoplasm that stained more darkly than others, perhaps reflecting a different pH. The only factor that most investigators appear to agree on is that mucous neck cells do not contain sulfated mucus (Gerard et al., 1967; Wattel et al., 1977; Sato and Spicer, 1980). In conclusion,
the role of these cells in producing protective mucus, if any, is still unknown.

In addition, little is known about stimuli to these cells. Jennings and Florey (1940) reported that vagal stimulation caused a slight discharge of mucus from the cells. Gerard et al. (1968) reported a discharge and increased synthesis after feeding and urecholine (a vagomimetic) administration. The observation that they do apparently respond to a stimulus by secretion may indicate that they help to protect the stomach lining.

If mucous neck cells represent a temporary phase in the development of another adult gastric cell type, we are no closer to understanding their fate than the function of their mucus. They have been named as the progenitor to every glandular cell type but the endocrine cells. Since they contain neutral glycoproteins as do some parietal cells, they may develop into this cell (Gerard et al., 1967). Wattel et al. (1979) suggested that since they incorporate only very small amounts of radioactively labeled precursors of mucus, and since they are morphometrically similar to chief cells in the lower neck, that they may, in fact, be young chief cells. These authors also considered the possibility that mucous neck cells may differentiate into "intermediate" cells and then into surface epithelial cells. The fact that degenerating mucous neck cells have never been reported under normal conditions may indicate that the cells truly are young transitional cells.
One new observation contributed by the present experiment is that mucus cells may fluctuate in size over the 24-hr period. Peaks in cellular area were observed at the mid-dark and early light timepoints in ad libitum-fed animals. One peak at the mid-dark sampling point was seen in fasted animals. Greatest values in the phase-shifted feeding animals were observed from the late dark to early light phase. A morphometric study served to confirm these trends. Thus, in all three experiments peak values in cell size occurred from the mid-dark to early light phase. Whether increased cell size indicates more mucus production, less secretion or a combination thereof remains unknown. A morphometric study of nuclei at various phases showed no discernible trends in nuclear volume. Unfortunately, the nuclei stained too darkly to distinguish nucleoli, making it difficult to perform a nucleolar count as had been done for the surface epithelial cells.

It does not appear that these cells respond to the presence of food, as peak values occurred at the end of the feeding phase in ad libitum-fed rats but before feeding in the phase-shifted feeding rats. While peak values in the former experiment occurred during histological indications of peak acid secretion, the opposite is true in the latter experiment (see data on parietal cell studies).

If the cells do participate in protection of the stomach lining, they do not appear to respond to the same stimuli as surface epithelial cells but are controlled by a factor independent of food intake.
Without further information on hormonal or neuronal actions on these cells, further speculation must wait. If the cells, on the other hand, are transitional stages in the development of parietal, chief or surface epithelial cells, then more information on circadian changes in the maturation of these cells is needed. Until that time, the function of the mucous neck cells remains obscure.
CHAPTER VII

DISCUSSION

In the preceding sections, the experiments on parietal and mucus-producing cells have been discussed separately. The purpose of this section is to integrate the knowledge and ideas acquired from these studies and apply them to some more general topics. These include the demonstration of rhythmic fluctuations in cell morphology, the relationship between structure and function in histological investigations, the effects of feeding schedule on the cellular morphology and physiological function of the stomach, the protective mechanisms of the stomach, the way gastric cell types vary at different glandular levels and the relationship between surface epithelial cells and mucous neck cells.

Are there demonstrable rhythms in cellular morphology?

There is evidence that many of the body's tissues display rhythmic fluctuations in cellular morphology. These variations may take the form of changes in cellular components of a tissue. For example, the number of mast cells seen in connective tissue is known to vary with a 24-hr period length (Polat, 1980). Changes also can occur within individual cells. In the neuroendocrine system, organelles and inclusions of
pinealocytes fluctuate in number and arrangement during the circadian period. These organelles include smooth and rough endoplasmic reticulum, ribosomes, secretory vesicles, microtubules and mitochondria (Welsh et al., 1979). A functionally related neural area, the superior cervical ganglion, also displays a morphological oscillation in nucleolar number (Pebusque et al., 1981).

Many areas of the gastrointestinal tract also display rhythms in cellular morphology. Scheving et al. (1978) demonstrated a rhythm in DNA synthesis by autoradiographic techniques. Regular fluctuations in stainable enzymes and organelles of hepatocytes have been reported (v. Mayersbach, 1965, Uchiyama, 1980). The amount of rough endoplasmic reticulum in salivary glands varies regularly (Albegger and Muller, 1973). The results of the present studies also demonstrate that regular fluctuations occur in the morphology of cells.

In the present studies, three cell types were examined over the 24-hr span in rats that were maintained on ad libitum or phase-shifted feeding regimens or were subjected to a fast. The stomachs of five animals were examined per timepoint. When the data were pooled, it became obvious that certain cellular characteristics fluctuated in each of the cell types, and that the changes were related to the time of day (the animals were subjected to identical light-dark cycles).

In the acid-producing parietal cells, four factors varied with time of day. Secretory canaliculi occupied as much as 20% of the
non-nuclear area at some timepoints and only about 5% at others. The amount of rough endoplasmic reticulum and microvesicles fluctuated in the cells over the 24-hr span, as did the number of multivesicular bodies.

Mucus-producing cells also manifested changes over the circadian period. Surface epithelial cells lining the gastric lumen varied in staining intensity and in the number of visible nucleoli. Mucous neck cells were distended with mucus at some timepoints and very narrow at others.

Thus, the results of these experiments show that there are demonstrable rhythms in the morphology and staining characteristics of three gastric cell types. These rhythms are present not only in animals feeding at will but also in rats under food-restricted conditions.

Can the cellular morphology of a tissue indicate its physiological state or function?

The concept that the physiological state of a cell can be reflected by its morphology is not new. A well-known example is Davies' (1963) demonstration that the contractile state of striated muscle can be determined ultrastructurally by its myofibrillar arrangement.

The gastric parietal cell has been studied extensively for morphological changes that occur in different physiological states. It is generally agreed that the resting, inhibited and hibernating states
are quite different from the food, drug, hormonally and neurally activated states (Rosa, 1963; Ito and Schofield, 1974; Ryabchikova and Vinogradova, 1979; Helander, 1964; Frexinos, 1974). It is also possible that the number of multivesicular bodies varies with physiological state although the function or origin of the organelle is not known (Hally, 1959; Rosa, 1963).

If, as the literature suggests, the actively secreting parietal cell has fewer microvesicles but more secretory canalicular area than the non-secreting cell, which has a replenished supply of microvesicles and collapsed canaliculi, then both states are represented in all three current experiments. The only instance when this reciprocal relationship did not persist was in the early dark phase of the phase-shifted feeding experiment. At this timepoint the canaliculi were quite distended, but there was an anomalous increase in microvesicles. Although the secretory canaliculi are generally expanded in the secretory state, (Helander, 1964), the increased number of microvesicles would seem to indicate either that the cells have ceased to secrete acid or that they are producing the stored form of acid more rapidly than they are releasing it.

Results of previous studies of the multivesicular body would seem to indicate that its number increases in actively secreting cells (Hally, 1959; Rosa, 1963). Using canaliculi and microvesicles as an indicator of acid secretion, however, the results of the current
experiments indicate that multivesicular bodies increase at the end of the quiescent phase or the very beginning of a new secretory phase. Their function remains obscure, although it does appear to be related in some way to food intake or the lack of it.

The results of the three experiments indicate that the feeding regimen does not influence the rhythmic variation in rough endoplasmic reticulum. Only fasting produced an effect on this organelle. In the absence of food the organelle varied little; its surface density remained low. Thus, the presence of food in the stomach may be necessary for an increased production of this organelle.

The functional significance of the change in the PAS-staining characteristics of the surface epithelial cells lining the stomach lumen is moot without further studies of the production, secretion and biochemical nature of their mucus and the manner of migration of the cells. There are, however, at least two possible reasons for the fact that sometimes cells lining the gastric lumen have pink and red-staining granules in the cytoplasm (after PAS-staining) while at other timepoints the mucous granules all stain dark red. The first possibility is that the cells produce mucus of a different composition at various timepoints. Wattel and Geuze's (1977) definition of red mucus as containing sulfate, and pink mucus as being sialic acid (sialidase susceptible) would indicate that at certain times the cells are producing sialic acid and at other times highly sulfated mucus. The
second possibility takes into account the often-made observation that the overall tendency of surface epithelial cells' mucus is to stain less intensely as the cells migrate towards the gastric surface (Wattel and Geuze, 1977; Gerard, et al., 1967; Zalewsky and Moody, 1979). If it were always the case that cells with red mucus were younger than cells with a pink mucus component, then the presence of cells on the gastric surface containing only red mucus could mean only one thing: that a relatively rapid migration had occurred. Since migration must certainly be preceeded by a mitotic division in progenitor cells to create new cells in the glands, the demonstration of Scheving et al., (1979) of periodic fluctuations in gastric cells' DNA synthesis may lend support to this hypothesis.

If the first explanation is correct, it is possible that the stomach requires mucus of different chemical composition for protection under various conditions. If the second hypothesis is correct, it would suggest that there is a periodic quickening in the isthmus-to-surface migration of surface epithelial cells. This might be due to an inherent rhythm or to a response to stress or stimulation, such as the passage of food through the stomach.

The surface epithelial cells also varied with respect to the number of cells displaying visible nucleoli. When 100 cells of the gastric lining were counted per animal, the number of nuclei displaying visible nucleoli varied among timepoints. Two explanations may account
for this phenomenon. First, the work of Adamstone and Taylor (1972) has shown that in comparable cells of the jejunal epithelium, which migrate from isthmus to villar tip, nucleoli undergo predictable changes. Early in the cell's life, the organelle is large. By the time the cell reaches the end of its migration (and life), the nucleolus is small, its parts fragmented and condensed. If the same is true of gastric surface epithelial cells, then the presence of fewer nucleoli at a given timepoint may indicate that the cells lining the stomach wall are relatively older, with small nucleoli either not visible or having less chance of being cut per section. A higher nucleolar count, then, would indicate that the nucleoli are larger in cells on the surface. The cells should be younger, indicating a recent migration. If this be the case, then a higher nucleolar count might be expected to correlate with a high number of cells containing only dark red mucus. There is no consistent correlation between these two variables in the three experiments, though.

A second possibility is indicated by the relationship between nucleoli and the activity of a cell. Nucleoli are reported to become larger when a cell is more active; i.e. producing more protein (Busch, 1974). In this case, a lower percentage of cells with visible nucleoli would indicate that the cells are quiescent and a higher count, that the cells are active. It is also of note that in the current experiments in every case, an increase in surface epithelial cell nucleolar count was accompanied by an increase in parietal cell secretory canalicular volume density, an indication of acid secretion.
While the mucous neck cells fluctuated in size at various timepoints, there is at present no additional information to allow an interpretation of the phenomenon. Larger cells may indicate an increase in mucus production or less secretion of the substance.

In conclusion, fluctuations in structure of two of the three studied cell types have given indications of probable changes in physiological state. The value of these studies is that they allow further understanding of cellular functions and the relationships between cell types. The studies also stress the observation that aside from the structural changes that occur during growth and aging and in pathological states, there are normal daily changes in healthy cells that reflect changes in the physiological state.

The effects of feeding regimens on structure and function.

The rat is a nocturnal animal; it eats primarily during the dark phase of a light-dark cycle. It also appears to have a specific pattern of feeding. The first half of the light phase is truly a resting phase, during which the rat eats very little if any food. During the second half of this phase the animal sometimes awakens and "snacks" lightly. Food consumption increases gradually throughout the first part of the dark phase, peaking in the middle of this phase. The animal eats less for the next few hours, and apparently consumes one last large "meal" just before the lights come on again (Siegel, 1961; Peraino et al., 1980).
The results of the study of parietal cells in ad libitum-fed animals indicate that changes in secretory canaliculi and microvesicles, the two structural indicators of acid secretion, reflect the eating habits of the rats. Indications of acid secretion decreased throughout the first half of the light phase. The cells appeared to be relatively quiescent by mid-light. There were indications of acid secretion in the late light phase, but the cells were again "quiescent" by early dark. A large increase in acid secretion was indicated at the mid-dark sampling point and again in late dark.

It is difficult to determine from these results whether food or feeding behavior has stimulated the acid secretion or whether there is an inherent rhythm in acid secretion arising from the cells themselves, and what factors might be mediating such a rhythm. If the secretion were due to the presence of food in the stomach, then fasting should remove the evident late dark phase increase in acid output. If the fluctuations are due to psychological factors such as "expectation" of feeding (Saito et al., 1976), one may attempt to reduce the factor by subjecting the animals to repeated feed-starvation phases.

In the present study, fasted animals were subjected to four consecutive feed:starvation phases of 8hr:40hr before being sacrificed. The results indicate that even in the absence of food, periodicity of acid output persisted. Peak indications of acid secretion were again from the middle to the late dark phase. The cells appeared to be
quiescent through the light phase. There was no activity in the late light phase as there had been in ad libitum-fed rats, but by early dark the cells appeared to be beginning another secretory phase. (The stomachs were completely empty of food at this time.) It appears, then, that a periodic output of acid persists even in the absence of a food stimulus. The source of this time cue is still not clear. Periodicity in cellular function may be inherent in the cells themselves. It is also quite possible that given the importance of psychological and neurologically-mediated factors such as sham feeding (Stendquist et al., 1980) and stress (Hoelzel, 1942) on acid output, that awakefulness alone stimulates secretion. The animals did display feeding behavior during the fast, as evidenced by their che,ring through a cage-restraining wire and the appearance of what appeared to be scales from tails in some rats' stomachs. These factors may have caused an increased vagal tone, effecting gastrin and acid secretion.

In the third experiment rats were habituated for 8 weeks to a phase-shifted feeding schedule wherein they were allowed food only during the mid-light phase (normally the resting phase). If the feeding schedule alone determines acid output, then an habituation to this new feeding schedule might be expected to reverse the acid secretion rhythm by approximately 180 degrees. The results were complicated by the fact that the animals responded to this regimen by gorging. Stomachs were distended with food during the mid-feeding sampling point, and they remained distended until mid-dark. The cause of this 12 hr retention of
food in the stomach may be either the quantity of food present or a lag in the initiation of digestive processes until the dark phase.

Quantification of the parietal cell canaliculi and microvesicles in this experiment indicate the greatest amount of acid was secreted during the second half of the light phase and the middle of the dark phase. This corresponds to the time that the greatest amount of food was present in the stomach. These data could suggest that the presence of food is the stimulus for acid secretion during these 12 hours. The interpretation of the results is complicated, however, by an increase of microvesicles in the early dark phase. The canaliculi did not decrease in area, but the vesicular proliferation is statistically significant. This result poses some interesting questions; e.g. what has affected the usual synchronization of the two cellular characteristics? One also wonders whether the rate of microvesicular production at this timepoint exceeds their rate of secretion. This investigator feels that the increase in microvesicles, occurring in the early dark as is also the case in ad libitum-fed animals, may indicate a change in the physiological state. Forte et al. (1977) have reported that the conformation of the secretory canaliculi can change before the number of microvesicles decreases. If the microvesicles are the true indicator of acid secretion in progress, then another interpretation may be found for the results of this experiment; i.e. there are two factors affecting acid secretion. The first may be the intake of food in the mid-light phase. The cells may then return to the resting state by early dark.
The second factor may be an inherent stimulus of acid output that increases during the animals' activity (dark) phase.

Fluctuations of the rough endoplasmic reticulum are evidently not related to food intake. Both ad libitum and phase-shifted feeding animals displayed peak amounts of the organelle from the mid-light phase until the early dark phase even though feeding was shifted about 180 degrees. The only effect of the feeding regimen appeared to be on the extent of this organelle's fluctuation. Fasting appeared to dampen the rhythm considerably. More distinct variations were evident in ad libitum and phase-shifted rats. The reason for this effect is not clear, unless the presence of food in the stomach is necessary to cause an increase in the production of rough endoplasmic reticulum.

Multivesicular bodies were clearly affected by feeding regimen. In both ad libitum and phase-shifted feeding animals, the organelle increased in number at the end of the "non-secretory" phase or just before the beginning of the feeding phase. Thus, shifting the feeding phase about 180 degrees also shifted the rhythm in multivesicular body numerical density by about 12 hrs. In fasted animals the organelle increased in number throughout the fast. A decrease was not seen during the timepoints sampled. Two interpretations of these data may be made. Either the multivesicular body is needed for the beginning of the secretory phase, or it represents a product that builds up during the resting phase. It has been suggested by Novikoff (1964) and others,
that the organelle is lysosomal in nature. Friend (1969) demonstrated acid phosphatase, a lysosomal enzyme, in the matrix of the structure in rat epididymal cells. Our acid phosphatase study showed reaction product in dense bodies of parietal cells (which did not occur in the presence of enzyme inhibitor). In no case, however, did we see evidence of the enzyme in multivesicular bodies. This does not, of course, negate their possible role as lysosomes in parietal cells; it merely indicates that the organelle does not demonstrate acid phosphatase in the parietal cell with the methods used. Further work is needed to understand the function and fluctuations of the multivesicular body.

When the results of all three feeding experiments are compared, it appears that feeding, per se, does not control the fluctuation in PAS-staining intensity of mucus in surface epithelial cells lining the gastric lumen. While the presence of food was correlated with a significant increase in red-staining mucus in the phase-shifted experiment and in the late dark phase of the ad libitum-fed rats, this was not the case in the late light phase of the latter experiment. At this time the stomachs' contents indicated that the animals had eaten recently, but the greatest number of slides showing pink mucus was seen at this timepoint. In addition, if feeding were the only stimulus to the appearance of red mucus in the surface cells, then the cells of the fasted animals should have contained only pink-staining mucus at all timepoints. This was not the case; even toward the end of the experiment, many cells were evaluated as having red mucus. These
results lead this investigator to believe that the presence of red mucus in the gastric surface cells may be due to a recent migration, bringing young cells with sulfated mucus to the apex of glands. If this is so, then there may be only one major migration per day in ad libitum-fed animals, that in the late dark phase. Rapid migration would appear to occur consistently from the mid-feeding (light) phase until the middle of the dark phase in phase-shifted feeding animals. The results of the fasted animals are capricious and at the present, inexplicable.

In spite of the changes that occurred in many of the variables in the three cell types, some characteristics remained constant in all three feeding experiments:

1. In all experiments the parietal cells of glandular necks had more dilated secretory canaliculi, fewer microvesicles, and more rough endoplasmic reticulum than cells of the glandular bases.

2. Surface epithelial cells of the upper foveolae generally contained the most mucus, followed by those on the gastric surface. Cells of the isthmus contained the least mucus.

3. Surface epithelial cells of the isthmus generally stained most darkly with PAS, followed by those on the gastric surface. Both stained more darkly than the mucous neck cells.

4. "Intermediate" cells, with characteristics of both surface epithelial cells and mucous neck cells were visible in all three experiments and at all timepoints sampled.
5. Although mucous neck cells were light pink after PAS staining, the cytoplasm was heterogeneous, with some areas staining slightly darker than others.

How does the stomach protect itself?

Stress and such drugs as alcohol and aspirin are commonly considered to be gastric irritants, sometimes leading to gastritis and ulcers. Even under non-stressed conditions, however, the stomach lining is exposed to potentially damaging substances and conditions. First, there is the passage of food through the stomach. The gastric walls are brought into contact with the chyme by peristalsis, which puts great mechanical stress on the lining cells. Secondly, hydrochloric acid and pepsinogen are secreted into the gastric lumen to aid in the digestive process. The former softens fibrous tissue and converts pepsinogen into pepsin, which digests proteins. Thus the stomach walls are in danger of being digested by the very substances that their glands produce.

The stomach possesses several known defences against these noxious conditions. There is a rapid cell renewal of the gastric lining, lubrication of the stomach walls and a physical barrier to autodigestion. The results of the present studies stress an amplification of these defensive mechanisms: a periodic increase in mucus production that offsets periodic increases in stress. All or most of these functions are provided by the surface epithelial cells.
Lubrication of the walls and a barrier to autodigestion are provided by the mucus secreted onto the stomach surface. A mucous layer that is clearly visible at the microscopic level forms on the cells lining the stomach lumen. Physiological studies have not succeeded in determining the effect of food on surface epithelial cell secretions. Any stimulus of mucus secretion causes a concurrent output of mucus from gastric pyloric and antral glands and possibly mucous neck cells. Histological studies showing physiological changes in the surface epithelial cells are a possible solution to this problem. Nucleolar changes may, for example, indicate the relative activity of the cells. By the definition of Busch (1974), a periodic increase in nucleolar count might indicate greater cellular activity. It is of note that statistically significant changes in nucleolar count in cells lining the stomach occurred in all three experiments; also, for every increase in nucleolar number there is a concurrent suggestion of increased parietal cell acid secretion as indicated by increased secretory canalicilar volume densities. As topical acid application is a stimulus to mucus secretion in some cells (Ivey and Oyama, 1921), the greater nucleolar counts suggest an increase of mucus production. Since no changes in cell size or mucous content were observed in any of the experiments, the mucus is probably secreted as rapidly as it is produced.

Periodic increases in cell renewal rates may be reflected in the staining properties of the surface epithelial cell mucus. Barring the possibility that cells of the gastric surface produce sialic acid at
some timepoints and sulfated mucus at others, the appearance of red mucus in the cells of the stomach lining may indicate rapid migration. If the presence of large amounts of food causes enough abrasion of the stomach surface to promote mitosis in the isthmus and migration of the cells to replace those lost from the surface, then the increase in red-staining cells seen from mid-light to mid-dark in the phase-shifted rats and in the late dark in ad libitum-feeding animals support the hypothesis. The small amount of food eaten in the late light phase in the latter experiment may not have been an adequate stimulus for migration. The results of the fasting experiment are inconclusive. If red mucus in surface cells does reflect recent migration, then the stress of fasting appears to have desynchronized the rhythm in the rats, as there was no cohesive pattern of change at different timepoints.

Finally, it is possible that sialic acid and sulfated mucus may serve different functions in the protection of the stomach. This has received scant consideration or investigation in the literature.

Differences in cells at different glandular levels.

The results of this investigation have shown that two gastric cell types, the parietal and surface epithelial cells change with respect to their glandular location. These results also suggest that their functions change.
Three of four studied parietal cell characteristics varied consistently among cells of the neck and base of the glands. The secretory canaliculi, for example, were more dilated in cells of the neck of glands. The same cells contained fewer microvesicles than cells of the base but a greater amount of rough endoplasmic reticulum per cytoplasmic area. These results raise some questions as to the cells' relative functions. It may be that if parietal cells arise from cells in the glandular neck and migrate basally, the greater amount of rough endoplasmic reticulum in the younger cells indicated increased production of proteins for growth and maturation. Another interpretation is that increased cellular activity requires an increase in protein production. The other two observed characteristics would support this. The consistently dilated canaliculi and sparse microvesicles of glandular neck cells suggest that they may produce more acid than cells in the base. In addition, the neck cells often appeared to react more quickly and to a greater degree and then to recover more slowly to a secretory stimulus than cells in the base.

These differences may be due to the cells' locations. Cells of the neck are more likely to be exposed to paracrine secretions of gastrin present in the gastric lumen. This is not only the case by virtue of their proximity to the stomach cavity but also because they abut on the glandular lumen while cells of the base are generally peripheral to the chief cells. Parietal cells of the base also may be less active because of their greater age.
In all experiments, a prominent difference in the amount of mucus was seen in the epithelial cells of different glandular locations. The smallest amount of mucus was observed in the youngest cells, deep in the isthmus. As the cells approached the gastric lumen they contained progressively more mucus reaching the maxima in the upper foveolae. Cells of the gastric surface, however, contained approximately two-thirds of the amount in cells of the foveolae. Zalewsky and Moody (1979) reported that exocytosis of individual mucous granules occurs from surface epithelial cells at all glandular levels. It may be that the cells of the foveolae produce more mucus than the cells in other locations. It is also possible that the cells on the gastric surface produce an equal amount but are exposed to more mechanical stress causing them to secrete more mucus.

These studies have confirmed the observations that two gastric cell types, the parietal and surface epithelial cells, differ histologically at various glandular levels. The changes are probably partly related to the age of the cell. There are also indications that the function of the cells is modified with location. Parietal cells appear to be most active while in the neck of glands, and less active in glandular bases. The function of surface epithelial cells in the isthmuses is not known. Their mucus may be for cellular protection from hydrochloric acid or for the cells' growth and differentiation or a combination of both. They do provide a source of new cells for the stomach surface, where cells are lost through trauma and normal cell
extrusion. Surface epithelial cells of the gastric lining almost certainly produce the mucus needed for protection against acid, pepsin and the abrasive action of food passing through the alimentary tract.

The relationship between mucous neck and surface epithelial cells.

Because both mucous neck and surface epithelial cells contain glycosaminoglycans which stain with PAS reagent, they are often considered together in discussions of the protection and function of the stomach. Helander (1964), for example, referred to "mucoid" cells of the stomach and did not distinguish between the two types. There is little justification for considering these cells together, however. The cells contain mucus of a different chemical nature (Ge.ard et al., 1967; Wattel et al., 1979), and it has not been demonstrated conclusively that they share a common origin. Finally, the current study was unable to find any histological characteristics that fluctuated synchronously in both cell types.

Both cells originate in the neck of gastric glands (Stevens and Leblond, 1953; Bertalanffy, 1960). Agreement on the source of the cells ends with this observation. While Stevens and Leblond and Bertalanffy reported mitotic figures in both mucous neck and surface epithelial cells, Wattel et al. (1979) stated that both develop from undifferentiated cells in the glandular neck and lower isthmus. The presence of "intermediate" cells in the glandular neck led Wattel and Geuze (1977) to suggest that surface epithelial cells may differentiate
into mucous neck cells or neck cells into surface cells (Willems et al., 1971).

The present study was not structured to add new information on the origin of the cells. The results did, however, suggest that the two adult cell types display circadian morphological oscillations that are non-synchronous. The changes that occur in the surface epithelial cells may be associated with such factors as mitotic rhythms and cell migration, food intake and acid secretion. Only one factor, however, was seen to fluctuate in the mucous neck cell. Its variation showed no relationship to fluctuations in food intake or acid secretion.

The non-synchronous nature of the circadian variations in characteristics of the two cell types raises the question of their respective functions. The current experiments tend to support the hypotheses that the surface epithelial cells help protect the stomach wall by responding to food and acid secretion by cell turnover and mucus output. The fluctuations in mucous neck cellular shape can be associated with no other factor in the stomach at present. Aside from the observation that mucous neck cells of the lower neck closely resemble chief cells, and thus may be their progenitor, the function of the mucous neck cell and its periodic fluctuation in size is still unknown. While surface epithelial cells and mucous neck cells may derive from a common origin (a logical candidate being the "intermediate" cell found between them in the glands), their eventually proved functions may be quite different.
SUMMARY AND CONCLUSIONS

The purpose of this study was to determine if there are histologically demonstrable changes in the characteristics of gastric mucosal cells and if these characteristics fluctuate rhythmically. The results were also studied to determine if they might yield information on the cells' functions and relationship to each other.

Adult male albino rats were housed under standardized light-dark conditions (12hr:12hr), and were subjected to one of three feeding regimens: ad libitum-feeding, fasting or phase-shifted feeding, in which they were allowed food only in the middle of the light phase when they would normally be resting. Three cell types of the gastric corpus were examined, the acid-producing parietal cell, and the mucus-producing surface epithelial and mucous neck cells. All displayed rhythmic fluctuations in morphology or histochemical staining characteristics.

In the ad libitum-feeding experiment, four parietal cell characteristics varied at the ultrastructural level. Secretory canaliculi were widely dilated in the second half of the dark phase and were dilated again in the late light phase. At other timepoints they occupied a significantly smaller area of the cells. Conversely, the
microvesicles occupied less of the cell area in the latter half of the
dark and late light phases, but they were more numerous in the middle of
the light phase and in the early dark. Rough endoplasmic reticulum
fluctuated with the same pattern as the microvesicles. The number of
multivesicular bodies was highest in the early dark phase and
significantly lower at other timepoints.

As noted by light microscopy, the mucus of surface epithelial
cells on the gastric surface stained most darkly with PAS reagent in the
late dark and contained more light pink-staining mucus in the late light
phase. Statistically significant peak values in the number of cells
containing visible nucleoli were observed in the late dark and late
light phases. Mucous neck cells fluctuated with respect to cellular
area. The cells displayed the largest profile areas at the mid-dark and
early light timepoints.

In the fasted rats, parietal cells' secretory canaliculi also were
expanded in the last half of the dark phase, narrower in the light phase
and dilated again at the beginning of the dark phase. Microvesicles
were least numerous in the late dark phase, increased throughout the
light phase and decreased in some cells in the early dark phase. Rough
endoplasmic reticulum was sparse in comparison to some timepoints in the
previous experiment. It fluctuated very little and appeared to be most
visible in the early and middle dark phase. Multivesicular bodies were
most numerous in the early dark.
The surface epithelial cells of fasted rats displayed no obvious trend of change in the PAS-staining properties. Staining intensity varied widely among timepoints and animals. There was one significant peak in the number of cells with visible nucleoli; this was in the late dark phase. Mucous neck cells appeared to be larger in the mid-dark phase.

When animals were allowed food only in the mid-light phase, cellular characteristics continued to fluctuate. Parietal cell secretory canaliculi remained dilated from the mid-light until the mid-dark phase. They were significantly narrower in the early light. Microvesicles were most numerous in the early light phase. They also increased in the early dark, but were significantly decreased in number in the mid-light and dark phases. Rough endoplasmic reticulum was most evident in the late light phase. Multivesicular bodies were most numerous in the mid-dark and early light phases.

The mucus of surface epithelial cells stained a dark red with PAS from mid-light through the middle of the dark phase and more pink in the late dark and early light. The number of cells with visible nucleoli was also significantly higher from mid-light to mid-dark and lower thereafter. The largest, most cuboidal-shaped mucous neck cells were observed in the animals sacrificed in the late dark and early light phases.
The results of all three experiments suggested that parietal cells of the neck of gastric glands are more active than cells of the base. The former cells contain more rough endoplasmic reticulum for protein production than cells deeper in the glands, their secretory canaliculi are more dilated and they contain consistently fewer microvesicles than cells of the glandular base. These characteristics have been demonstrated to be related to acid secretion (Helander, 1964; Tarnowski et al., 1980). In contrast to the findings of Hally (1959) and Rosa (1963), the current studies indicate that multivesicular bodies increase in number as acid secretion decreases.

Using the dilation of secretory canaliculi and depletion of microvesicles as indicators of acid secretion, parietal cells displayed a rhythm in acid secretion in all three feeding experiments. While the secretion appeared to be related to food intake in ad libitum-feeding animals, an oscillation in secretion appeared to persist in the absence of food. In phase-shifted feeding animals, acid secretion was indicated from the mid-light phase, when the animals are normally quiescent, until the mid-dark phase, when they normally are secreting actively. The microvesicles suggested that the cells may secrete less in the early dark phase (as was the case in ad libitum-feeding animals). These results indicate that there may be two factors affecting acid secretion: food intake and the activity cycle of the animal.
The variation in staining intensity of surface epithelial cells lining the gastric lumen may indicate one of two functional changes. As a dark-red mucus is highly sulfated and a light pink mucus is said to indicate a high sialic acid content, it is possible that the stomach lining cells produce different types of mucus related to the time of day or to the stress of food digestion. Gerard et al. (1968), however, reported that cells of the surface produce sialic acid while younger cells in the isthmus contain sulfated mucus. Given the migration of cells from isthmus to surface of the stomach, the results of the current studies may indicate that there is a rhythm in the rate of migration of cells. Red-staining mucus on the surface would indicate a periodic quickening in migration, bringing younger cells to the surface. In animals allowed food, this state was correlated with the greatest amounts of food content of the stomachs.

Variations in nucleolar count of these cells may reflect a change in the size of the organelles. Larger nucleoli would have a greater chance of being sectioned. An increase in the size of nucleoli has been related to cellular activity. It is of note that by this interpretation of increase in visible nucleoli, mucus production in these cells may increase in all experiments whenever acid secretion is indicated by the parietal cell data.

Fluctuations in the mucous neck cell's area are evidently not related to food intake. They appear to be influenced by other factors.
It is possible that they do not participate significantly in the stomach's protection. They may, instead, be immature cells. Mucous neck cells and chief cells are similar in morphology and are located in contiguous areas. It is possible, then, that the mucous neck cells develop into chief cells.
BIBLIOGRAPHY


APPENDIX I. Tissue processing for electron microscopy.

Reagents and solutions

Karnovsky's fixative (modified)

8% paraformaldehyde . . . . . . . 5 ml
calcium chloride (anhydrous). . . 5 mg
25% glutaraldehyde. . . . . . . . 2 ml
distilled water . . . . . . . . . . 3 ml
0.2M Cacodylate buffer. . . . . . 10 ml

Add paraformaldehyde to calcium chloride. Then add the remaining reagents in the order listed.

Paraformaldehyde solution

paraformaldehyde powder . . . . 2 gm
distilled water . . . . . . . . . 25 ml

Add water to paraformaldehyde and mix at room temperature; result is a milky solution. Add 3 to 4 drops 1N NaOH. Stir until almost clear. Store in the dark overnight. Filter before using.
Cacodylate buffer

- distilled water: 250 ml
- cacodylate salts (3H2O): 10.7 gm
- 1N HCl: about 2 ml

Combine water and salts; adjust pH to 7.2 - 7.4 with HCl. Store refrigerated.

Osmium tetroxide solution

- OsO4 crystals: 1 gm
- 0.2M cacodylate buffer: 50 ml
- 0.44M sucrose in distilled water: 50 ml
### Tissue Processing Schedule

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Embed tissue.
APPENDIX II
APPENDIX II. Morphometric formulae.

Mathematical formulae in the morphometric studies of parietal cells were taken from Weibel and Bolender (1973).

\[
\text{Volume density} = V_v = \frac{P}{P_t}
\]

\[
\text{Surface density} = S_v = \frac{2(I_i)}{L_t}
\]

\[
\text{Numerical density} = N_v = \frac{N}{P_t}
\]

\[P = \text{test points within structure}\]

\[P_t = \text{test points in non-nuclear cytoplasm and secretory canaliculi}\]

\[I_i = \text{line intersections with structure}\]

\[L_t = \frac{1}{2} \text{number of endpoints in non-nuclear cytoplasm and secretory canaliculi} \times \text{line length (1.5 cm)}\]

\[N = \text{number of structures}\]
APPENDIX III
APPENDIX III. Modified Barka and Anderson technique for acid phosphatase.

The function of the multivesicular body in parietal cells is unknown. Friend (1969) demonstrated acid phosphatase, a lysosomal enzyme, in the matrix of the organelle in rat epididymal cells. The purpose of the current study was to determine whether parietal cells' multivesicular bodies also contain the enzyme, which would suggest that it functions as a lysosome.

Stomachs were harvested as in other experiments. Two rats each were decapitated at 0700 and 1800. Tissue from each stomach was trimmed into small rectangular blocks and divided into 3 groups: the experimental (buffer with substrate), Control 1 (buffer with no substrate) and Control 2 (buffer and substrate with acid phosphatase inhibitor).

Reagents and solutions

Tris acid maleate

tris (hydroxymethyl) amino methane ... 24.2 gm
analytical grade anhydrous maleic acid ... 23.2 gm
Tris maleate buffer

Sol. A. 0.2M NaOH . . . . . . . . . . . . 7 ml
Sol. B. 0.2M tris acid maleate . . . . . .50 ml

Combine Sol. A and Sol. B. and bring to 100 ml with distilled water. At this point, solution is 0.1M. Adjust pH to 5 with 0.5N NaOH.

Gomori solution

1.25% n beta-glycerophosphate

(adjusted to pH 5 with 1N HCl . . . 10 ml
distilled water . . . . . . . . . . . . 10 ml
tris maleate buffer . . . . . . . . . . . .10 ml

Combine above solutions. Add 20 ml of 0.2% lead nitrate (pH5) (drop by drop) with constant stirring. Adjust pH to 5.000 with 0.5N NaOH.

Working solutions

Experimental solution - Gomori solution, room temperature, constant agitation.

Control 1 - tris maleate buffer, room temperature, constant agitation.

Control 2 - 0.25 ml 0.1M sodium fluoride : 25 ml Gomori solution, 37 degrees C.
Tissue processing schedule

<table>
<thead>
<tr>
<th>total time</th>
<th>change of solution</th>
<th>solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>exposure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 hr</td>
<td>0</td>
<td>4% glutaraldehyde in cacodylate buffer</td>
</tr>
<tr>
<td>1/2 hr</td>
<td>1</td>
<td>cacodylate buffer</td>
</tr>
<tr>
<td>2 hr</td>
<td>0</td>
<td>experimental or control solution</td>
</tr>
<tr>
<td>15 min</td>
<td>0</td>
<td>cacodylate buffer</td>
</tr>
<tr>
<td>1 hr</td>
<td>0</td>
<td>1% osmium tetroxide in cacodylate buffer</td>
</tr>
<tr>
<td>10 min</td>
<td>0</td>
<td>50% acetone</td>
</tr>
<tr>
<td>10 min</td>
<td>0</td>
<td>75% acetone</td>
</tr>
<tr>
<td>20 min</td>
<td>1</td>
<td>90% acetone</td>
</tr>
<tr>
<td>30 min</td>
<td>2</td>
<td>100% acetone</td>
</tr>
<tr>
<td>12 hrs</td>
<td>0</td>
<td>3 pt acetone : 1 pt epon</td>
</tr>
<tr>
<td>overnight</td>
<td>0</td>
<td>1 pt acetone : 1 pt epon</td>
</tr>
<tr>
<td>overnight</td>
<td>1</td>
<td>100% epon</td>
</tr>
</tbody>
</table>

Embed tissue.
Results

Experimental - tissue was well fixed; reaction product was found in dense bodies, often showing a polar distribution (Fig. 1). Some scattered reaction product was also found in nuclei and more sparsely in the cytoplasm. Reaction product was never seen associated with multivesicular bodies. (Fig. 2).

Control 1 - no precipitate that could be confused with reaction product was seen (Fig. 3).

Control 2 - reaction product was found scattered in nuclei. None was seen in multivesicular bodies or in dense bodies (Fig. 4).

Discussion

The results of this experiment indicate that multivesicular bodies contained very little if any acid phosphatase in either timepoint sampled. Dense bodies appeared to contain the enzyme and may thus function as lysosomes. The reaction product found in nuclei and scattered in the cytoplasm may have been non-specific stain.

The first control tissue (with no substrate) ascertained that no precipitate from the fixation process that could have been mistaken for reaction product was present.

The second control tissue, which contained sodium fluoride, an enzyme inhibitor, did not prevent the reaction product from forming in
Fig. 1. Tissue incubated with reagent (beta-glycerophosphate). Reaction product is concentrated in a dense body (RD). Scattered reaction product is also seen in the cytoplasm (NR). G = matrix granule of a mitochondrion. (X 71,000)
Fig. 2. Tissue incubated with reagent (beta-glycerophosphate). The reaction product is scattered in both the nucleus (NR) and the cytoplasm, but is not associated with the multivesicular body (MVB). (21,700)
Fig. 3. Control 1. Buffer with no beta-glycerophosphate. No reaction product is visible. C = chief cell; DB = dense body; M = mitochondrion; MVB = multivesicular body; N = nucleus. (X 33,300)
Fig. 4. Control 2. Buffer with beta-glycerophosphate and sodium fluoride. Non-specific reaction product is seen in the nucleus (NR) and cytoplasm, but is not associated with dense bodies (DB) or multivesicular bodies (MVB). (X 71,000)
the nuclei, indicating that this was a non-specific reaction. The inhibitor did eliminate the reaction in dense bodies. Since it had occurred in the experimental solution, the results indicate that dense bodies contain acid phosphatase. It is highly likely that they function as lysosomes. The function of multivesicular bodies in this cell, however, remains unknown. While the lack of acid phosphatase does not negate their potential role as lysosomes (they may contain less of the enzyme, or may contain another enzyme entirely), they may also serve a different function or may represent a waste product in the cells.
APPENDIX IV
APPENDIX IV. Periodic acid schiff (PAS) reaction for epon-embedded tissue.

Reagents

Sodium metaperiodate solution
sodium metaperiodate powder . . . 0.8 gm
distilled water . . . . . . . . . . 30 ml
100% ethanol . . . . . . . . . . .70 ml

Reducing solution
potassium iodate . . . . . . . . .3 gm
sodium thiosulfate . . . . . . .3 gm
distilled water . . . . . . . . .60 ml
100% ethanol . . . . . . . . . .90 ml
2 N HCl . . . . . . . . . . . .1.5 ml

N.B. This is a saturated solution.

Schiff's reagent
Sol. A: pararosaniline
hydrochloride . . . . . . .0.5 gm
1 N HCl . . . . . . . . . .15 ml
Sol. B: potassium metabisulfite . . . 0.5 gm
distilled water . . . . . . . 85 ml

Combine solutions A and B. Store overnight in the dark. Add 0.3 gm activated charcoal, agitate for 2 min. Filter 2 to 3 times, until solution is almost clear. Store at 4 degrees C. Discard when red.

Sulfite water
sodium metabisulfite
(10% in distilled water) . . . 12 ml
1 N HCl . . . . . . . . . . . . . . . . . 10 ml
distilled water . . . . . . . . . . . 200 ml

Procedure

1) float sections on water on slide. Allow to heat (115 degrees C) for at least 2 min after dry, or incubate overnight at 40 degrees C.

2) 10 min in sodium metaperiodate solution.

3) rinse in 70% ethanol.

4) 5 min in reducing solution.

5) rinse in 70% ethanol.
6) 1 hr in Schiff's reagent at 37 - 40 degrees C.

7) 3 changes, 2 min each, in sulfite water.

8) 10 min in running tap water.

9) coverslip in xylene-base medium.
The dissertation submitted by DANIELLE M. JACOBS has been read and approved by the following committee:

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Nov. 30, 1981
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