ABSTRACT

The renewal of various types of epithelial cells was examined in the descending colon of adult mice given ^{3}H-thymidine. Semi-thin Epon sections were stained with PA-Schiff-iron-hematoxylin and radio-autographed.

The existence of three cell lines was established: vacuolated-columnar, mucous and enterochromaffin. In the vacuolated-columnar cell line, only vacuolated cells divide. A vacuolated cell at the cryptal base and its progeny would go through three mitotic cycles in the lower two-thirds of the crypt before becoming columnar cells. In the mucous cell line, cells originate from vacuolated cells in the lower one-third of the crypt and undergo one or two mitotic cycles before ceasing to divide. Enterochromaffin cells do not divide, but renew by differentiation of vacuolated cells. The proliferative rate of epithelial cells undergoes diurnal variation, which affects mucous cells more seriously than vacuolated cells. All epithelial cells migrate upward along the cryptal wall, and eventually are lost from the surface.

Name: William Wei-Lien Chang

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EPITHELIAL CELL RENEWAL IN MOUSE DESCENDING COLON

by

W. W-L. Chang

RENEWAL OF THE EPITHELIUM OF THE DESCENDING COLON OF THE MOUSE

by

William Wei-Lien Chang

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Anatomy
McGill University
Montreal, Quebec, Canada.

July, 1970.

To my wife

Delphine

and

daughters

Phyllis

and

Bernice

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GENERAL INTRODUCTION : HISTORICAL DEVELOPMENT OF THE CONCEPT THAT THE INTESTINAL EPITHELIUM IS A RENEWING POPULATION

The mucosa of the small intestine of mammals contains a number of finger-like or leaf-shaped projections, villi, extending into the lumen, and as many blind-ended tube-like structures, crypts of Lieberkühn, festooned in the lamina propria (Macklin and Macklin, 1932; Patzelt, 1936; Toner, 1968). In the large intestine, villi are present only in the developing colon of embryos and disappear in the later stages of embryonal development (Patzelt, 1882; Patzelt, 1936); therefore, the mucosa in the adult colon is composed of smooth surface and numerous crypts which open into the colonic lumen. Both villi or surface and crypts are lined by a single layer of epithelial cells. Since there are similarities in origin, structure and function between small and large intestines, and many important works on the renewal of the epithelial cells in the mammalian intestine have been performed in the small intestine, the cell renewal of these two parts of the intestine is considered together in this historical note.

According to Leblond and his associates (Leblond and Walker, 1956; Leblond, Messier and Kopriwa, 1959; Messier and Leblond, 1960; Leblond, 1964), the cell populations of the adult organisms can be divided into three groups on the basis of their proliferative activity: static, expanding and renewing cell populations. The intestinal epithelium has

been classified as a renewing cell population, because it has a high proliferative activity which is largely balanced by a cell loss without obvious changes in the size of its component structures.

Historically, there has been an interesting course of development from the realization of renewal of cells in the intestinal epithelium to the detailed kinetic analysis of cell behavior in this process of renewal. Based on the availability of experimental tools and the ingenious approaches made by the investigators in the field, this course of development of the knowledge on cell renewal can be stated according to the following stages:

(I) Regeneration of intestinal epithelium

Toward the end of the 19th century, the cryptal and villus epithelium of small intestine were generally considered separately. Heidenhain (1888) observed, however, that epithelial cells of the crypt did not differ significantly from those of the villus, whether columnar or mucous cells were considered. It was Paneth (1888), who demonstrated the common embryological origin of the epithelial cells of both crypts and villi. These authors also noted that mitotic figures were abundant in the cryptal epithelium, but their significance was not fully appreciated by them.

In studying the development of the mucosa in the large intestine in the mammalian embryos, Patzelt (1882) described that the formation of new epithelial cells occurred from the so-called "mother cells" (Brutzellen) located at the base of the crypts and also that epithelial

cells perishing in the course of time were replaced by epithelial cells pushed upwards from the base of the crypts. Moreover, he observed that on the walls of the crypts and villi of the developing colon, the formation of mucous cells took place, which started with the appearance of small mucous globules between the nucleus and the free apical margin of columnar cells.

However, credit was generally given to Bizzozero (1888, 1889, 1892, 1893) for formulation of the theory of regeneration of the intestinal epithelium in the adult animals. On the basis of the distribution of mitotic cells and cellular appearance in the epithelium of colon and rectum as well as of duodenum in several species of mammals, he reasoned that epithelial cells were exclusively formed in the crypts and migrated up to the surface or villi to replace the damaged or lost cells. Since mitotic figures were observed in both columnar and mucous cells in the crypt, this regenerative activity was presumably carried out not only by columnar cells but also by mucous cells (Bizzozero, 1889, 1892).

(II) From regeneration to constant renewal

In the beginning of the 20th century, it was generally believed that epithelial cells lining the intestinal villi or surface were frequently damaged and lost in the course of the digestive activity or by bacteria or toxins (see Patzelt, 1936). However, Leblond and Stevens (1948) found that adequately fixed preparations of rat small intestine usually showed no signs of cell damage and even so mitoses were abundant in the crypts. Since they noticed cell streamers or gaps at the villus tips,

they reasoned that the cells formed from mitoses in the crypts migrated along the side of villi and were extruded "alive" at the tips of villi. This would indicate a constant physiological process of cell production in the crypts, migration along the villi and loss at the villus tips rather than a regenerative process to replace damaged cells. Therefore, a term "renewal" was introduced to substitute "regeneration".

Concomitant with the change in the concept, Leblond and Stevens (1948) introduced first the concept of the steady state in this process of cell renewal; that is to say, the production of newly formed cells should be balanced by a loss of cells, since there was no evidence of growth of villi or crypts in the adult intestine. Of further importance, their work was the first attempt to calculate the turnover time of the whole intestinal epithelium based on the mitotic index (1.57 and 1.35 days respectively for duodenum and ileum) and to estimate the duration of mitosis of intestinal epithelial cells using colchicine (1.13 hours). These methods were adopted by McMinn (1954) in cat, Bertalanffy (1960) in rat and Bertalanffy and Nagy (1961) in man to estimate the parameters of cell production and turnover in the intestinal epithelium. The use of these classic techniques for the study of cell renewal was critically re-evaluated by Leblond (1959) and Stevens-Hooper (1956, 1961).

Supporting evidence for this theory of cell renewal was the finding by Ramond (1904) of a large number of epithelial cells in the intestinal lumen, thus indicating that cells were lost in this direction. This was confirmed by Wright et al. (1940), who found desquamated epithelial cells

in the intestinal secretions collected from fistulas, and also recently by Pink et al. (1970), who identified both columnar and mucous cells in the washings of the intestinal contents with electron microscope. The fact that the presence of epithelial cells in the intestinal contents was not solely due to damage of epithelium was suggested by the observations that the closed loops of intestine became filled with desquamated cells (Stevens-Hooper, 1956), and that cells were continuously desquamated into the intestinal lumen in starved animals (Leblond and Stevens, 1948; Stevens-Hooper and Blair, 1958). Creamer et al. (1961) observed directly the shedding of cells from the villus tips in the dog; they found that this process was intermittent and was not related to the activity of the villi. That the villus tips were the main site of cell extrusion in the epithelium of small intestine was confirmed by many other investigators (McMinn, 1954; Bertalanffy and Nagy, 1961; O'Connor, 1966; Imondi and Bird, 1966, among others). Furthermore, Enesco and Altmann (1963) estimated a figure of 1.58×10^9 cells lost daily from the small intestine of a rat alone.

(III) Confirmation of migrational activity of intestinal epithelium by markers

The first experimental evidence of cell migration from crypt to villus in the intestinal epithelium was obtained by Friedman (1945), who made mucous cells of the crypt swollen by exposing them to mild X-ray radiation and traced them from crypt to villus.

Following the development of radioautographic techniques (Belanger and Leblond, 1946), Leblond, Stevens and Bogoroch (1948) observed following ^{32}P injection that cryptal cells became labeled within two hours, and after 18-24 hours these labeled cells became part of the epithelium on the side of the villi. Similar migrational activity of epithelial cells in the direction of the villus tips was also observed after administration of methionine-^{35}S (Belanger, 1956; Leblond, Everett and Simmons, 1957) or adenine-^{14}C (Pelc and Howard, 1956; Walker and Leblond, 1958). Walker and Leblond (1958) demonstrated graphically the advancing flow of newly formed cells ascending the villi of the small intestine, and they also showed for the first time the similar process of cell production and migration in the large intestine.

(IV) Kinetic analysis of cell renewal

Of particular importance in the investigation of the renewing cell populations was the introduction of tritium (^3H)-labeled thymidine to the biological sciences. Because of its specificity of incorporating into the DNA molecules of cells (Taylor et al., 1957; Amano et al., 1959), its stability to a variety of histological procedures after incorporation into DNA (Cleaver, 1967) and the reasonably short range of beta-particles emitted from tritium, high resolution radioautography was feasible using ^3H-thymidine to determine the precise localization of the cells.

As a first result, the previous studies on the site of proliferation and the mode of migration of epithelial cells in the intestine were confirmed by many investigators using ^3H-thymidine (Hughes et al., 1958;

Leblond and Messier, 1958; Quastler and Sherman, 1959; Messier and Leblond, 1960; Messier, 1960; Creamer et al., 1961; Lesher et al., 1961a; Lipkin and Quastler, 1962; Fry et al., 1961, 1962, 1963; Lipkin et al., 1963a, 1963b; Lipkin, 1965a, 1965b, 1966; McDonald et al., 1964; Shorter et al., 1964; Sawicki et al., 1968 and others).

A most important consequence was the introduction of methods for the kinetic analysis of the renewing cell populations. Based on the concepts developed by Leblond and Stevens (1948), Quastler and Sherman (1959) introduced a concept of compartments: crypts being the "progenitor" compartment and villi the "functional" one. Important parameters in the compartmental analysis were defined and evaluated. This method of compartmental analysis was further extended and generalized by Quastler (1960, 1963).

A very important contribution made by Quastler and Sherman (1959) was the method of labeled mitosis curve, by which an estimation of the durations of presynthetic (G_1) phase, DNA synthesis (S) phase, and postsynthetic (G_2) plus mitotic (M) phases, and also of the cell cycle was made possible. In the mouse ileum, they found the mean cell cycle time to be 18 3/4 hours, the duration of S phase to be 7 1/2 hours, that of G_2 and M to be 1 3/4 hours, and that of G_1 to be 9 1/2 hours. Although a majority of cryptal cells went from DNA synthesis to mitosis after a short and comparatively constant interval, there was a considerable variability in the duration of G_1 (Quastler and Sherman, 1959). Using the labeled mitotic curve method, Cameron and Greulich (1963) found the

average duration of the S phase to be 7 hours in the renewing epithelia of the mouse. Similarly, in the mouse colon, the duration of the S phase and that of cell cycle were estimated to be 4.5 - 8 hours and 16 hours respectively by Lipkin and Quastler (1962) and to be 8 hours and 19 hours respectively by Thrasher (1967). In the human intestines, the duration of the DNA synthesis phase appeared to be longer (10 - 15 hours) (Lipkin et al., 1963a, 1963b; Lipkin, 1965a, 1965b). Comparative rates of cell proliferation in different areas of the gastrointestinal tract of newborn hamster were investigated by Lipkin and Deschner (1968), who showed that there was more rapid proliferation in small intestine than in stomach and colon. Of particular interest was the demonstration by Cairnie et al. (1965a) that the cell cycle time was about 14 hours long at the bottom of the crypt and shortened to 10 hours in the upper portion of the crypt, most of the change being in the duration of G_1 phase and partly in the duration of S phase. They also noted that cells in the upper portion of the crypt exhibited a considerable uniformity in the length of the periods of the cell cycle. The cell cycle time was found to be increased with age (Lesher et al., 1961b; Thrasher and Greulich, 1965; Thrasher, 1967a, 1967b) and also in the germ-free animals (Lesher et al., 1964).

For the analysis of cell migration in the villi, Quastler and Sherman (1959) estimated the progress of labeled cells on villi by plotting distance between the base of villi and leading edge of labeled cells in percent of villus height against time between labeling and sacrifice. In

this way, the transit time of villi of mouse ileum was found to be 46 1/2 hours on average, although considerable differences between animals were noted. On the other hand, Lesher et al. (1961a) and Fry et al. (1961, 1962, 1963) estimated the total transit time of the intestinal epithelium by calculating the time taken for labeled cells to reach 50% of the extrusion zone of the villi, and the crypt transit time by calculating the time in which 50% of the villi contained labeled cells at their bases. The difference of these two transit times would be the villus transit time. They found that (1) the total transit time increased with age; and (2) the total transit time appeared to be longer in duodenum and jejunum than in ileum, but the crypt transit time was longer in ileum than in duodenum and jejunum.

For the analysis of the migrational activity of cells in the crypt where cell proliferation takes place, Cairnie et al. (1965a) devised two methods. In the first method, the velocity of cryptal cells was estimated by plotting the cell position in the crypt at which the labeling fell to half the plateau level at various times after giving ^{3}H-thymidine. The second method was based on an assumption that cell migration up the cryptal walls was due to the population pressure created by the formation of new cells below. Thus the migrational velocity of a cell at a particular position of the crypt was equal to the cumulative proliferative rate of the cells up to that position. The cell velocity in the upper portion of the crypt calculated by two methods matched fairly nicely and was 1.2 - 1.4 cell positions per hour. These methods were adopted by Sawicki et al.

(1968) in their study of cell renewal in the ascending colon of the guinea pig. In accordance with the results of Cairnie et al. (1965a), the cell velocity increased towards the mouth of the crypt where it was 0.7 - 0.8 cell positions per hour (Sawicki et al., 1968).

The work of Quastler and Sherman (1959) indicated the presence of "critical phase" of the cell cycle in the transition from the dividing state to the non-dividing state at the top of the crypts in the mouse ileum. Detailed analysis of this transition was carried out by Cairnie et al.(1965b). From the analysis of the distribution of cells synthesizing DNA and cells in mitosis in the crypts, they postulated the slow cut-off model of the crypt. This model allowed for a gradual changeover of cells from divisions producing two mitosable daughter cells to divisions producing two non-mitosable daughter cells over a wide range of cell positions while cells ascended the cryptal walls. This has described the phenomenon of the transition from the dividing to the non-dividing cells very nicely, although the reasons or the mechanism of this transition are still unknown.

(V) Renewal of individual cell types in the intestinal epithelium

Four cell types are present in the epithelium of the small intestine: columnar (or absorptive), mucous (or goblet), enterochromaffin (or argentaffin) and Paneth cells (Macklin and Macklin, 1932; Patzelt, 1936; Trier and Rubin, 1965; Trier, 1968; Toner, 1968). However, in the large intestine, only the first three cell types have been recognized (see Chapter I). In most studies on cell renewal as described previously,

no distinction was made of individual cell types known to be present in the intestinal epithelium. Since columnar cells are a great majority in the cell population of intestinal epithelium (Quastler and Sherman, 1959), it may be assumed that the cell renewal thus studied would reflect the behavior of columnar cells in general. Even though this assumption is likely to be true, some doubt remains until the behavior of all the other cell types present in the intestinal epithelium is clarified.

Renewal of mucous cells

At the time when Bizzozero (1888, 1889, 1892, 1893) developed the theory of regeneration of intestinal epithelium, he noticed some mitotic figures in both columnar and mucous cells; thus he postulated that both columnar and mucous cells were formed in the crypt and migrated up to the surface or villi to replace the damaged cells. However, the dividing capacity of mucous cells has been doubted by a number of workers in the field, who asserted that cell division and cell specialization such as mucogenesis were antagonistic (Macklin and Macklin, 1932). Therefore, by demonstrating cells intermediate between mucous cells and other epithelial cells, the latter, particularly columnar cells, were postulated as a precursor of mucous cells (see Chapter III for details).

After the availability of ^{3}H-thymidine, Leblond and Messier (1958) demonstrated that both columnar and mucous cells in the rat small intestine became labeled by 8 hours after giving ^{3}H-thymidine and thus renewed themselves. Thrasher and Greulich (1966) confirmed the ob-

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servations of Leblond and Messier (1958), and analyzed the distribution of mucous cells synthesizing DNA in the murine duodenal crypts. Moreover, they estimated the duration of various phases of cell cycle of mucous cells, which was of the same order as that of columnar cells. On the other hand, Cairnie (1970) could not find any mucous cells in mitosis in the rat small intestine, and classified mucous cells as a non-proliferating population which was presumably originated in columnar cells located in the lower portion of the crypt. In fact, he found 2% of mucous cells labeled at one hour after injection of ^{3}H-thymidine, which were attributed to "false positivity" of the radioautographic reaction. Approximately the same percentages of mucous cells were found to be labeled by Merzel and Leblond (1969) soon after giving ^{3}H-thymidine. At the same time, these authors found mitotic figures in some mucous cells containing a small amount of mucous globules (designated as oligomucous cells), but not in mucous cells in a typical goblet form which became labeled only at later times. Although oligomucous cells fitted the requirements for the precursor of mucous cells in a typical goblet form, not enough of them were present to account for the renewal of mucous cells. Based on the calculation of the turnover times of columnar and mucous cells, they concluded that oligomucous cells originated in undifferentiated columnar cells in the lower portion of the crypt on the one hand, and gave rise to typical goblet mucous cells on the other.

Renewal of enterochromaffin cells

Information on the renewal of enterochromaffin cells is very

scanty. Although there has been a great deal of speculation on the origin of enterochromaffin cells in the literature (see Chapter IV), it is not known whether or not these cells undergo renewal. Deschner and Lipkin (1966) claimed to have observed enterochromaffin cells labeled one hour after administration of ^{3}H-thymidine and also in mitosis, contrary to the observations made by classic histologists (Patzelt, 1936); thus they asserted that these cells were capable of self-renewal, but at a slower rate. On the other hand, Ferreira (1970) could not find any labeled enterochromaffin cells at one hour or one day following the start of continuous infusion of ^{3}H-thymidine, but he demonstrated that these cells became labeled steadily starting around 36 hours after giving ^{3}H-thymidine, indicating that enterochromaffin cells might belong to a renewing cell population.

Renewal of Paneth cells

Opinions on the renewal behavior of Paneth cells in the mammalian small intestine were divided mainly into two groups depending on whether or not Paneth cells were seen in mitosis (Patzelt, 1936; Cheng, Merzel and Leblond, 1969). Thrasher and Greulich (1966) observed frequently labeled Paneth cells soon after injection of ^{3}H-thymidine and they claimed that Paneth cells were capable of self-renewal with the cell cycle time of 80 hours. Similar observations were made in man by Deschner (1967). However, since Leblond and Messier (1958) pointed out that Paneth cells never incorporated labeled thymidine, evidence has been accumulated in favor of this non-dividing property of Paneth cells

(Hampton, 1968; Leblond, Merzel and Cheng, 1968; Troughton and Trier, 1969; Cheng, Merzel and Leblond, 1969; Cairnie, 1970). Label appeared first on the Paneth cell nuclei 1 - 3 days after giving ^{3}H-thymidine (Troughton and Trier, 1969; Cheng, Merzel and Leblond, 1969; Cairnie, 1970), and thereafter the number of labeled Paneth cells increased steadily with time in animals given continuous infusion or serial injections of ^{3}H-thymidine (Cheng, Merzel and Leblond, 1969; Cairnie, 1970). It appeared that Paneth cells would turn over in three weeks or so in the mouse small intestine (Cheng, Merzel and Leblond, 1969). Although Hampton (1968) and Cheng, Merzel and Leblond (1969) suggested that Paneth cells had their origin in undifferentiated columnar cells at the base of the crypt, Cairnie (1970) hinted that these cells had their precursor outside the intestinal epithelium.

CHAPTER 1

EXISTENCE OF THREE CELL POPULATIONS IN THE DESCENDING COLON OF THE MOUSE

INTRODUCTION

It is usually agreed that three types of epithelial cells are present in the large intestine of mammals: columnar or absorptive cells, mucous or goblet cells, and enterochromaffin or argentaffin cells.

Other cell types have been mentioned, however. Paneth cells have been reported in the cecum but not in the rest of the colon of various species: mouse (Martin, 1910), horse and donkey (Trautmann, 1910) and man;[1] but reviewers believe that these occurrences of Paneth cells have little significance. (Macklin and Macklin, 1932; Patzelt, 1936; Toner, 1968).

Recently, an additional cell type, referred to as vacuolated cell, was described in the rectum of rodents and man by Hollmann (1965),

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[1] In man, Bloch (1903a, 1903b) found Paneth cells in the colonic epithelium of infants but only before the age of two years. Some investigators described Paneth cells in appendix only (Schmidt, 1905; Hertzog, 1937) or in appendix and cecum only (Kerr and Lendrum, 1936; Watson and Roy, 1960), while others saw small numbers of these cells in the remaining parts of colon in adults (Hamperl, 1928; Feyrter, 1931; Paterson and Watson, 1961; Laurén, 1961; Lewin, 1968, 1969), especially in some pathological conditions: appendicitis (Kitagawa and Takahashi, 1958), tuberculous typhlitis (Hertzog, 1937), ulcerative colitis (Watson and Roy, 1960; Paterson and Watson, 1961; Verity et al., 1962), or benign tumors (Thorel, 1898; Schmidt, 1905; Laurén, 1961; Holmes, 1965; Lewin, 1968), and in the vicinity of carcinoma (Black and Ogle, 1948; Laurén, 1961).

who considered it as a precursor of mucous cells. Meanwhile, using histochemical techniques, Spicer (1960, 1965) described what is probably the same cell as a sialomucin-containing, non-goblet, mucus-filled epithelial cell in the rectum and descending colon of rodents. Wetzel, Wetzel and Spicer (1966) considered this cell as a precursor of ordinary columnar cells.

Finally, Silva (1966) reported the presence of what he termed as multivesicular cell in the distal colon of the mouse. This cell, which occurred infrequently, was characterized in the electron microscope by long microvilli on the apical surface and a number of granule-containing vesicles in the cytoplasm.

With so many claims for new epithelial cells, it was decided to reexamine the number of cell types present in the descending colon of the mouse and investigate whether they belonged to the same or different populations. Radioautography was used at various times after an injection of ^{3}H-thymidine. The results at the one-hour interval revealed which cell types were capable of division. Later results revealed the transformation of cells of one type into another.

At the outset, two difficulties were encountered. First, differences were observed between the cryptal cells of ascending and descending colon (Martin, 1961), and even between those from the various parts of the ascending colon. It was, therefore, decided to restrict the present investigation to an area of the descending colon occupying one cm above the pelvic rim. This area was found to be representative of both

descending colon and rectum. Furthermore, the number of cells of each type present in this area was found to be reasonably constant.

Secondly, the cells were so crowded in the walls of the crypts that it was difficult to identify them with precision in paraffin sections. This problem was solved by using semi-thin (1-μ) Epon sections in the light microscope for routine work, with occasional reference to thin sections in the electron microscope.

MATERIALS AND METHODS

Experiments

In Experiments I and II, the animals were sacrificed at various times after a single injection of ^{3}H-thymidine. In Experiment I, eight female Swiss albino mice, approximately four months of age, received a subcutaneous injection at 08:30 h of 4 μCi of ^{3}H-thymidine per gram body weight (Schwarz BioResearch, specific activity 11.0 Ci/mM). Under ether anesthesia, the animals were sacrificed in pairs 1, 36, 72 and 144 hours later by perfusion of a 4% paraformaldehyde solution in Millonig's buffer (Millonig, 1961) at pH 7.2 - 7.4 through the left ventricle of the heart.

In Experiment II, twenty mice of the same strain, sex and age as above were given a subcutaneous injection of 1 μCi of the same ^{3}H-thymidine as above per gram body weight at 12:00 h. At various time intervals thereafter (1, 9, 15, 24, 36, 48, 72, 96, 120 and 168 hours), they were perfused with 4% paraformaldehyde as above except that 0.1 M

Sörensen's phosphate buffer at pH 7.2 - 7.3 was used as buffer.

Experiment III was designed to examine the effect of diurnal variation on ^{3}H-thymidine uptake. Mice of the same strain, sex and age as above, weighing 28-32 gm were caged in groups of 5-6 animals in a conventional animal room exposed to natural light (August-September). To avoid possible variations arising from the estrous cycle, vaginal smears were examined daily at 08:00 - 10:00 h for at least a week. On the first day of diestrus (Allen, 1922), they were randomly assigned to one of the following four groups. Each of the four groups consisting of five animals each was given a single subcutaneous injection of 1 μCi of ^{3}H-thymidine (New England Nuclear, specific activity, 16.0 Ci/mM) per gram body weight at 03:00, 09:00, 15:00 and 21:00 h respectively, and one hour later anesthetized with ether. A segment of the descending colon was removed from the area located within one cm above the pelvic rim and placed in normal saline at room temperature. If free of fecal materials (scybala), the segment was immediately transferred to a 4% paraformaldehyde solution in 0.1 M Sörensen's buffer (pH 7.2 - 7.3) for a four-hour immersion fixation. If the segment contained a scybalum, a sharp cut was made towards its center and the scybalum was squeezed out by the contraction of the muscular coat in the colonic wall. Fixation was then done as above.

Method for light microscopic radioautography

In the first two experiments, perfusion was followed by further fixation of the colonic segment in the perfusion fluid for 2-4 hours. The segment was cut transversely into five or more ring-like pieces, each 1-2 mm high. After dehydration through graded concentrations of acetone, these pieces were embedded in Epon 812 (Luft, 1961) in such a way that the colonic wall would be cut in cross section (Fig. 1-1).

Six consecutive, one-micron thick Epon sections of the entire cross section of the colon were cut using the serial sectioning technique of Merzel and Leblond (1969). After having trimmed off 10 microns from the same Epon block, the next six consecutive sections were cut and mounted on the next slide. This procedure was repeated until six slides were prepared from each Epon block.

The glass slides with sections were placed on a hot plate at 60^{o}-80^{o}C for at least ten minutes to ensure good adhesion. They were then immersed into 1% aqueous periodic acid (PA) solution for one hour, washed in tap water for 10 minutes and stained for one hour with the Schiff reagent (prepared according to Tomasi, 1936; see Pearse, 1960). The slides were washed in running tap water for at least ten minutes, rinsed in distilled water and dried. They were then mordanted for ten minutes at 60^{o}C in 5% ferric ammonium sulfate and stained for the same length of time at the same temperature in Regaud's hematoxylin (Schantz and Schecter, 1965). The prestained Epon sections were radioautographed by the coating technique of Kopriwa and Leblond (1962) using

NTB-2 emulsion from Eastman Kodak. After exposure of one week in Experiment I, four weeks in Experiment II, and three weeks in Experiment III, the slides were developed, fixed, air-dried, and mounted in Permount.

Method for electron microscopic radioautography

After perfusion and fixation in paraformaldehyde, small pieces of the descending colon from Experiment I were postfixed in 1% osmium tetroxide solution in the Millonig's buffer solution for 1-2 hours, and then dehydrated and embedded in Epon. Ribbons of silver to gold Epon sections were mounted on celloidin coated slides and dipped in diluted liquid Ilford L-4 emulsion (Kopriwa, 1967); after exposure of 4 to 12 weeks, they were developed, stripped and mounted on 200 mesh copper grids. For staining, the grids were floated, with the emulsion side down in darkness, over a drop of saturated uranyl acetate (Watson, 1958) for 20-30 minutes and then over the lead citrate solution (Reynolds, 1963) for an hour in a chamber free of carbon dioxide. The preparations were examined in a Hitachi HS-7 electron microscope.

Measurements of the number of cells in longitudinal sections of crypts

In the present investigation, the term "cryptal column" was used to designate the cells located on one side of a longitudinally sectioned crypt (Fig. 1-2). For enumeration of the cells of a cryptal column, the counts were started from the midpoint at the base of the crypt, continued upward along the cryptal wall, and ended at the crypt-surface

junction. The size of the crypt was expressed by the number of epithelial cells present in the cryptal column, excluding lymphocytes and other migrating cells, particularly the cell with large crystal-like granules referred to as "crystal-containing cell" by Silva (1967). Similarly, the size of the corresponding surface epithelium was estimated by counting the number of epithelial cells present between the crypt-surface junction and the zone of cell extrusion.

Only crypts sectioned along their longitudinal axis were used; and these were defined as showing at least three quarters of the length of the lumen. At the same time, the base of the crypt had to be in apposition to the muscularis mucusae; and the cryptal column as well as the corresponding surface epithelium had to be cut in such a way as to show a single layer of epithelial cells.

The crypt-surface junction was determined by drawing an imaginary line making a 45^{o} angle with the longitudinal axis of the crypt, and going approximately from the crypt mouth to the upper portion of the lamina propria (Fig. 1-3).

The cell counts on the surface were done from the crypt-surface junction to the site of cell extrusion. This site was indicated either by the presence of an extruding cell (Figs. 1-10 and 1-11); or by the dimple left on the surface by a previous cell extrusion (Fig. 1-9 right); or by a nucleus located higher than other epithelial nuclei, since such a nucleus is believed to belong to the next cell to be extruded. Finally, it was found that the area of cell extrusion was frequently in apposition

to a capillary located at the top of the lamina propria just beneath the basement membrane of the surface epithelium. Failing to encounter any of these clues, the middle point between two adjacent crypts was used as the point where the count of surface cells was stopped.

Cryptal size and normalization of crypts

For quantitative analysis, twenty crypts, that is, forty cryptal columns, were examined in each animal in Experiments I and III, and ten crypts or twenty cryptal columns in Experiment II. For analysis, each cell in the cryptal column and in the corresponding surface epithelium was examined, and a record was kept of its type, its position from the base, and the phase of cell cycle.

The cryptal size as expressed by the number of epithelial cells per cryptal column varied from 18 to 42 cells (Fig. 1-4) in 400 cryptal columns from twenty animals (Experiment II). The overall distribution of the cryptal sizes essentially followed a normal distribution with mean value of 29 cells per cryptal column. Further analysis was carried out to find out whether this variation was due to a variation within animals or among animals. The mean cryptal size for individual animals ranged from 21.8 to 35.3 cells per cryptal column. The standard deviation in each animal was of the order of 10% of the mean. One-way analysis of variance (Freund et al., 1960) revealed that there was a significant difference in cryptal sizes among animals (see Appendix 1-1).

When a cryptal column was composed of 30 cells, it was divided into 10 segments of three cells each, as shown in Figure 1-3. When

the number of cells in the cryptal column was greater or smaller than 30, the number of cells per segment was made greater or smaller than three, in proportion to the number of cells in the column. This procedure, called normalization, was carried out as suggested by Cairnie and Bentley (1967), that is, each cell in each cryptal column was converted proportionally to one of 100 units in the standard column; these 100 units were then divided into ten equal segments, each with ten units. The cell was then assigned to the proper segment.

For convenience, the segments were numbered from the base of the crypt, where segment 1 was located, to the mouth of the crypt where segment 10 was situated (Fig. 1-3). Since there was an average of nearly 30 cells in one cryptal column, each segment of the crypt would be made up of three actual cell positions. All the epithelial cells in each segment of the crypt were pooled for each experimental group and classified for the analysis of cell distribution and behavior in each segment of the crypt.

RESULTS

The mucosa of the descending colon may be smooth or folded around the lumen (Fig. 1-1) according to whether the tunica muscularis is relaxed or contracted. The mucosa is filled with straight test-tube like crypts whose blind end is usually in apposition to the muscularis mucosae. The shape, length and diameter of the crypts are fairly uniform (Fig. 1-1), although some variation is encountered.

Types of epithelial cells in mouse descending colon

The free surface and the crypt walls are lined by a single layer of epithelial cells (Fig. 1-2). All cells abut on the basement membrane and their nucleus is next to it, except for mitotic cells whose nucleus undergoes a temporary migration toward the cryptal lumen. The cells may be grouped into two main classes, according to whether or not they stain with PA-Schiff.

(A) The PA-Schiff negative class comprises several cell types:

(1) vacuolated cells, the cytoplasm of which contains unstained vacuoles. At the base of the crypts (segment 1), these cells have a rather small number of vacuoles (Figs. 1-5, 1-7), but higher up (segments 2 to 7) their apical cytoplasm is filled with partly confluent vacuoles (Figs. 1-5 and 1-23). These vacuoles may be observed whether fixation is carried out with glutaraldehyde, paraformaldehyde or osmic acid, and, therefore, are not artefactual. In the electron microscope, the vacuoles are partly bound by a membrane and contain very fine fibrils in varying amounts (Fig. 1-23). Mitochondria are scanty, and free ribosomes numerous. The apical surface of these cells may be smooth or contain a few microvilli. Vacuolated cells are not present in segments 8-10 nor on the surface.

(2) columnar cells, which are observed above segment 8 (Fig. 1-8) and on the surface (Fig. 1-9). They have a lighter nucleus than that of vacuolated cells and an apical surface covered by a striated

border (Fig. 1-8, top), which in the electron microscope is seen to be composed of packed microvilli (Fig. 1-25). They contain more numerous mitochondria but fewer ribosomes than vacuolated cells. Some cells, particularly in segment 8, may show a few vacuoles as well as a striated border. Such cells appear to be intermediate between vacuolated and columnar cells, and are termed "transitional cells " (Fig. 1-8).

The columnar cells on the surface are rather narrow. Their nucleus is less basally located and has more indentations and peripheral clumps of chromatin than in columnar cells located in segments 8-10 (Fig. 1-10). Occasionally on the surface a columnar cell has a pyknotic nucleus and dark cytoplasm, partly separated from the adjacent surface epithelial cells. Such cell is interpreted as being extruded (Fig. 1-10).

(3) enterochromaffin cells, which may be observed along the whole length of the crypt, but differ according to location. In segments 1 through 4 most enterochromaffin cells appear roughly triangular with a broad base abutted on the basement membrane (Figs. 1-16 and 1-17). When the cell apex approaches the cryptal lumen, it narrows and shows a thick brush border. However, most enterochromaffin cells observed in sections do not reach the lumen. The nucleus is rather light, round or ovoid, occasionally indented, with finely granulated chromatin and one to three nucleoli at the periphery. The cytoplasm is comparatively pale and contains a

varying amount of very fine, darkly stained granules predominating at the base (Figs. 1-16 and 1-17). A few cells, in which granules are present within vesicles but are not readily seen display a broad apex containing a filamentous bundle which appears to be continuous with the brush border. Like enterochromaffin cells, they have a light nucleus and pale cytoplasm (Fig. 1-15). These appear to be the multivesicular cells described by Silva (1966) in the distal colon of the mouse and will be classified here as enterochromaffin cells.

Enterochromaffin cells seen above segment 5 of the crypt usually have a long foot process which is filled up with fine granules (Fig. 1-12) (see Schofield, 1952; Schofield and Silva, 1968). The foot process, which is better appreciated in serial sections, extends along the basement membrane toward the bottom of the crypt; but the apex of the cells can be visualized only with much difficulty even in serial sections. The multivesicular cells of Silva may also be seen in this region (Fig. 1-14).

In the surface epithelium, enterochromaffin cells tend to have a fusiform shape (Fig. 1-13). Most but not all contain abundant fine granules, and their nuclei tend to have densely stained fine chromatin granulations with occasional chromatin flakes. A long brush border is again evident in these cells.

In conclusion, there are three types of PA-Schiff negative cells: vacuolated, columnar and enterochromaffin cells.

(B) PA-Schiff positive cells : the mucous cells

These cells contain a variable number of globules of mucus stained with PA-Schiff. In segments 1-2, mucous cells are small. Some have only few isolated mucous globules in the supranuclear, presumably Golgi, region (Fig. 1-7), while others have the globules collected into a narrow theca (Fig. 1-5). Assessment of the amount of mucous globules requires serial sections. In some instances, the very identification of mucous cells requires serial sections, since this is the only procedure by which isolated mucous globules may be detected in the supranuclear area. The PA-Schiff staining of mucous globules is less intense in the low segments than in segments 7 through 10. Usually the periphery but not the center of mucous globules appears to be stained, giving the theca a honey-combed appearance (Figs. 1-5 and 1-7). This may be an artefact since, when the colon is postfixed in osmic acid for electron microscopy, the content of mucous globules is homogeneous, consisting of a feltwork of fine granules and fine fibrils (Fig. 1-24). This is in contrast with the vacuoles of vacuolated cells which contain no fine granules. The remaining cytoplasm of mucous cells tends to be darker than in adjacent vacuolated cells, and its staining intensity parallels that of the nucleus.

Going from segments 2 to 5, mucous cells contain an increasing amount of mucous globules, but the theca remains narrow and cylindrical (Fig. 1-5), while the nuclei become darker. The rim of peripheral cytoplasm is darkly stained by iron-hematoxylin. In segments 5 and 6,

the theca is enlarged, with increase in staining of mucus with PA-Schiff, and of nucleus and cytoplasm with iron-hematoxylin (Fig. 1-6).

In the upper four segments of the crypt, the swollen theca gives mucous cells a typical goblet form (Fig. 1-9). The nuclei are dark and pushed to the foot of the goblet. The mucous globules are stained more intensely and homogeneously than those in the lowest segments.

On the surface, most mucous cells are narrow and contain a small amount of mucus (Fig. 1-9). The nuclei are dark and tend to elongate along the long axis of the cells (Fig. 1-10). In some mucous cells, the staining intensity of the cytoplasm with iron-hematoxylin seems to decrease in comparison with mucous cells in segments 7 through 10 of the crypt. Rarely present in the surface epithelium is a mucous cell with a pyknotic nucleus, dark cytoplasm and a small amount of mucous globules, separating partly from the adjacent columnar cells (Fig. 1-11). This cell is interpreted as being extruded.

Distribution of the four types of epithelial cells in the crypt and on the surface

The four main types of epithelial cells were enumerated in each segment and on the surface. The results (Table 1-1) showed that columnar cells were present only in the highest segments, and vacuolated cells only in the lower. Adding up the percentages of vacuolated, transitional and columnar cells in segments 6 through 9 yielded 80-85% at each level, that is, approximately the same as the percent of vacuolated cells in segments 1 through 5 and the percent of columnar cells in segment 10.

However, the percent of columnar cells went up to 89 on the surface.

Enterochromaffin cells made up 1-2% of the population at all levels, except in segments 1 and 2 (about 4%) and in segments 10 and on the surface where they became scarce.

Mucous cells amounted to about 16% in all segments, although their number was significantly smaller in segment 1 and on the surface (see Appendix 1-2).

Sites of cell proliferation and pathways of migration as shown after a single injection of ^{3}H-thymidine

The location of label in animals sacrificed one hour after injection of ^{3}H-thymidine pinpointed the cells where DNA synthesis took place prior to mitosis, whereas their location at later intervals indicated the pathways of migration (Experiments I and II).

(A) PA-Schiff negative cells

Of these, only vacuolated cells were labeled at one hour after ^{3}H-thymidine injection (Figs. 1-18, 1-19, 1-23). The frequency of labeling was high in segments 1 through 4 only. In segment 1, the dividing cells often had only few vacuoles.

By 36 hours, the labeled vacuolated cells increased in number and some of them were located higher up than at the 1-hour interval (Fig. 1-26). At the same time, some of the transitional and columnar cells in segments 7 through 10 of the crypt became labeled (Figs. 1-20 and 1-26). By 72 hours or later, the number of labeled vacuolated, transitional and columnar cells was increased in the crypts, and some

columnar cells in the surface epithelium were labeled (Fig. 1-22).

From these observations, it was concluded that labeled cells migrated from the lower segments to the surface and that, in so doing, those labeled cells which at first were of the vacuolated type transformed into columnar cells through the stage of transitional cells. The columnar cells reached the surface, where occasionally a labeled one was seen to be extruded. In other words, vacuolated cells and columnar cells constituted a single cell line, in which vacuolated cells represented the dividing compartment and columnar cells, the non-dividing compartment.

Finally, of all enterochromaffin cells examined, none was labeled at one hour after injection of ^{3}H-thymidine. By 36 hours or later, a few were (Fig. 1-27). These labeled enterochromaffin cells were located in segments 1 and 2 of the crypt; at later times, some of those located higher in the cryptal walls also became labeled. While enterochromaffin cells because of their characteristic morphology constituted a distinct cell line, they were not labeled at one hour and therefore should originate from another cell type.

(B) PA-Schiff positive cells : The mucous cells

At one hour after injection of ^{3}H-thymidine, mucous cells were often labeled in the lower four segments of the crypt, seldom in segments 5 and 6, but never in the highest segments. The mucous cells labeled in segments 1 to 4 contained either scattered globules of mucus or a small theca (Fig. 1-24). Hence proliferative activity was encountered

in mucous cells only when the amount of mucous globules present was rather small.

By 36 hours or later after injection of labeled ^{3}H-thymidine, silver grains were observed over the nuclei of mucous cells with swollen theca in the upper segments of the crypt (Fig. 1-21); and by 72 hours or later, over the nuclei of narrow mucous cells in the surface epithelium (Fig. 1-22).

From these observations, it was concluded that the mucous cells arising in the lower portion of the crypt migrated upwards, and in doing so, underwent morphological changes.

DISCUSSION

The renewal of the epithelial cells of the large intestine was first observed independently by Hughes et al. (1958) and Walker and Leblond (1958) in the mouse. From these and subsequent works (Messier and Leblond, 1960; Messier, 1960; Cole and McKalen, 1961; Lipkin and Quastler, 1962; Lipkin et al., 1963a, 1963b; MacDonald et al., 1964; Shorter et al., 1964; Lipkin, 1965a, 1965b; Lipkin, 1966; Thrasher, 1967a; Sawicki et al., 1968), it was learned that epithelial cells proliferate in the lower portion of the crypt and migrate toward the surface from which they are eventually extruded. These conclusions were fully confirmed in the present investigation, and, in addition, the cell types involved in proliferation and migration were identified.

Validity of techniques

The histological technique selected had to be such as to reveal all cell types clearly. This goal could not be achieved in paraffin sections regardless of the stain used, but was accomplished using 1-micron thick Epon sections. Furthermore the staining technique had to be such as to neither activate nor inhibit the emulsion used for radio-autography. Pilot tests revealed that there were no differences between the grain count of unstained and PA-Schiff-iron-hematoxylin stained radio-autographs. This staining procedure was, therefore, adopted for the investigation.

A key technique was the arbitrary subdivision of crypts into ten equal segments. Since the average crypt column consisted of 29, that is, nearly 30 cells, it was considered that each one of the ten segments included three cells on the average. When the number of cells was greater or smaller, a proportionate correction was carried out to "normalize" the numbers of crypt cells to 30. Cairnie and Bentley (1967) compared short and long crypts in the rat small intestine and concluded that "a long crypt resembles, at least in so far as proliferation is concerned, a short crypt stretched to the same length." A similar conclusion was reached in the crypts of the colon since we found, for instance, that the location of transitional cells was in or near segment 8, whatever the number of cells in the crypt column was.

The four cell types and their ability to divide

(1) The vacuolated cells occupied the lower three-quarters of the crypts. They were characterized by vacuoles which stained neither with PA-Schiff nor with PA-silver (Hollmann, 1965), and therefore were presumed not to contain glycoprotein. They probably contained protein material and, according to Wetzel et al. (1966), they would include some acidic carbohydrate. In the present investigation vacuoles were seen protruding into the cryptal lumen, suggesting discharge in a manner similar to the release of mucous globules from mucous cells. With regard to their ability to proliferate, vacuolated cells were often seen in mitosis; and many of them were labeled at one hour after ^{3}H-thymidine injection, thus indicating that they were about to divide.

(2) The columnar cells had a striated border believed to indicate absorptive properties. These cells were never seen in mitosis, nor were they labeled at one hour after ^{3}H-thymidine injection.

(3) The mucous cells varied in appearance according to the region of the crypts. Some were found in mitosis in the lower six segments and some became labeled soon after injection of ^{3}H-thymidine. Hence, there was no doubt that mucous cells could divide. The dividing capacity of mucous cells was questioned by a number of investigators in the field, who claimed that cell division and cell specialization such as mucogenesis were two antagonistic processes which could not take place at the same time (Macklin and Macklin, 1932). However, mucous cells with mitotic figures were described by several workers (Bizzozero, 1889, 1892;

Sacerdotti, 1894; Zipkin, 1903; Leblond and Messier, 1958; Thrasher and Greulich, 1966; Merzel and Leblond, 1969). The present work supported the capability of mucous cells to undergo mitosis, but mitosis was mainly seen in those mucous cells which contained small amounts of mucous globules and which were located in the lower portion of the crypt. Therefore, a certain degree of mucogenesis did not necessarily interfere with cell division. Yet the dividing capacity decreased as more and more mucous globules were accumulated in the cells, and it eventually disappeared, as cells ascended the cryptal wall. In addition, since the segments where mitosis was found were the same for vacuolated and mucous cells, it was possible that environmental factors exerted some control on the division of both.

(4) Enterochromaffin cells with their characteristic granules were never seen in mitosis nor were they ever labeled at one hour after ^{3}H-thymidine injection.

In conclusion then, only two of the four cell types, vacuolated and mucous cells, had the ability to divide, at least in the lower six segments of the crypts. In contrast, columnar and granule-containing enterochromaffin cells did not have the ability to enter into mitosis.

Epithelial migration

When radioautographs of the epithelium were examined 36 hours and later after ^{3}H-thymidine injection, vacuolated and mucous cells labeled at the time of the injection had completed division and it was possible to trace the fate of their labeled daughter cells.

Progeny of vacuolated cells

By 36 hours after ^{3}H-thymidine injection, the label appeared in transitional cells. Since these cells retained a few small vacuoles (which, according to Wetzel et al., 1966, have the same histochemical affinities as those in vacuolated cells), their acquisition of label was attributed to a transformation of the labeled daughter cells of vacuolated cells. The later appearance of the label in columnar cells was attributed to further evolution in this direction.

That the three cell types — vacuolated, transitional and columnar — correspond to three stages in the evolution of the cells of a single population was confirmed by the fact that, if the numbers of these three cells were added for each segment (Table 1-1), the total was always between 80 and 85% (except 89% on the surface).

It was concluded that the three cell types belonged to a single line. The loss of vacuoles and the appearance of a striated border as cells migrated upwards suggested that the functional emphasis was at first on secretion but shifted later to absorption as the cells reached the upper part of the crypt and the surface.

Progeny of dividing mucous cells

By 36 hours the label appeared in some of the larger mucous cells located around segment 8 (Fig. 1-21) and by 72 hours, in the narrower mucous cells approaching the surface (Fig. 1-22). The morphological gradation between the narrow cells at the base of the crypt and the large ones toward segment 8, as well as the progressive

change at the surface completed the demonstration that mucous cells constituted a single cell line.

Some of the mucous cells at the bottom of the crypt were hardly distinguishable from vacuolated cells, since they had a small amount of vacuoles in the supranuclear region in addition to a few PA-Schiff positive mucous globules. This may suggest that both mucous cells and vacuolated cells have the same origin in the lower portion of the crypt. The lower frequency of mucous cells in segment 1 of the crypt gave some support to this possibility. Hence, the present investigation supported a theme similar to that of Merzel and Leblond (1969), that is, mucous cells would originate from poorly differentiated vacuolated cells, but would still have a limited capacity to divide.

Finally, since both production and discharge of mucus were continuous (Neutra and Leblond, 1966), the swelling of mucous cells to a goblet shape as they reached the upper half of the crypt was attributed to a predominance of mucus production over discharge, whereas the narrowing of these cells as they reached the surface would be due to the opposite.

Origin of enterochromaffin cells

In addition to the typical enterochromaffin cells, it was decided for the time being to include in this cell line the cells with a large bundle of filaments in their apical region, the multivesicular cells of Silva (1966), even though their granules were poorly visible. That such granules existed was indicated by the electron microscope pictures of

Silva (1968). It was tentatively assumed that these cells were a special differentiation of enterochromaffin cells.

Enterochromaffin cells differed from the other two cell lines not only by their morphological features, but also by the absence of dividing cells. Those enterochromaffin cells containing granules were never labeled at one hour after ^{3}H-thymidine injection. Yet since some of these cells became labeled by 36 hours and later, perhaps enterochromaffin cells arose from the poorly differentiated vacuolated cells at the bottom of the crypt.

Existence of three cell lines:

Based on the morphological characteristics, the distribution and the renewal behavior of the cells, the present work reveals that there are three basic cell lines in the epithelium of the descending colon in the mouse: vacuolated-columnar, mucous, and enterochromaffin cell lines. The additional cell types described: vacuolated cells (Hollmann, 1965; Spicer, 1960, 1965; Wetzel, et al., 1966) and multivesicular cells (Silva, 1966) appear to belong to the three main cell populations. Paneth cells were absent.

An attractive hypothesis based on the observations was that the three main cell lines had their source in the same poorly differentiated cells. Thus, the three cell lines would not be independent and capable of self-renewal, as Thrasher and Greulich (1966) claimed for columnar cells, mucous cells and Paneth cells in the mouse small intestine. Instead, the mucous and enterochromaffin cell lines would be dependent

on the same poorly differentiated cells at the base of the crypt from which the vacuolated-columnar cell line originates.

SUMMARY

The epithelium of the descending colon in the mouse was examined using one micron thick Epon sections of paraformaldehyde-fixed tissues stained with PA-Schiff and iron-hematoxylin, and radioautographed.

From morphological observations and the labeling of cells after injection of ^{3}H-thymidine, it is established that there are three cell lines in the epithelium of the descending colon: vacuolated-columnar, mucous, and enterochromaffin cell lines.

Vacuolated-columnar and mucous cells arise from mitosis in the lower portion of the crypt; the two intermingled populations migrate toward the surface, and after reaching it, are extruded to the colonic lumen. In the first cell line, vacuolated cells divide to produce more vacuolated cells, but as they migrate upwards, they lose their vacuoles while ceasing to divide and become typical columnar cells. The mucous cells making up the second cell line arise from mitosis of cells with a small amount of mucus. As daughter cells migrate upwards, their theca swells, presumably because production of mucus exceeds release. Conversely on the surface, the theca becomes narrow as release predominates. In the third cell line, the enterochromaffin cells identified by the presence of fine granules do not divide and appear to originate from another cell type.

Table 1-1. Distribution of the various types of epithelial cells in the crypt and on the surface in the descending colon of the mouse.*

	Vacuolated cells	Transitional cells	Columnar cells	Mucous cells	Entero-chromaffin cells
Surface	-	-	89.3	10.5	0.2
Crypt segment 10	-	-	84.5	15.3	0.2
9	0.1	10.3	72.3	16.5	0.8
8	15.5	52.6	14.9	16.0	1.0
7	66.2	15.9	0.2	16.5	1.2
6	82.4	0.1	-	16.5	1.0
5	81.5	-	-	17.1	1.4
4	80.0	-	-	19.0	1.0
3	80.1	-	-	17.9	2.0
2	81.1	-	-	15.0	3.9
1	83.3	-	-	12.7	4.0

*Data are expressed in percentage of each cell type in each segment of the crypt and are mean values for the twenty animals in Experiment III.

Fig. 1-1. A cross section of the descending colon of the mouse in a state of contraction. From outside in, one may see a dark band of smooth muscle, a narrow light area (submucosa), a thin band of muscularis mucosae and the mucosa itself with its numerous crypts of Lieberkühn (and a lymphatic nodule). x 47.

This and all photographs taken in the light microscope were of sections stained with the PA-Schiff technique and iron hematoxylin.

(1)

Fig. 1-2. Two longitudinally sectioned but slightly curved crypts in the descending colon. x 420.

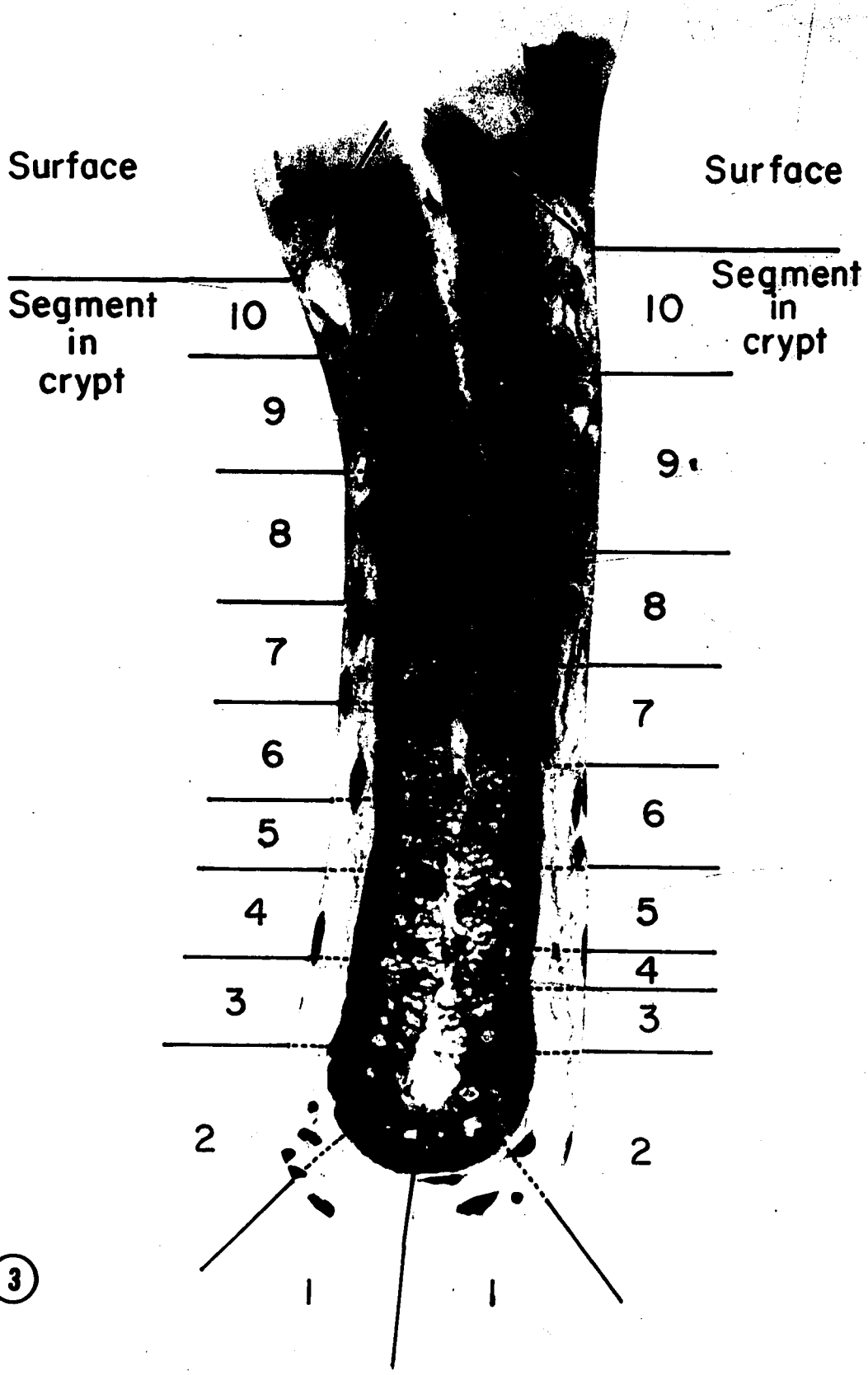
In the upper half of the crypt, large mucous cells are seen; the mucus has a honey-combed appearance. In the lower half, mucous cells are also present, but they are small and can hardly be recognized at this magnification. Among the PA-Schiff negative non-mucous cells, ordinary columnar cells with uniformly stained cytoplasm are located on the surface and in the upper part of the crypt, whereas those cells in the lower two-thirds of the crypt have a foamy appearance which at a higher magnification proves to be due to PA-Schiff negative vacuoles. They are referred to as vacuolated cells.


2

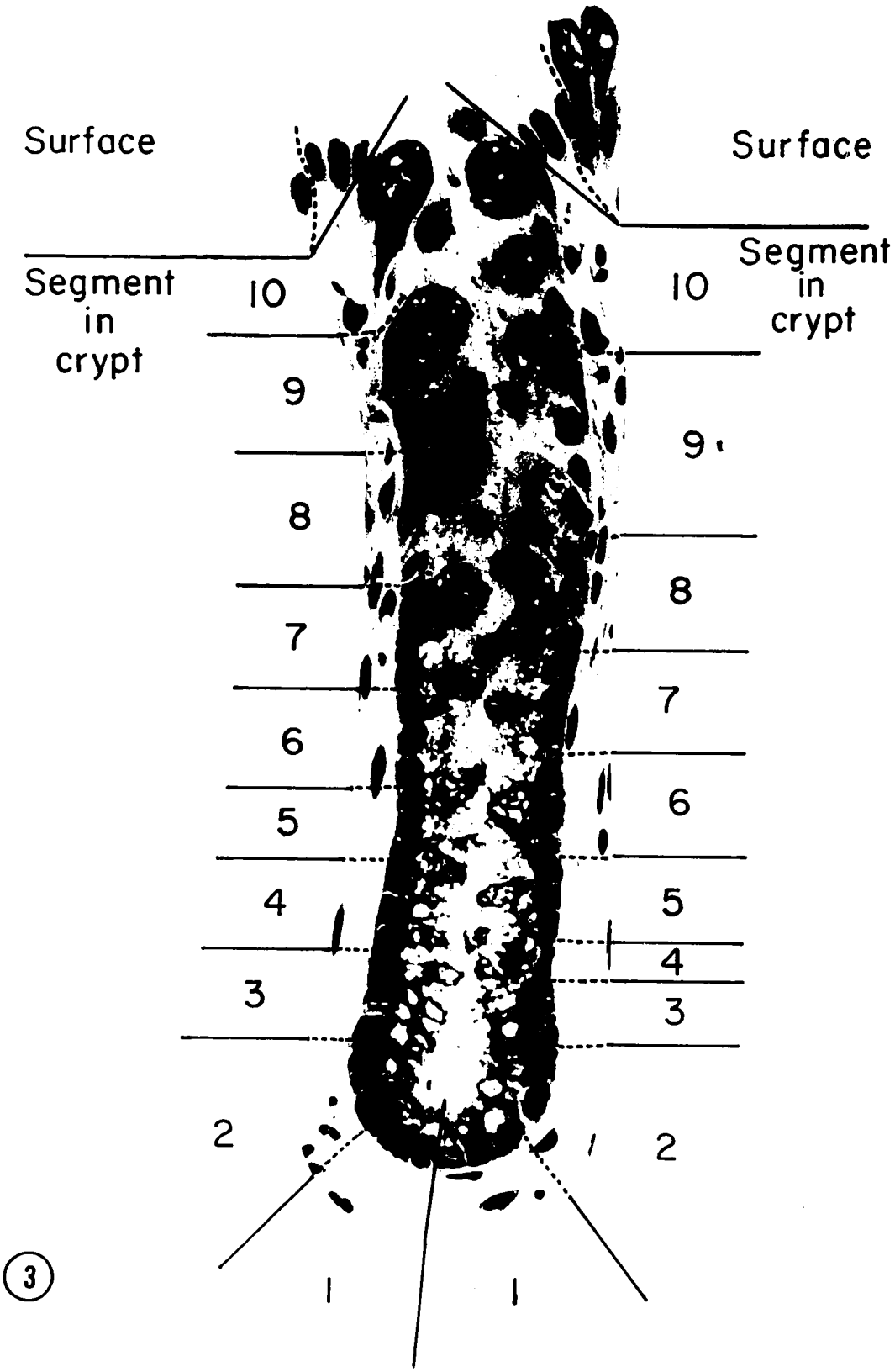
Fig. 1-3. A longitudinally sectioned crypt in the descending colon of the mouse, in which the segments in the crypt and the surface epithelium are drawn.

There are thirty cells on the left side of the cryptal wall from the center at the base of the crypt (solid line) to the crypt-surface junction (solid line) (designated as cryptal column), and three cells in the corresponding surface epithelium from the crypt-surface junction to the site of cell extrusion. Each segment of the crypt contains three cells. The segments are numbered from the base of the crypt (segment 1) to the crypt-surface junction (segment 10).

In the right cryptal column, there are thirty-one cells. Each segment has three cells, except for segment 9 where four cells are included. The corresponding surface epithelium contains three cells.

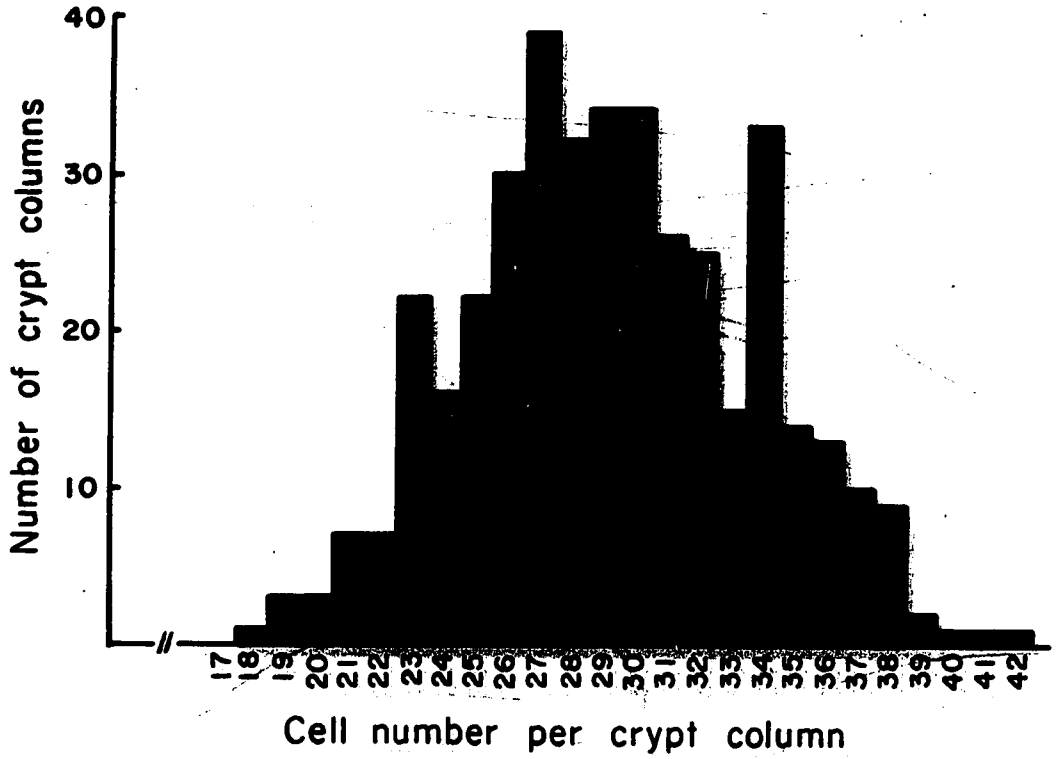

Surface
Surface
Segment in crypt
Segment in crypt
10
10
9
9
8
8
7
7
6
6
5
5
4
4
3
3
2
2
1
1

(3)

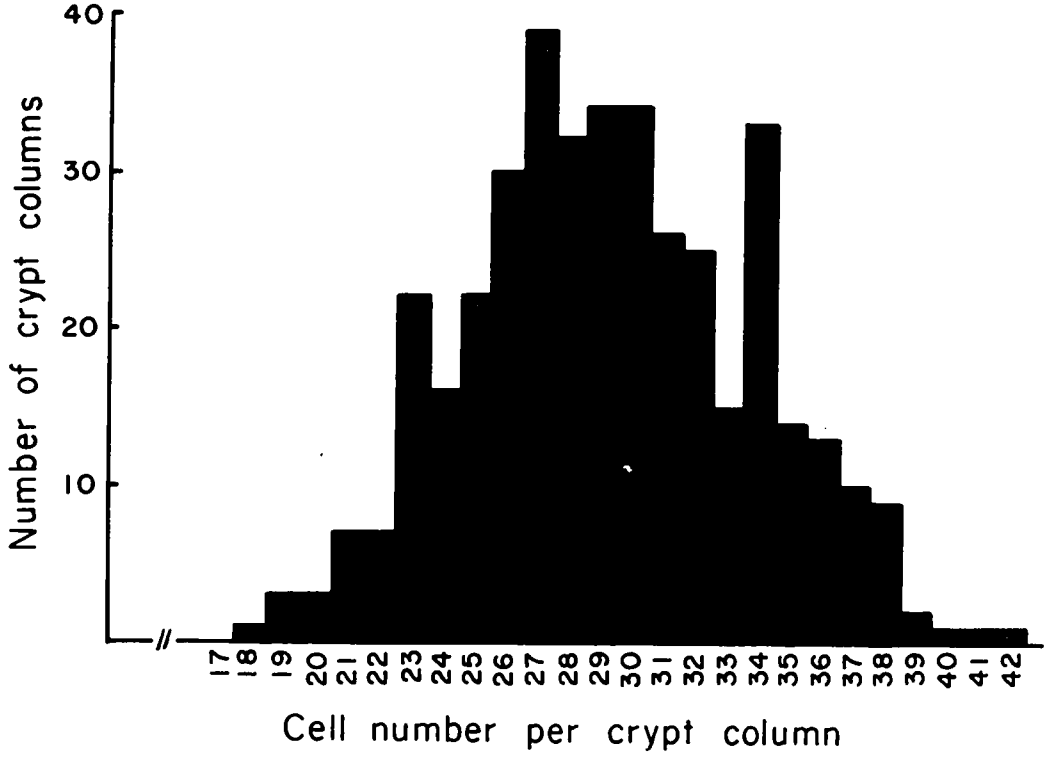

Surface
Surface
Segment in crypt
Segment in crypt
10
10
9
9
8
8
7
7
6
6
5
5
4
4
3
3
2
2
1
1

(3)

Fig. 1-4. Histogram showing the distribution of measurements of cryptal size expressed as the number of epithelial cells per cryptal column. The sample consists of 400 cryptal columns from 20 animals. (Experiment II).


40
30
20
10
Number of crypt columns
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
Cell number per crypt column

4


40
30
20
10
Number of crypt columns
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
Cell number per crypt column

(4)

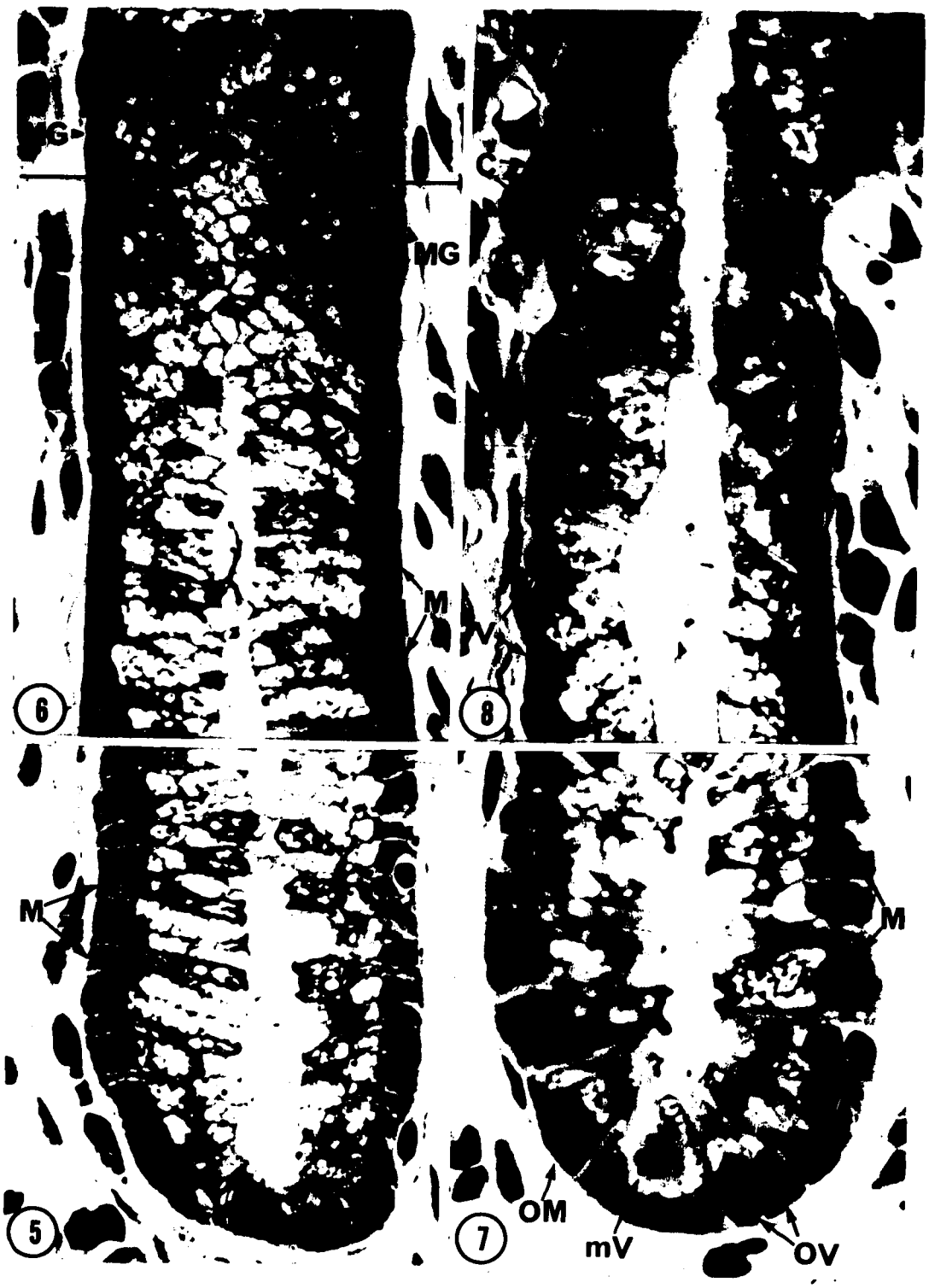
Figs. 1-5 to 1-8. These pictures represent the base (Figs. 1-5, 1-7) and middle portion (Figs. 1-6, 1-8) of crypts.

Fig. 1-5. The lower portion of a crypt. A few PA-Schiff positive mucous cells with small theca (M) are seen among the numerous PA-Schiff negative vacuolated cells. x 1300.

Fig. 1-6. Most of the picture represents the middle portion of a crypt; the horizontal lines near the top limit the upper portion. The PA-Schiff positive mucous cells in the middle portion of the crypt are cylindrical in shape (M), whereas those in the upper portion of the crypt assume a goblet form (MG). All the PA-Schiff negative cells are vacuolated cells in the middle portion of the crypt; transitional and columnar cells in the upper portion of the crypt are not clearly depicted. x 1300.

Fig. 1-7. The base of a crypt. A mucous cell with a few mucous globules (OM) is seen next to a mitosis of a vacuolated cell (mV). Mucous cells with a small theca (M) are seen at right. Cells containing a small amount of PA-Schiff negative vacuoles are present at the base (OV), next to the mitosis mentioned above. x 1700.

Fig. 1-8. The upper portion of a crypt showing vacuolated (V), transitional (T), and columnar (C) cells. The transitional cell (T) contains many small discrete vacuoles or vesicles in both the supranuclear and apical area. Columnar cells have no vacuoles in the apical region (but a few may be present in the supranuclear area corresponding to the Golgi region); they are further characterized by a prominent brush border. Vacuolated cells have both the supranuclear and apical areas filled up with vacuoles, and they have no brush border. Nuclei are darker in vacuolated than columnar cells. x 1700.


G
MG
M
6
8
M
M
5
7
OM
mV
OV

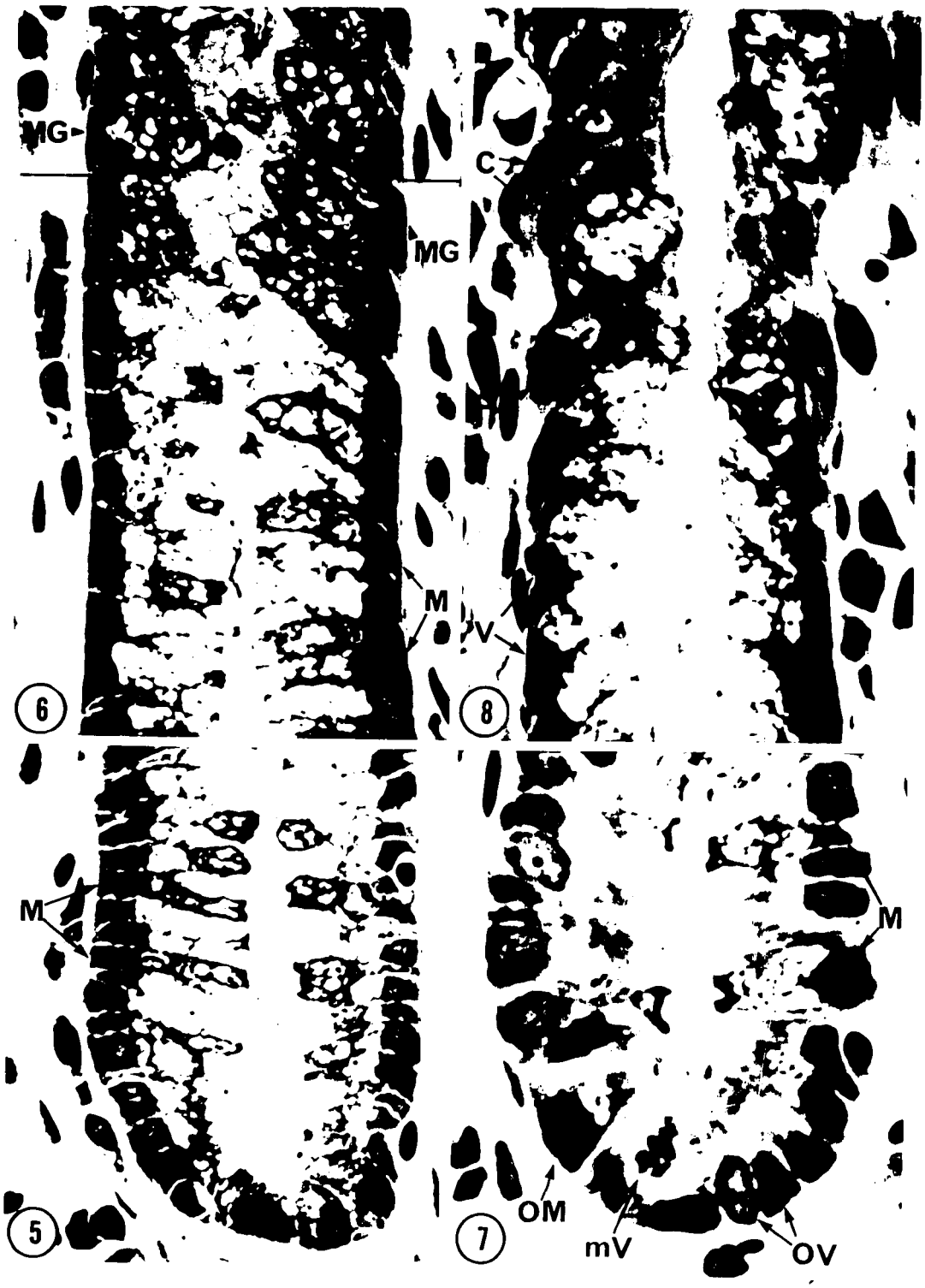

MG
MG
M
6
C
V
8
M
M
5
OM
mV
7
OV

Fig. 1-9. The upper portion of a crypt and the surface epithelium. The mucous cells in the upper portion of the crypt have a typical goblet form with dark nuclei in the foot of the goblet and darkly stained mucus in the swollen theca. The PA-Schiff negative cells are all columnar. x 1300.

Fig. 1-10. The mouth of a crypt and the surface epithelium. In comparison with columnar cells in the crypt, the columnar cells in the surface epithelium tend to have dark, narrow nuclei located in the middle instead of at the base of the cells. On the surface, a columnar cell with a pyknotic nucleus and dark cytoplasm (arrow) is seen to be partially separated from the adjacent columnar cells; this cell is interpreted as being extruded. x 1300.

Fig. 1-11. In the surface epithelium, a mucous cell is occasionally seen with a pyknotic nucleus and dark cytoplasm (arrow), which is also interpreted as being extruded. x 1700.

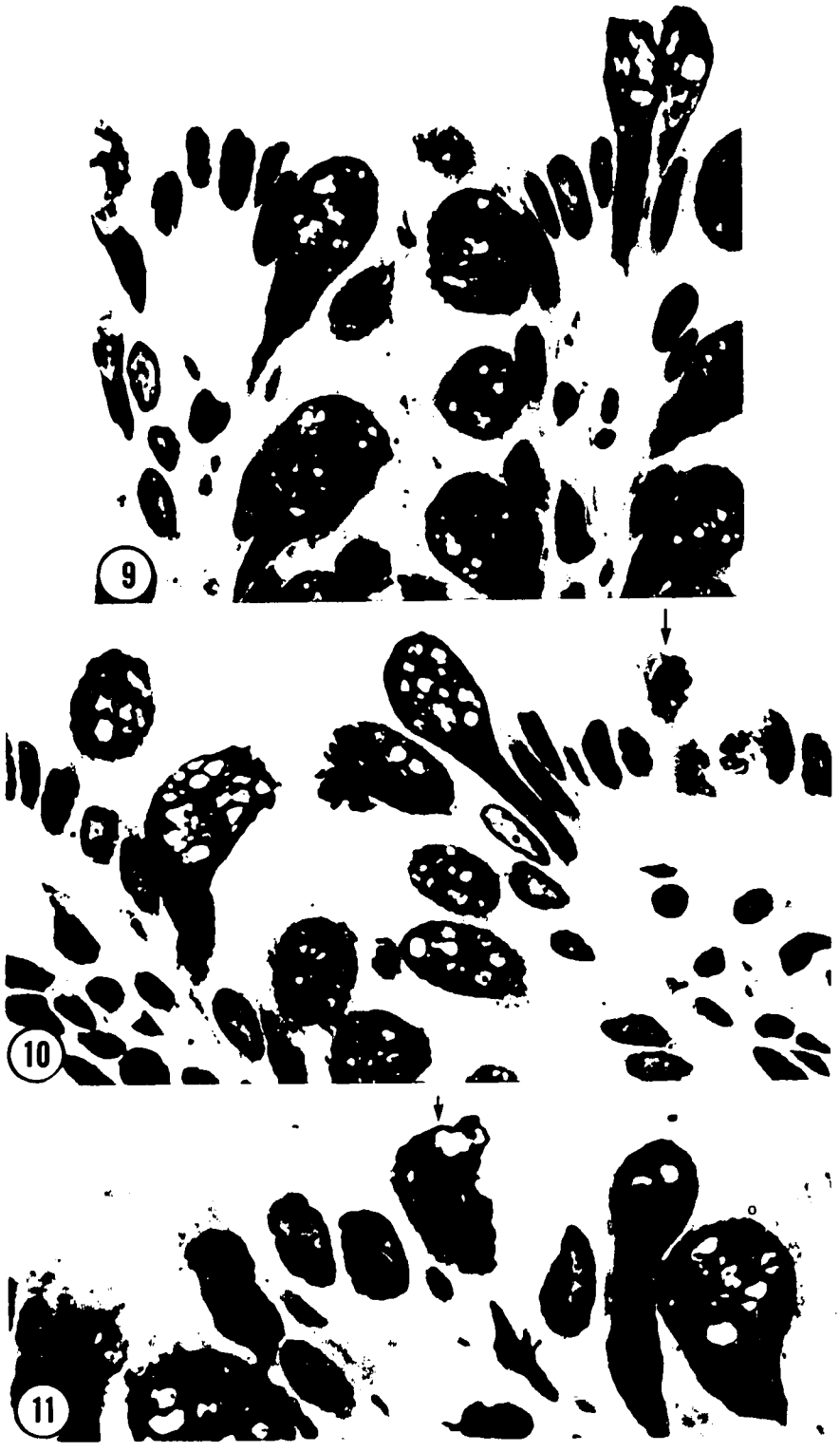

9
10
11

Fig. 1-16. In the lower portion of a crypt is a triangular shaped enterochromaffin cell with its apical portion extending narrowly to the cryptal lumen. The dark area at the lower left of the nucleus is due to a group of enterochromaffin granules. These are often present in the basal region of the cell, as is the case here. This is a radioautograph prepared from a mouse given ^{3}H-thymidine continuously for 5 days; other epithelial cells are labeled. x 1700.

Fig. 1-17. The lower portion of a crypt. An enterochromaffin cell is seen on the right side of the cryptal wall. Clumps of granules are present at the base of the cell and in the perinuclear area. The pale area above the nucleus is the Golgi region. A small brush border is present at the narrow apex. x 1700.

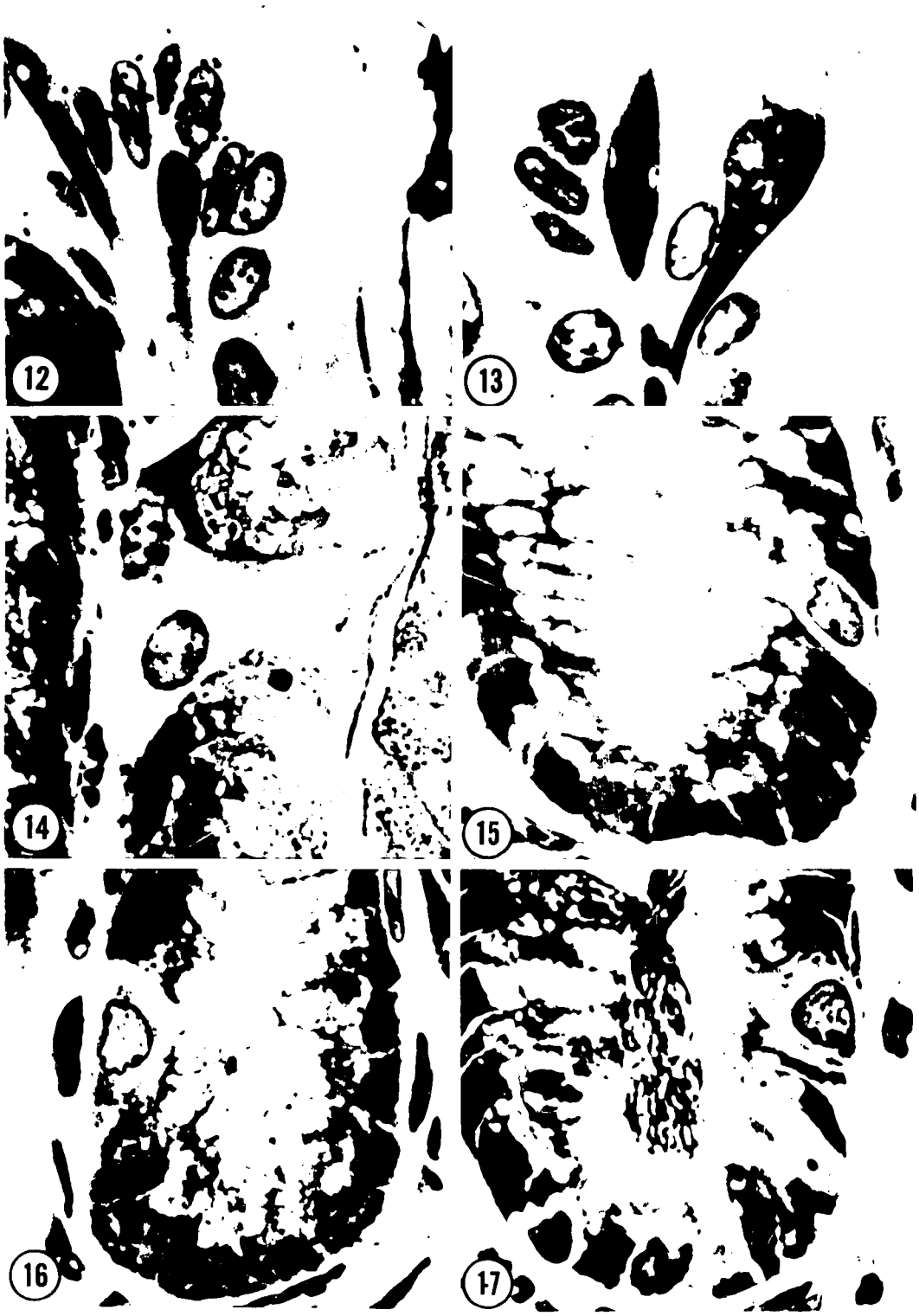
Figs. 1-12 to 1-17. Enterochromaffin cells.

Fig. 1-12. The mouth of a crypt and the surface epithelium. An enterochromaffin cell with a long basal process filled up with darkly stained granules is located near the crypt-surface junction. Silver grains are present over the nuclei of many columnar cells, since this radioautograph is prepared from an animal given ^{3}H-thymidine continuously for 5 days. x 1700.

Fig. 1-13. Present in the surface epithelium is a fusiform-shaped enterochromaffin cell containing darkly stained granules throughout the cytoplasm. x 1700.

Fig. 1-14. The upper portion of a crypt. In the center of the picture, a large enterochromaffin cell shows an ovoid nucleus containing mainly fine chromatin granulations. The cytoplasm is comparatively pale with perinuclear granules which are not distinguished in the photograph. This cell has a well developed brush border, from which some filamentous structures are extending into the apical cytoplasm of the cell. This picture is taken from a radioautograph prepared from an animal given ^{3}H-thymidine continuously for 8 days; all other epithelial cells are labeled. x 1700.

Fig. 1-15. Near the base of a crypt, there is an enterochromaffin cell with pale cytoplasm in which granules are hardly recognizable. A long brush border with filamentous structures is seen in the apical region. x 1700.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17

Figs. 1-18 to 1-22. Light microscopic radioautographs from mice sacrificed at various times after a single injection.

Fig. 1-18. One-hour interval. This is the lower portion of a crypt with a few labeled vacuolated cells. Some of the cells at the bottom of the crypt contain only few vacuoles, but they may also be labeled. x 1300.

Fig. 1-19. One-hour interval. A few labeled vacuolated cells and a mitotic vacuolated cell (mV) are seen in the lower portion of a crypt. x 1300.

Fig. 1-20. 36-hour interval. Upper portion of a crypt. Two columnar cells (C) and one transitional cell (T) are labeled. x 1300.

Fig. 1-21. 36-hour interval. Upper portion of a crypt showing one labeled columnar cell and one labeled mucous cell (arrow). x 1300.

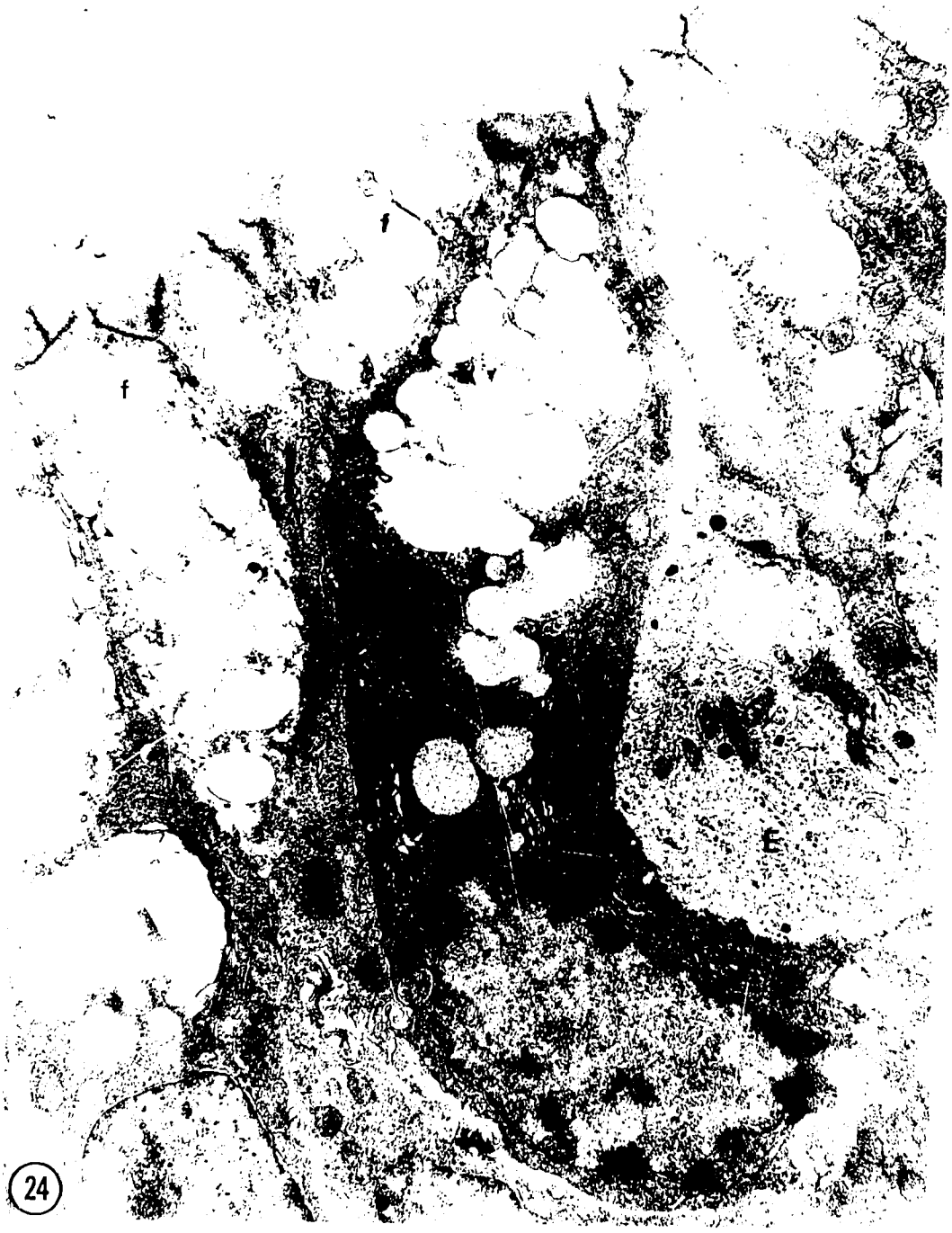
Fig. 1-22. 72-hour interval. A few columnar cells on the surface as well as in the upper portion of the crypt are labeled. Also labeled are a narrow mucous cell in the surface epithelium (one arrow) and a mucous cell at the mouth of a crypt (two arrows). x 1300.


72 h
22
36h
C
C
T
20
21
1 h
1 h
mV
18
19

Fig. 1-23. Electronmicroscopic radioautograph of the middle portion of a crypt from a mouse killed one hour after injection of ^{3}H-thymidine, stained with both uranyl acetate and lead citrate, as are the following electronmicroscopic radioautographs. The nucleus at the lower left center is overlaid with silver grains indicating uptake of ^{3}H-thymidine. This nucleus belongs to a vacuolated cell as shown by the presence of vacuoles in the supranuclear (Golgi) region and in the cell apex. Only few microvilli (miv) are present at the surface. The vacuoles are partly membrane-limited and partly confluent. Mitochondria are scanty and free ribosomes numerous, as may be seen in this and other vacuolated cells at right. At upper left center, the theca of a mucous cell is located within rather dark cytoplasm. Mucous globules stain more than the vacuoles of nearby cells. x 11,800.

(23)

Fig. 1-24. Electronmicroscopic radioautograph of a labeled mucous cell located in the lower portion of a crypt, obtained from a mouse sacrificed one hour after ^{3}H-thymidine injection. The mucous cell is cylindrical in shape and has a small theca. It has a well developed Golgi complex and rough endoplasmic reticulum and numerous free ribosomes. Its apical cytoplasm contains many partly membrane-limited, partly confluent globules of mucin; these contain a fine stippling of dots with a few slender fibrils. A few short microvilli are present on the apex of the cell. Its cytoplasm is darker than that of vacuolated cells. The vacuoles of vacuolated cells contain only fine fibrils (f). All other cells are vacuolated cells except for a part of cytoplasm of an enterochromaffin cell (E) next to the labeled mucous cell. x 13,000.



(24)

Fig. 1-25. Electronmicroscopic radioautograph of a labeled columnar cell in the upper portion of a crypt, from a mouse killed 72 hours after injection of ^{3}H-thymidine. The columnar cell has numerous microvilli (miv) on the surface and its lateral plasma membrane shows extensive interdigitations with that of adjacent cells. The Golgi apparatus and rough endoplasmic reticulum are moderately developed; mitochondria are many and free ribosomes are scanty. No vacuoles are present in the cytoplasm; instead, a few small partly membrane-bound vesicles are seen in the apical cytoplasm. x 10,800.

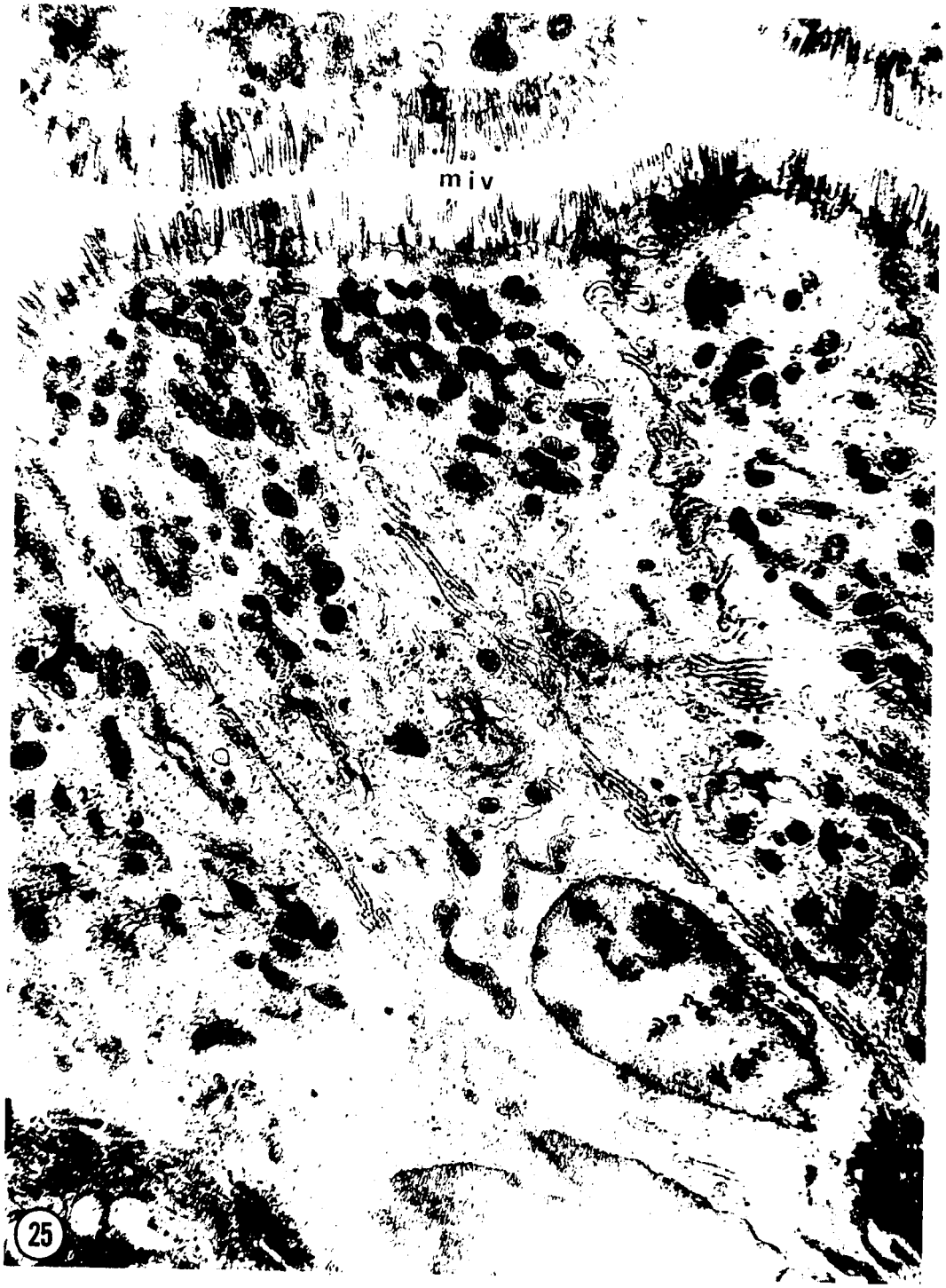

miv
25

Fig. 1-26. The distribution of labeled cells in the vacuolated-columnar cell line at three different time intervals after administration of ^{3}H-thymidine (Experiment II). The crypts are divided equally into ten segments. The horizontal lines indicate the presence of a particular cell type in a particular segment. A black rectangle on the horizontal line represents the fraction of labeled cells in the segment, the distance occupied by each segment being taken as a unit. (The number of vacuolated cells in segment 9, of transitional cells in segment 7, and of columnar cells in segment 8, is too small to give an adequate estimate of labeled fractions). (Data are listed in Appendix 1-3.)

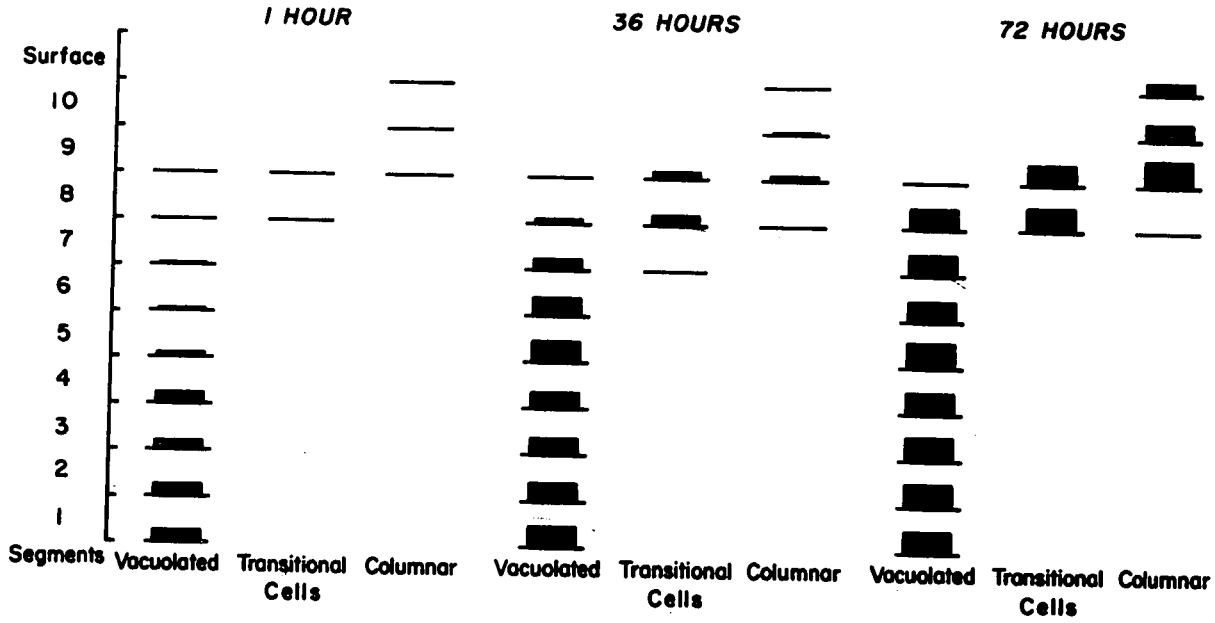
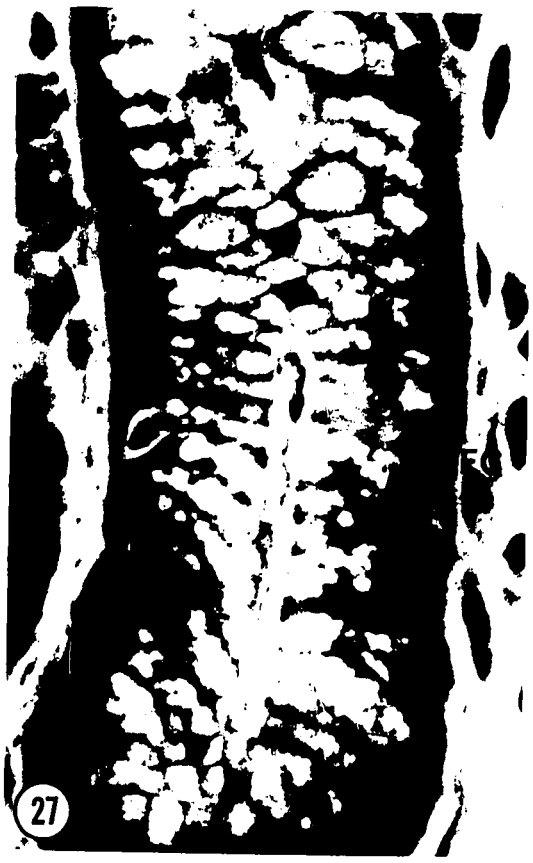

1 HOUR
36 HOURS
72 HOURS
Surface
10
9
8
7
6
5
4
3
2
1
Segments
Vacuolated
Transitional Cells
Columnar
Vacuolated
Transitional Cells
Columnar
Vacuolated
Transitional Cells
Columnar

Fig. 1-27. Light microscopic radioautograph prepared from a mouse sacrificed 6 days after a single injection of ^{3}H-thymidine. Silver grains are present only over the nucleus of an enterochromaffin cell with pale cytoplasm (EC). x 1300.


27

Appendix 1-1. Analysis of variance table for the analysis of cryptal sizes between animals.

Source of variation	Degree of freedom	Sum of squares	Mean squares	F	P
Between animals	19	4,751.21	250.06	29.25	$P < 0.01$
Error	380	3,250.23	8.55		
Total	399	8,001.44			

Appendix 1-2. Analysis of the mucous cell populations between animals and between segments of the crypt and the surface.

(A) Analysis of variance table.

Source of variation	Degree of freedom	Sum of squares	Mean squares	F	P
Between segments	10	1,125.95	112.60	11.85	$P < 0.01$
Between animals	19	1,329.13	69.95	7.36	$P < 0.01$
Error	190	1,805.61	9.50		
Total	219	4,260.69			

(B) The Duncan's multiple range test for the segments.

Segments	4	3	5	6	7	9	8	10	2	1	S
% mucous cells	19.0	17.9	17.1	16.5	16.5	16.5	16.0	15.3	15.0	12.7	10.
$\alpha = 0.05$	———									——	—

Appendix 1-3. Percents of labeled cells in the vacuolated-columnar cell line in each segment of the crypt and on the surface at three different times after single injection of ^{3}H-thymidine.*

	Time after injection of ^{3}H-thymidine								
	1 hour			36 hours			72 hours		
	V	T	C	V	T	C	V	T	C
Surface	-	-	0	-	-	0	-	-	24.8
Segment in crypt 10	-	-	0	-	-	0.9	-	-	34.4
9	-	0	0	-	14.3	10.7	-	42.9	56.6
8	0	0	-	10.3	20.4	-	45.1	50.0	-
7	1.2	-	-	23.4	-	-	45.7	-	-
6	3.6	-	-	40.7	-	-	43.2	-	-
5	10.4	-	-	46.0	-	-	52.0	-	-
4	24.1	-	-	37.6	-	-	48.1	-	-
3	20.7	-	-	37.4	-	-	50.0	-	-
2	27.6	-	-	40.7	-	-	49.4	-	-
1	28.0	-	-	47.1	-	-	48.1	-	-

V = Vacuolated cells

T = Transitional cells

C = Columnar cells

*Data of Fig. 1-26.

CHAPTER II

KINETICS OF THE VACUOLATED-COLUMNAR CELL LINE OF THE EPITHELIUM IN THE DESCENDING COLON OF THE MOUSE

INTRODUCTION

Bizzozero (1888, 1889, 1892, 1893) explained the mitotic activity of intestinal crypts by a "regeneration" of the intestinal epithelium, which would yield the cells needed to replace those dying or damaged by intestinal toxins and bacteria. However, Leblond and Stevens (1948) could not find signs of cell damage and death in the epithelium, but noticed cell streamers or gaps at the tips of villi; they reasoned that instead of regeneration, there was a constant physiological renewal of the epithelium, with the mitotic activity of the crypts balanced by cell extrusion at the villus tips. Radioautography after ^{14}C-adenine or ^{3}H-thymidine injection confirmed the existence of a continuous renewal of epithelial cells in the small and large intestine of mammals (Walker and Leblond, 1958; Leblond and Messier, 1958; Hughes et al., 1958; Quastler and Sherman, 1959; Lipkin and Quastler, 1962; Cairnie et al., 1965a; Sawicki et al., 1968; and others).

Maintaining a balance between the production and loss of cells would insure a steady state of the intestinal epithelium (Leblond and Stevens, 1948). This concept made possible a detailed analysis of cell proliferation and migration in the small intestine (Cairnie et al., 1965a)

and in the large intestine (Sawicki et al., 1968). However, these and other investigations (Messier and Leblond, 1960; Quastler and Sherman, 1959; Lipkin and Quastler, 1962; Lipkin et al., 1963a, 1963b; Lipkin, 1965a, 1965b; Lipkin, 1966; Thrasher, 1967a; Lesher et al., 1961a, 1961b; Fry et al., 1961, 1962, 1963) were based on counts of all nuclei present without discriminating the various cell types. The assumption was that since a high proportion of epithelial cells was columnar, the turnover rate calculated from counts on all epithelial cells would approximate the turnover rate of columnar cells. To reconsider this assumption, it was decided to make a separate examination of the three cell populations identified in the descending colon of the mouse (Chapter I), that is, the vacuolated-columnar, mucous, and enterochromaffin cell lines. Concomitant analysis of the three cell populations would provide baseline information on the renewal behavior of the separate components of the colonic epithelium, and also would clarify the relationship, if any, between these cell populations. The vacuolated-columnar cell line, which includes 80-85% of the total epithelial population, will be examined in the present chapter. For the sake of completeness, the kinetic parameters calculated for all epithelial cells (including the three cell lines) are presented in Appendices 2-1 and 2-2. The details of the techniques for calculation are similar to the following.

MATERIALS AND METHODS

Three experiments were considered: (1) a group of adult mice sacrificed at various times after a single ^{3}H-thymidine injection (Experiment II, reported in detail in Chapter I); (2) four groups of five adult mice each sacrificed one hour after ^{3}H-thymidine injection but at four different times of the day (Diurnal variation experiment or Experiment III, also reported in Chapter I); (3) a group of adult female mice of the same strain as in the other two experiments, that were sacrificed at various times after the beginning of a continuous infusion of ^{3}H-thymidine (Experiment IV). In Experiment IV, three and a half month old mice were kept in individual plastic cages with lids and disposable filter caps (Carworth Co., New York City) under natural light supplemented by artificial light between 09:00 and 17:00 h. (The experiment was carried out from November to January.) Vaginal smears were checked daily between 08:30 and 10:00 hours for at least three weeks and those mice that had a 4-8 day relatively regular cycle were selected for continuous infusion at a time when they entered metestrus II (Allen, 1922). According to the technique devised by Cheng et al. (1969), a polyethylene tube (Clay-Adams Intramedic PE20) was placed in the subcutaneous tissue of the back and right lateral abdomen, so as to protrude at one end into the abdominal cavity, while the other end came out of the body at the occipital region. The latter was enclosed in the sheath of a shielded cable and was long enough to be connected to a B.D. 1 ml tuberculin syringe with Luer-lok through a B.D. adapter No. 3021F. Six animals were under treatment

at the same time; six syringes were held in a plastic holder, in such a way that the pistons of syringes could be pushed by a mobile plastic plate which in turn was pushed by a Sage infusion pump. The infusion pump was adjusted in such a way that the syringes delivered 1 ml of fluid every day. First, the infusion was done with normal saline for three days. The actual ^{3}H-thymidine infusion started on the fourth post-operative day, so that the animals had recovered from the operation and become used to the presence of the tubing. One ml of the ^{3}H-thymidine solution (New England Nuclear Corp., specific activity 18.9 Ci/mM) in the concentration of 50 μCi/ml of sterile saline was filled in daily. Since at the time of infusion the mice were approximately 30 grams in weight, the dose could be calculated to be about 1.7 μCi per gram body weight per day. The experiment was carried out at room temperature (75-80^{o} F). Mice thus treated were sacrificed in pairs at various times (1 hour, 1, 2, 3, 4, 5, 8 and 12 days) following the initiation of ^{3}H-thymidine infusion. The descending colon was fixed in 4% paraformaldehyde with 0.1 M Sörensen's phosphate buffer. The tissues were processed to 1 micron thick Epon sections, stained with periodic acid-Schiff and iron-hematoxylin, and radioautographed. The details of the histological and radioautographic methods as well as quantitation of epithelial cells were described in Chapter I. (Twenty crypts, or forty cryptal columns and their corresponding surface epithelia were examined in each animal in Experiment IV).

DIRECT AND DERIVED RESULTS

The proliferative activity of cells in the vacuolated-columnar cell line

In the vacuolated-columnar cell line, only vacuolated cells proliferate (Chapter I). This proliferation may be measured by the labeling index one hour after injection of ^{3}H-thymidine. The data on diurnal variation (Experiment III) yielded the following figures for the labeling index: 9.5% at 04:00 h, 10.6% at 10:00 h, 6.0% at 16:00 h and 8.9% at 22:00 h. These four values were averaged and yielded a mean labeling index of 8.8%. This figure may be used to calculate the turnover rate of the vacuolated-columnar cell line, provided that the DNA synthesis time is known. Lipkin and Quastler (1962) using samples from both proximal and distal colon of the mouse and counting all cell types indiscriminately obtained a mean of 6.5 hours by the labeled mitosis curve method. Thrasher (1967a) applying the same procedure to the mouse descending colon derived a duration of 8 hours. Using this last figure, the overall turnover rate of the vacuolated-columnar cell population was found to be $1.1\%\left(\frac{8.8}{8}\right)$ cells per hour (26.4% cells per day).

Labeling indices of vacuolated-columnar cells in individual segments

From the diurnal variation experiment, it was also possible to estimate the mean labeling index for each segment of the crypt, that is, the mean percent of vacuolated-columnar cells in a given segment which are labeled (Table 2-1, column I.). It was thus found that labeling was equally high in segments 1 through 4 and tapered off from segment 5 to 7. Transitional and columnar cells located in segment 8 or above were

never labeled at one hour after injection.

The labeling index of each segment in relation to the whole vacuolated-columnar cell population could also be calculated, that is, the percent of the whole vacuolated-columnar cell population which consists of labeled vacuolated cells in a given segment. Since each segment contained approximately the same number of cells, this index could be estimated by dividing the figures of column I in Table 2-1 by the number of segments, 11 (that is, ten in crypts and one equivalent segment on the surface). The results were listed in column II in Table 2-1.

Birth rate

The cell birth rate per hour was computed for each segment by dividing the labeling indices in column II of Table 2-1 by the DNA synthesis time (eight hours according to Thrasher, 1967a). The birth rate was highest in segments 1 through 4.

Cell fluxes

Cell fluxes were estimated on the assumption that the migration of a cell from one position to the next resulted from population pressure exerted by the mitosis of one cell located at a lower level in the crypt. In segment 1, there was no influx of cells (assuming that there was no extra-epithelial origin of epithelial cells), but one mitosis would add one cell to the population and thus cause one cell to move out of segment 1 into segment 2. In other words, the birth rate of segment 1 was equal to the efflux of this segment; and this in turn was the influx of segment 2. Thus, 0.25 cells per 100 cells in the total vacuolated-columnar cell

population would constitute the efflux of segment 1 and also the influx of segment 2 per hour (see columns IV and V, Table 2-1). In segment 2, 0.23 cells were produced every hour for 100 cells. When this number was added to the influx into this segment, a figure of 0.48 cells per 100 cells per hour was obtained, which was the efflux of segment 2 and also the influx of segment 3. Similarly, the influx and efflux calculated for other segments were listed in columns IV and V (Table 2-1). Because from segment 8 up, no cells were produced, the influx and efflux of these non-dividing segments of the crypt and surface were the same as the efflux of segment 7, which was 1.17 cells per 100 cells per hour.

The last value would also be the turnover rate of the whole vacuolated-columnar cell population which might also be estimated directly from the mean labeling index and was found above to be 1.1% cells per hour, similar to 1.17% cells per hour.

Mean number of mitotic cycles in segments 2-7

The number of generations that a cell and its progeny go through in ascending the cryptal walls from segment 2 to segment 7 may be estimated by calculating the number of mitotic cycles relating the generations. The mean number of these mitotic cycles may be calculated for each successive segment by making use of the relationship between the influx (K_{in}) and the efflux (K_{out}) demonstrated by Cleaver (1967) for a dividing compartment in which there is no cell death (as seems to be the case for segments 1 to 10):

$$K_{out} = K_{in} \cdot 2^{d}$$

where d is the mean number of mitotic cycles. This formula is converted to the following by taking the logarithm of both sides of the equation:

$$d = 3.322 \cdot \log_{10} \frac{K_{out}}{K_{in}}$$

The results (Table 2-1; column VI) revealed that vacuolated cells would go through about one mitotic cycle in segment 2, but through fewer and fewer cycles as they migrated up the cryptal walls. Adding up the values for segments 2-7 yielded the total number of mitotic cycles from segment 2 to segment 7, that is, 2.26. However, Cleaver's technique could not apply to segment 1, for which a method devised by N.J. Nadler was used (see next section).

Mean number of mitotic cycles in segment 1 and estimation of cell cycle time: (method devised by N.J. Nadler)

Since the labeling index in segment 1 was 22.4%, the turnover time of this segment would be $\frac{100}{22.4} \times 8 = 35.7$ hours, taking the DNA synthesis time to be 8 hours (Thrasher, 1967a) and assuming that all vacuolated cells in this segment were in the proliferative cycle. Therefore, the mean number of mitotic cycles would be $\frac{t_c}{35.7}$, where t_c is the cell cycle time.

The value of t_c could be estimated by considering the migrational velocity of the cells. Assuming that the increment of cells in the cryptal wall is the result of mitotic activity, the velocity increment of cells in each segment in terms of proportion of segment per hour would be equal to the number of mitotic cycles divided by the cell cycle time in hours.

The actual velocity in a particular segment would be the cumulative velocity increment up to that segment. Now the transit time in the non-dividing segments (7-10) of the crypt was estimated to be 9.6 hours per segment (see below); therefore, the migrational velocity of cells in the non-dividing segments would be $\frac{1}{9.6}$ or 0.104 segment per hour. Hence,

$$\frac{\sum_{1}^{s} d}{t_c} = 0.104$$

For segment 1, $d = \frac{t_c}{35.7}$ (from above); for segments 8-10 and the surface, d = 0. Accordingly (from Table 2-1),

$$\frac{t_c/35.7}{t_c} + \frac{0.94 + 0.64 + 0.39 + 0.20 + 0.08 + 0.01}{t_c} = 0.104$$

whence, t_c = 29.7 hours.[1]

---

[1]Two explanations are available why the calculated turnover time (35.7 hours) in segment 1 could be longer than the calculated mean cell time for all dividing cells in the vacuolated-columnar cell population (29.7 hours).

(1) Cairnie et al. (1965a) demonstrated in the rat small intestine that the cell cycle time was longer at the base of the crypt than in the remaining portion of the crypt. Therefore, if in the mouse colon the cell cycle time in segment 1 were also longer than the mean cell cycle time of the cryptal cells, this would explain why the turnover time of segment 1 is longer than the mean cell cycle time. Indeed, segment 1 is the "stem cell" compartment of the crypt, where mucous cells and enterochromaffin cells may also arise from poorly differentiated vacuolated cells. Hence, there is a possibility of long G_1 or even G_0 phase in the cell cycle of the transforming cells.

(2) If, on the other hand, the cell cycle time were not significantly different throughout the crypt, the following explanation is available (suggestion from Dr. N.J. Nadler). Since there are three cell positions in segment 1, on the average, the time taken to replace these three cell positions (turnover time) can be estimated in relation to the cell cycle time in the following situations (Fig. 2-6):

(a) Suppose that counting is made after completion of division of the cell in position 1 from the bottom of the crypt, it will take one cell cycle time (t_c) to replace all three cells; i.e., the turnover time is equal to t_c.

(b) If counting starts after division of the cell in position 2 (its daughter cells are in positions 2 and 3), the cell in position 1 will divide next in the time q_1 and its two daughter cells occupy positions 1 and 2. The cell in position 3 will divide next, but it needs one cell cycle time from the time q_1 to replace a third cell. Therefore the turnover time is $q_1 + t_c$.

(c) Suppose that counting starts after division of the cell in position 3, two possibilities arise:

First, if the cell in position 2 divides first and takes positions 2 and 3, the cell in position 1 will divide next in the time q_2 to push one cell up. However, it needs one cell cycle time from the time q_2 to replace a third cell. Thus the turnover time is $q_2 + t_c$.

Second, if the cell in position 1 divides first in the time q_3, the cell in position 3 from position 2 will divide next, but one cell cycle time from q_3 is necessary to replace a third cell. Therefore, the turnover time is $q_3 + t_c$.

From the above analysis, it is obvious that the mean turnover time in segment 1 would be longer than the cell cycle time.

As a corollary to the second explanation, if all the proliferating epithelial cells in each dividing segment of the crypt really have the same cell cycle time, they would end up in synchronization with regards to their cell cycle. However, this is found to be not the case. The explanation is that the cell cycle time of individual cells in upper segments do change (see Chapter V, data on diurnal variation), as they ascend the cryptal wall, perhaps due to environmental factors. Accordingly, these cells undergo asynchronous mitotic divisions on ascending the cryptal wall.

From this value of cell cycle time, the mean number of mitotic cycles in segment 1 was estimated to be $\frac{29.7}{35.7}$ or 0.83.

The total number of mitotic cycles that a vacuolated cell and its progeny went through from the bottom of the crypt to the transformation into columnar cells was $\sum_{1}^{s} d$, that is 3.09. The evidence of about three mitotic cycles implied the existence of about four generations as cells went up through the proliferative compartment of the crypt.

Mode of migration

Turnover time of the vacuolated-columnar cell population based on proliferative rate

From the overall turnover rate of 1.1% per hour of the whole vacuolated-columnar cell population, the turnover time of this population may be calculated to be $\frac{1}{1.1} \times 100$ or 91 hours.

This approach may be used for only the first 7 segments (dividing compartment). Since these segments contained 7/11ths of the cell population, their contribution to the turnover time was $\frac{1}{1.1} \times \frac{7 \times 100}{11} = 58$ hours. By difference, the contribution to the turnover time of the non-dividing compartment was 33 hours. In the non-dividing compartment, turnover time would be equivalent to transit time, so that it took 33 hours to go from the beginning of segment 8 to the extrusion zone, that is, an average of $\frac{33}{4} = 8.3$ hours per segment (based on the data of Experiment III).

Transit time in the non-dividing segments (8-10 and surface)

Because of the absence of mitosis, the cells were assumed to migrate at a constant speed in the non-dividing segments. One assessment of this speed was just given. Two others were also examined.

If the highest segments in which labeled cells could be found at each time interval after injection of ^{3}H-thymidine were plotted against various times after injection, there appeared to be a linear relationship between them (Fig. 2-1). The slope of these leading edges of labeled cells was a measure of the cell velocity and calculated by the method of least squares to be 0.08 segment per hour. The inverse of this cell velocity, 1/0.08 or 12.4 hours per segment, was the mean transit time per segment (data of Experiment II).

Data from the continuous infusion experiment (Experiment IV) were also used as follows. The percent labeled vacuolated-columnar cells for each segment was estimated at the various time intervals (Fig. 2-2). The curves of percent increment of labeled cells in these non-dividing segments were joined on the graph by a line drawn at the 50% labeling level. The times measured on this line between the curves for each segment were 9.0, 10.2 and 9.6 hours respectively for the distances of segments 7-8, 8-9 and 9-10 (Table 2-2). These were the transit times. The mean of these figures was 9.6 hours (data of Experiment IV). The time for a 50% labeling level to go from segment 10 to the surface was also estimated and found to be 17.4 hours. Even though this figure may be high because of possible loss of labeled cells, it might indicate a slowing down of the

rate of migration after the cells reached the surface.

In conclusion, data on the transit time per segment were obtained by different methods in the three experiments: 8.3 hours in Experiment III, 12.4 hours in Experiment II and 9.6 hours in Experiment IV. The calculation for the first figure implied assumption on the duration of DNA synthesis, while the migration of leading edges appeared to be easily influenced by environmental factors. The last figure implied no assumption and was probably the most accurate.

Transit time in the dividing segments (1-7)

According to the method devised by N.J. Nadler, the transit time in a given segment in the dividing compartment of the crypt is estimated from the velocity of that segment, which is obtained as the cumulative velocity increment up to that segment. The velocity increment in a given segment is equal to the number of mitotic cycles divided by the cell cycle time. From the number of mitotic cycles calculated for each segment (Table 2-1) and the cell cycle time of 29.7 hours as estimated previously, the velocity and transit time of each segment were calculated and listed in Table 2-3, and depicted in Fig. 2-3. The cells moved slowly at the base of the crypt and faster and faster as they ascended to the non-dividing segments where the migration of cells was at a constant speed.

Comparison of turnover rates based on transit time and on proliferation rate

In Table 2-4, the turnover rate of vacuolated-columnar cells was estimated in two different ways.

First, the turnover rate was calculated from the transit times estimated for segments 7-10 (non-dividing compartment). It was reasoned that in these segments the transit time t was constant, so that in a crypt with segments composed of three cell positions for instance, these three cells must be replaced in a time t; and, therefore, the turnover of a number of cells equal to that of the whole crypt and the surface should take a time of $\frac{t \times 33}{3} = 11t$. In one hour, the percentage of cells turning over, i.e., the turnover rate, will be $\frac{100}{11t}$. This method was used to calculate the figures in the upper part of Table 2-4.

Second, the turnover rate was calculated as done previously on the basis of proliferative (birth) rate, i.e., from the labeling index assuming the DNA synthetic duration of 8 hours. The results were presented in the lower part of Table 2-4.

Comparison revealed that the turnover rate calculated from the labeling index in Experiments II, III, and IV was substantially higher than that calculated from transit times. This would lead to an overproduction of vacuolated-columnar cells in the colonic epithelium. To reconcile the existence of a steady state, it would be necessary, assuming no cell loss, that some of the vacuolated-columnar cells be transformed into another cell line. Consideration of the increase in the number of labeled cells with time on continuous infusion with ^{3}H-thymidine (Fig. 2-4) confirmed that this was actually the case. At the one-hour interval there was a great difference between the labeling of the two cell lines. If the two cell lines had been independent (with each one producing

its own cells), the two curves depicting the increase in labeling with time would have diverged. However, they were parallel, indicating that the low labeling of mucous cells at one hour must have compensated by a contribution from the vacuolated-columnar cell line to the mucous cell line.

DISCUSSION

Three types of assumptions were made in support of the kinetic analysis of the vacuolated-columnar cell population in the descending colon of the mouse. First, the widely accepted view was adopted that cells taking up ^{3}H-thymidine were engaged in DNA synthesis and would divide within the next few hours. Second, it was assumed that no cells were contributed from extra-epithelial sources; while Andrew (1957, 1965) suggested that invading lymphocytes might transform into epithelial cells, the present observations were in favor of the classical opinion that epithelial mitoses in the crypts were the sole source of new epithelial cells. Third, cell migration was a uni-directional upward movement toward the surface, and the girdle of crypts did not change under physiological conditions; meanwhile no cell was lost in the crypt, but at the surface cells were lost in a limited region, the extrusion zone (Chapter I).

Cell proliferation

Proliferation was maximal in segments 1 through 4, tapered off in segments 4 through 7, and was absent from segment 8 up. In the

present work, the total number of mitotic cycles of vacuolated-columnar cells from their birth at the base of the crypt to their state of non-proliferation in segments 8-10 was estimated to be about three. From the number of mitotic cycles per segment (Table 2-1), it was clear that the first cycle took place mostly in segment 1 (83% of the cells) and was completed in segment 2 (100 - 83 = 17%). This would be the "stem cell" division yielding on the average one cell remaining in situ and one moving up. The second mitotic cycle would occur partly in segment 2 (94 - 17 = 77%) and be completed in segment 3 (100 - 77 = 23%) yielding more vacuolated cells. The third and last mitotic cycle took place between segments 3 and 7, and gave rise to cells which would then cease to divide (Fig. 2-5).

This interpretation seemed at first glance to be in keeping with that offered for the crypts of the small intestine under the name of "slow cut-off" model (Cairnie et al., 1965b). These authors proposed that there was a gradual changeover from divisions giving rise to two dividing cells to divisions producing two non-dividing cells, so that over a range of cell positions the probability of the latter type increased up to 100%. A slightly different view was that, even though mitosis was not differential and gave rise to two identical daughter cells, their eventual mitotic behavior depended on their position in the crypt. For instance, in the middle third of the crypt, the lower daughter cell of a division might go into mitosis, while the upper one remained in interphase (Leblond, Clermont and Nadler, 1967). In this regard, it may be pointed

out that three mitotic cycles were not observed in a certain fraction of cells at all times of day, but rather the number of the cycles varied from two to four according to the time of day. Such variation would be readily explained by the theory that, of the two daughter cells of mitosis after completion of the second mitotic cycles, either two, one or none may divide again, according to position in the crypt. Perhaps differences in the microenvironment account for this relation between position and ability to divide.

Cell migration

Cell migration up the cryptal walls was explained by the population pressure created by the formation of new cells below. A satisfactory model based on this assumption was devised for a stratified squamous epithelium by Leblond, Greulich and Pereira (1964). Similarly Cairnie et al. (1965a) and Sawicki et al. (1968) calculated the migrational velocity of a cell at a particular position of the crypt as being equal to the cumulative proliferation rate of cells up to that position.

However, the demonstration by Grad and Stevens (1950) of the reduction of the number of cells per villus following mitotic arrest of cryptal cells produced by a single large injection of ^{32}P was taken as evidence that the migration and loss of cells in the small intestine could occur in the absence of population pressure from accumulating newly formed cells. Intervention of factors other than the pressure in the small intestinal epithelium was further supported by the experimental results on the effect of starvation on the intestinal epithelium by Stevens-

Hooper and Blair (1958), which showed that the rate of cell extrusion could be altered independently of the rate of cell production in the intestinal epithelium. This view was shared by Sherman and Quastler (1960), and was confirmed by O'Connor (1966) in the developing intestine of the mouse from late fetal life to maturity.

On the other hand, the present observations demonstrated that the migrational velocity of epithelial cells in the non-dividing segments of the crypt in the murine descending colon was fairly constant. In a study of healing of artificial mucosal lesions in the cat, McMinn and Mitchell (1954) and McMinn (1954) noticed that the epithelium grew over the floor of a defect at a rate similar to that at which it normally passed along the sides of the villi.

In conclusion, even though various factors may play a role in the migration of epithelial cells in the large intestine, population pressure is probably the main one.

Transformation of cells from the vacuolated-columnar cell line to other cell lines

Comparison of turnover rates based on transit time and on proliferative rate disclosed that, on the basis of a steady state, there was an overproduction of cells in the vacuolated-columnar cell line. As pointed out previously, cells overproduced in this cell line could contribute to the mucous cell line, since the rate of increment of labeled cells in both the vacuolated-columnar and mucous cell lines was similar in spite of generally low labeling of mucous cells at one hour after injec-

tion of ^{3}H-thymidine (also see Chapter III).

The transformation of "undifferentiated" columnar cells of the intestinal crypts to mucous cells has been suggested on morphological grounds by the demonstration of intermediate forms between them (Paneth, 1888; Nassonov, 1923, 1924; Clara, 1926; Chlopkow, 1928; Florey, 1932; Freeman, 1962, 1966; Hollmann, 1963; Palay, 1958 and others). Recently a quantitative assessment of this possibility has been documented. By estimating the turnover time of both columnar and mucous cells in the murine small intestine, Merzel and Leblond (1969) demonstrated that poorly differentiated columnar cells give rise to oligomucous cells, which in turn differentiate to mucous cells in a typical goblet form. Cairnie (1970) came to a similar conclusion using a kinetic analysis of mucous cell labeling in the rat small intestine; namely, mucous cells renew by differentiation from cryptal columnar cells.

Since enterochromaffin cells were labeled later but not soon after ^{3}H-thymidine injection and since enterochromaffin cells were not found in mitosis (Chapters I and IV), it is possible that vacuolated-columnar cells contribute also to enterochromaffin cells.

The kinetic parameters for the cells in the vacuolated-columnar cell line would have different values than calculated here if transformation to the other two cell lines takes place. However, since the actual fraction of the total vacuolated-columnar cell population which would be necessary to transform into mucous cells is very small in order to rescue the mucous cell population (see Chapter III), the kinetic parameters of

this cell line presented in Table 2-1 and Table 2-3 would not show any significant change in their values if cell transformation were considered in quantitative terms.

SUMMARY

Proliferation and migration of cells in the vacuolated-columnar cell line in the descending colon of the mouse were investigated in Epon embedded materials using ^{3}H-thymidine.

In the normalized crypts which had ten equal segments, labeled vacuolated cells at one hour after injection of ^{3}H-thymidine were encountered in the lower four segments and in decreasing numbers in segments 5 through 7. From the percent labeled cells in the segments of the crypt, the birth rate and cell fluxes were computed. Moreover, it was found that a cell in this cell line would undergo three mitotic cycles on the average from its birth at the cryptal base to its extrusion from the surface; of these three cycles, the last one which took place from segment 3 to 7 appeared to be a changeover from dividing cells to non-dividing cells, in accordance with the "slow cut-off" model of Cairnie et al. (1965b).

In ascending the cryptal walls, cells moved much slower at the base of the crypt, and accelerated toward the upper portion of the crypt, but they migrated at a constant speed in the non-dividing segments of the crypt.

Comparison of the turnover rates based on transit time and on proliferative rate indicated that, on the basis of a steady state, there was an overproduction of cells in this cell line. It is concluded that this would contribute to the mucous and enterochromaffin cell lines.

Table 2-1. The proliferative activity and related parameters in each segment of the crypt in the vacuolated-columnar cell line.*

Segment	For 100 vacuolated-columnar cells in each segment	For 100 cells in the total vacuolated-columnar cell population				Mean Number of mitotic cycles (VI)
	% cells labeled at 1 hour (I)	% cells labeled at 1 hour (II)	Birth rate per hour (III)	Influx of cells per hour (IV)	Efflux of cells per hour (V)	
10	-	-	-	1.17	1.17	-
9	-	-	-	1.17	1.17	-
8	-	-	-	1.17	1.17	-
7	0.8	0.07	0.01	1.16	1.17	0.01
6	5.1	0.46	0.06	1.10	1.16	0.08
5	11.9	1.08	0.14	0.96	1.10	0.20
4	20.1	1.83	0.23	0.73	0.96	0.39
3	22.1	2.01	0.25	0.48	0.73	0.64
2	20.5	1.86	0.23	0.25	0.48	0.94
1	22.4	2.04	0.25	-	0.25	(0.83)

* See results for details.

Table 2-2. Time taken for vacuolated-columnar cells to migrate from segment to segment in the non-dividing area of the crypt as measured from the distance between curves in Fig. 2-2 at the 50% labeling level. (Experiment IV).

Segments considered	Transit time (in hours)	Mean transit time per segment (in hours)
7 to 8	9.0	
8 to 9	10.2	
9 to 10	9.6	
10 to S	17.4	
7 to 10	28.8	9.6
7 to S	46.2	11.6

Table 2-3. Velocity and transit time of vacuolated-columnar cells in each segment of the crypt.*

Crystal segment	Mean number of mitotic cycles	Velocity increment in segment	Velocity in segment		Transit time (in hours)	
10	-	-		0.104		9.6
9	-	-		0.104		9.6
8	-	-		0.104		9.6
7	0.01	0.01/29.7	3.09/29.7	0.104	29.7/3.09	9.6
6	0.08	0.08/29.7	3.08/29.7	0.104	29.7/3.08	9.6
5	0.20	0.20/29.7	3.00/29.7	0.101	29.7/3.00	9.9
4	0.39	0.39/29.7	2.80/29.7	0.094	29.7/2.80	10.6
3	0.64	0.64/29.7	2.41/29.7	0.081	29.7/2.41	12.3
2	0.94	0.94/29.7	1.77/29.7	0.060	29.7/1.77	16.8
1	0.83	0.83/29.7	0.83/29.7	0.028	29.7/0.83	35.7

* See results for details.

Table 2-4. Turnover rate in the vacuolated-columnar cell line.

(A) Calculated from transit times of non-proliferative segments.

Experiment	Transit time (hour per segment)	Turnover rate of the whole population (% cells per hour)
II	12.4 [a]	0.73
IV	9.6 [b]	0.95
	11.6 [c]	0.78

(B) Calculated from labeling frequency of proliferative cells.

Experiment	% cells labeled at 1 hour	Turnover rate of the whole population (% cells per hour)
II	9.6 [d]	1.20
III	8.8 [e]	1.10
IV	10.5 [f]	1.31

a = Estimated from the leading edge of labeled cells at various times after a single injection of ^{3}H-thymidine (Fig. 2-1).

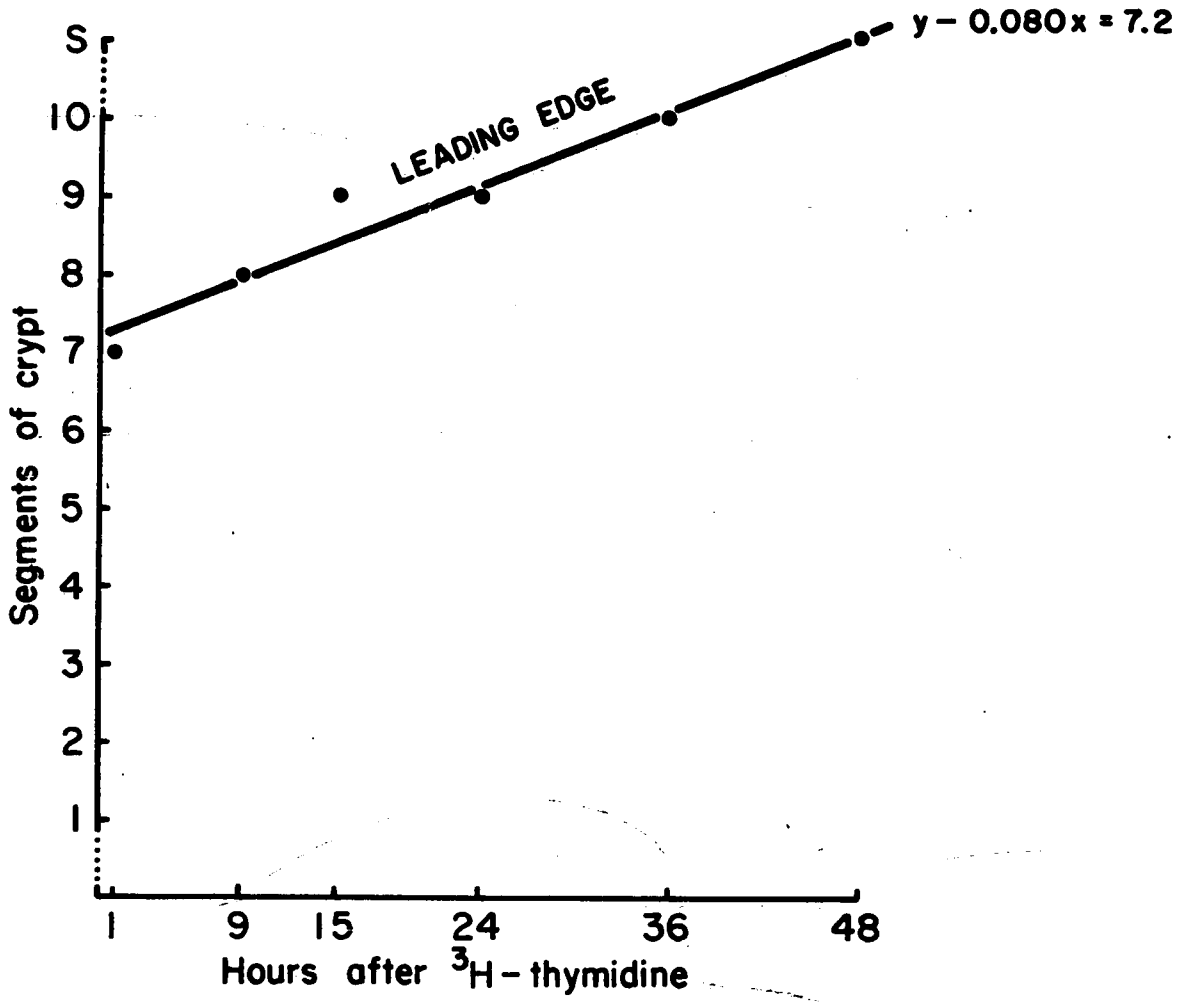
b and c = Estimated from the distance between curves in Fig. 2-2 at the 50% labeling level: b and c are the means per segment (Table 2-2), b being the third of the distance between segments 7 and 10 curves; and c the fourth of the distance between segment 7 and surface curves.

d = Animals sacrificed at 13:00 hour.

e = The mean labeling index of the four groups of animals in the diurnal variation experiment.

f = Animals sacrificed at 12:00 hour.

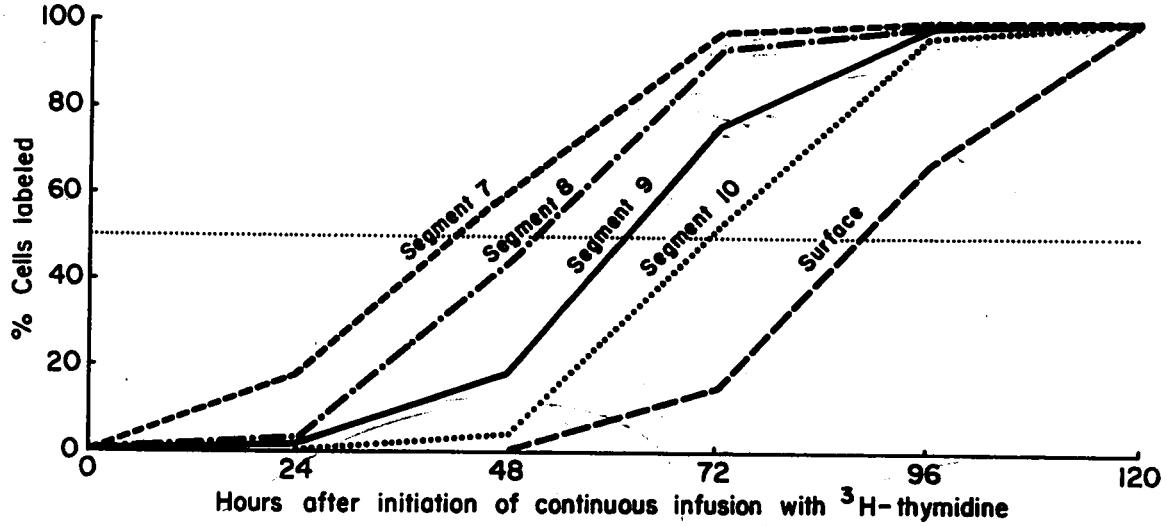
Fig. 2-1. Highest segments of colonic crypts with labeled cells at various times after a single injection of ^{3}H-thymidine.


y – 0.080x = 7.2
LEADING EDGE
Segments of crypt
S
10
9
8
7
6
5
4
3
2
1
1
9
15
24
36
48
Hours after ^{3}H – thymidine

(1)

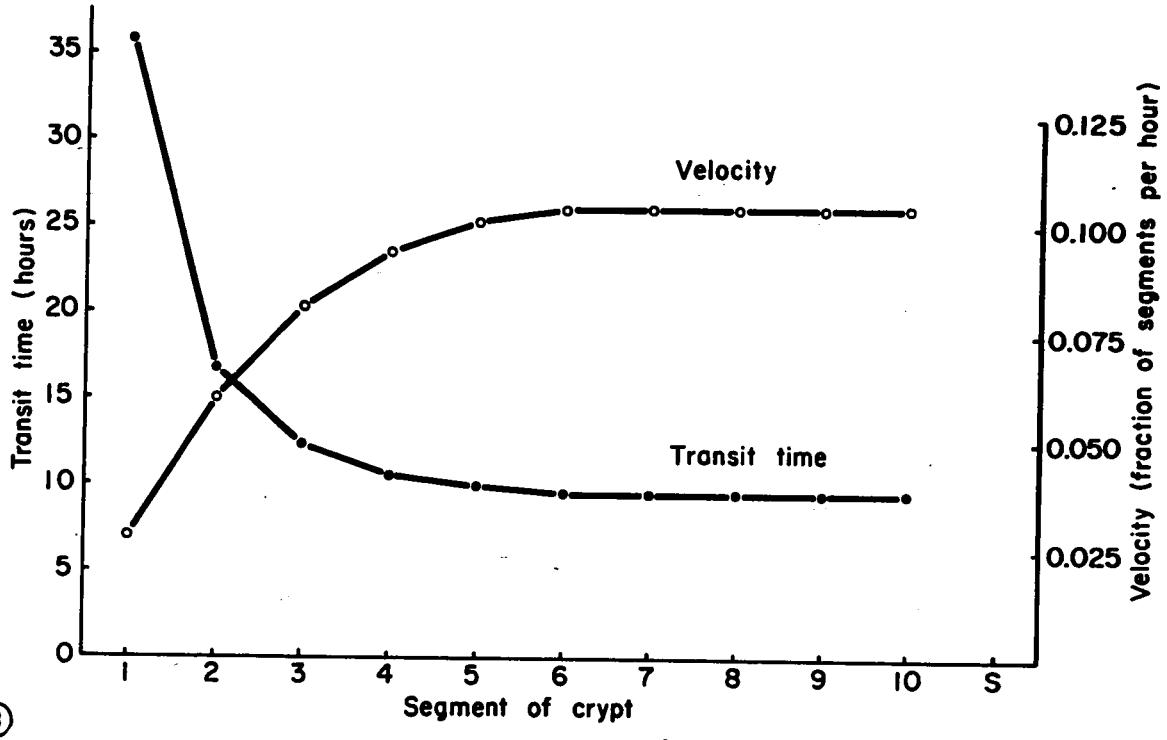
Fig. 2-2. The increment in the percent labeled vacuolated-columnar cells in individual non-proliferative segments of colonic crypts at various times after the start of continuous infusion of ^{3}H-thymidine.

The vacuolated - columnar cell line



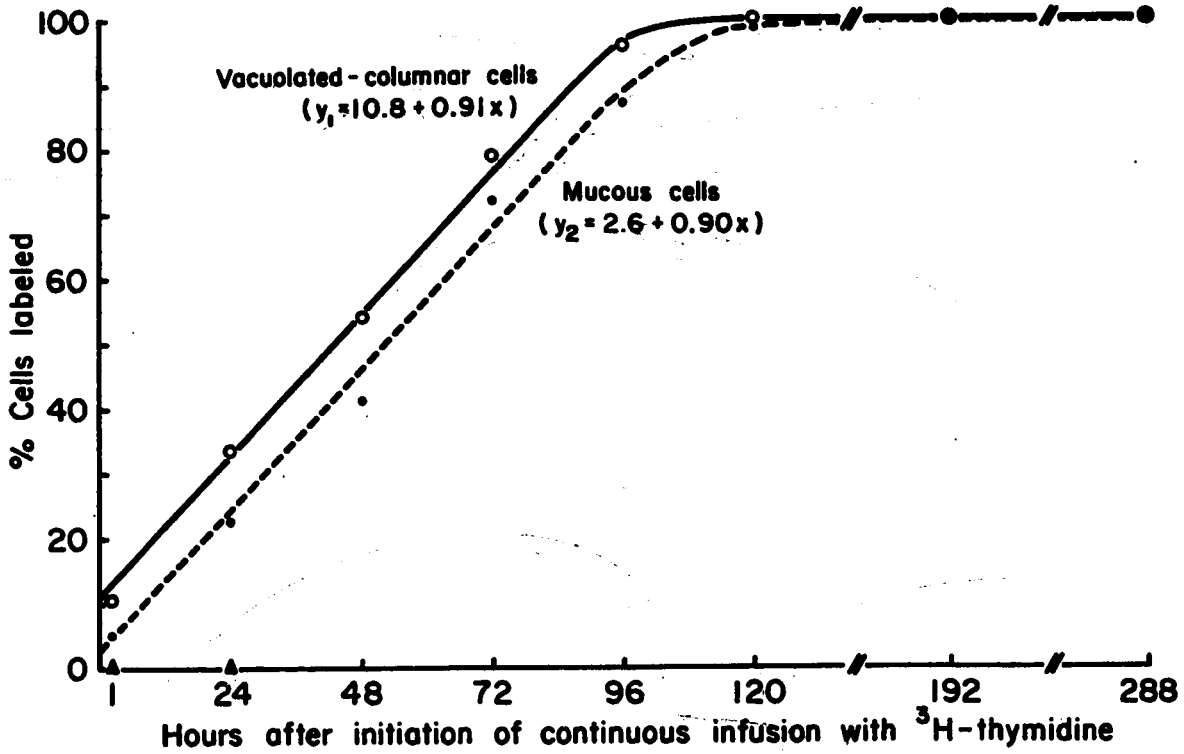
(2)

Fig. 2-3. The velocity and transit time in the segments of the crypt.


35
30
25
20
15
10
5
0
Transit time (hours)
Velocity
Transit time
0.125
0.100
0.075
0.050
0.025
Velocity (fraction of segments per hour)
1
2
3
4
5
6
7
8
9
10
S
Segment of crypt

③

Fig. 2-4. The increment in the percent labeled vacuolated-columnar cells and mucous cells in the whole epithelium at various times after the initiation of continuous infusion of ^{3}H-thymidine.


100
80
60
40
20
0
% Cells labeled
Vacuolated - columnar cells
(y_1 = 10.8 + 0.91x)
Mucous cells
(y_2 = 2.6 + 0.90x)
1
24
48
72
96
120
192
288
Hours after initiation of continuous infusion with ^{3}H-thymidine

4

Fig. 2-5. The number of mitotic cycles and the site of mitotic divisions in the crypt.

The vacuolated - columnar cell line

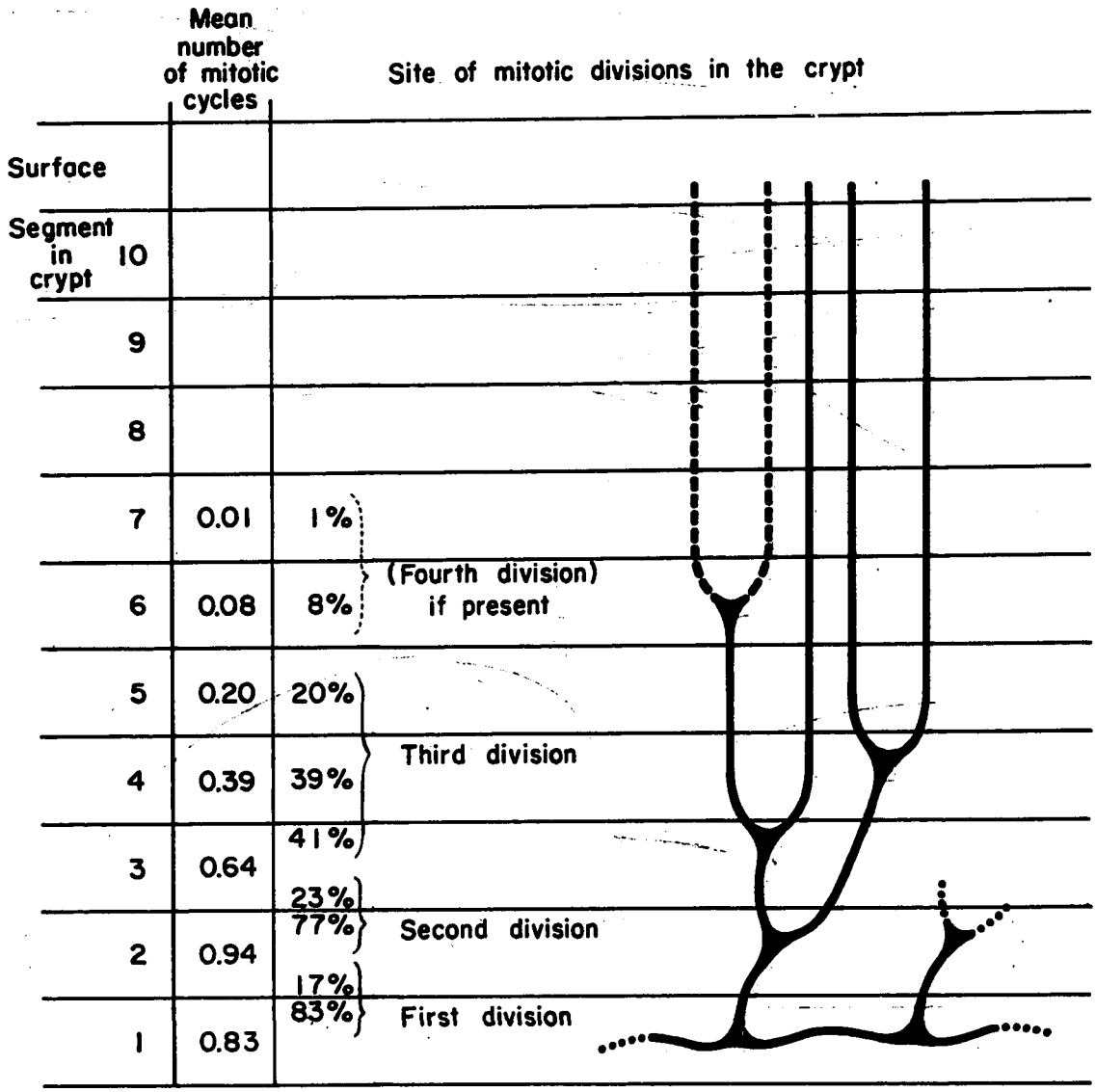
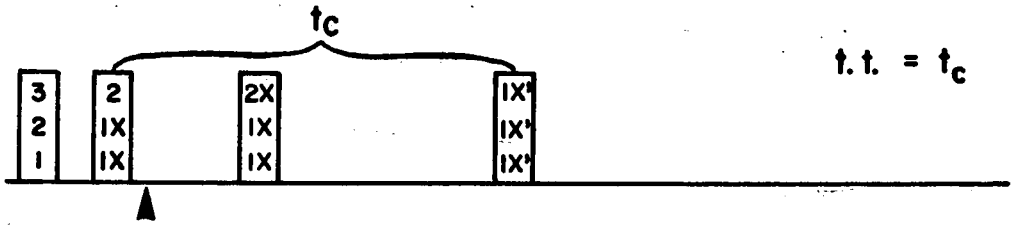


Fig. 2-6. Diagram showing the relationship between the turnover time and the mean cell cycle time of cryptal cells in segment 1 of the crypt.

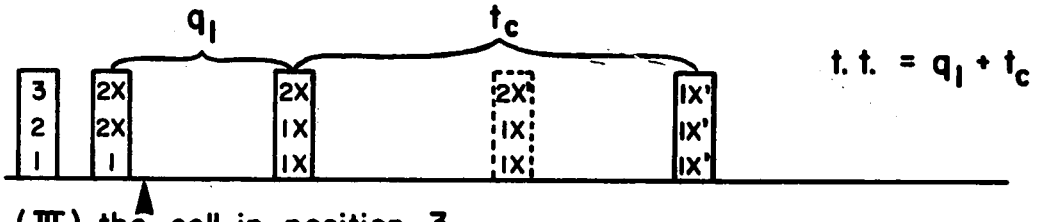
Turnover time and cell cycle time in segment I of the crypt

Counting starts after division of

(I) the cell in position 1

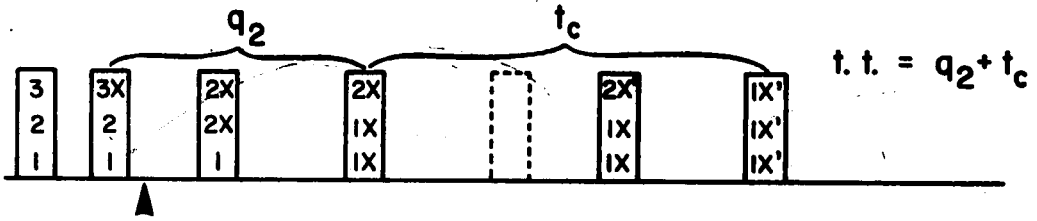


(II) the cell in position 2

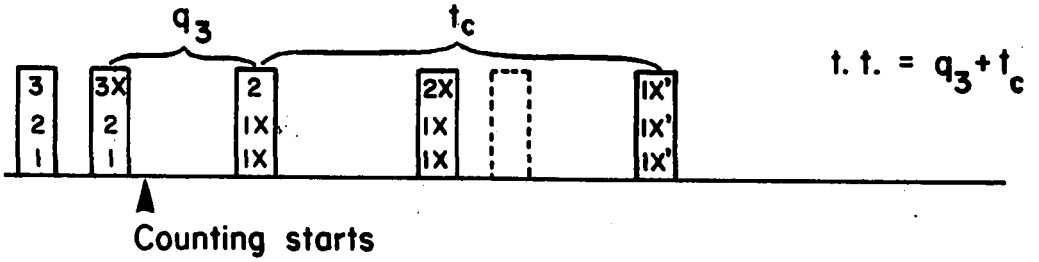


(III) the cell in position 3

(A) the cell in position 2 divides first.



(B) the cell in position 1 divides first



X : first division, X': second division,
t.t. : turnover time, t_c : cell cycle time

Appendix 2-1. The proliferative activity and related parameters in each segment of the crypt for all epithelial cells (including the three cell lines).

Segment in crypt	For 100 epithelial cells in each segment	For 100 cells in the total epithelial cell population				Mean Number of mitotic cycles (VI)
	% cells labeled at 1 hour (I)	% cells labeled at 1 hour (II)	Birth rate per hour (III)	Influx of cells per hour (IV)	Efflux of cells per hour (V)	
10	-	-	-	1.07	1.07	-
9	-	-	-	1.07	1.07	-
8	-	-	-	1.07	1.07	-
7	0.7	0.06	0.01	1.06	1.07	0.01
6	4.3	0.39	0.05	1.01	1.06	0.07
5	10.3	0.94	0.12	0.89	1.01	0.18
4	18.0	1.64	0.21	0.68	0.89	0.38
3	20.1	1.83	0.23	0.45	0.68	0.59
2	19.0	1.73	0.22	0.23	0.45	0.97
1	20.5	1.86	0.23	-	0.23	(0.72)

Appendix 2-2. Transit time of epithelial cells including the three cell lines in each segment of the crypt.

Segment in crypt	Mean number of mitotic cycles	Velocity increment in segment	Velocity in segment		Transit time (in hours)	
10	-			0.104		9.6
9	-			0.104		9.6
8	-			0.104		9.6
7	0.01	0.01/28.2	2.92/28.2	0.104	28.2/2.92	9.7
6	0.07	0.07/28.2	2.91/28.2	0.103	28.2/2.91	9.7
5	0.18	0.18/28.2	2.84/28.2	0.101	28.2/2.84	9.9
4	0.38	0.38/28.2	2.66/28.2	0.094	28.2/2.66	10.6
3	0.59	0.59/28.2	2.28/28.2	0.081	28.2/2.28	12.4
2	0.97	0.97/28.2	1.69/28.2	0.060	28.2/1.69	16.7
1	0.72	0.72/28.2	0.72/28.2	0.026	28.2/0.72	39.2

CHAPTER III

KINETICS OF THE MUCOUS CELL LINE OF THE EPITHELIUM IN THE DESCENDING COLON OF THE MOUSE

INTRODUCTION

A number of works have been performed on the renewal of epithelial cells in the large intestine of rodents (Walker and Leblond, 1958; Hughes et al., 1958; Messier and Leblond, 1960; Messier, 1960; Lipkin and Quastler, 1962; Thrasher, 1967; Sawicki et al., 1968) and man (Cole and McKalen, 1961; Lipkin et al., 1963a, 1963b; Lipkin, 1965a, 1965b; 1966; MacDonald et al., 1964; Shorter et al., 1964), yet, in these studies, no distinction was made of different cell types present in the colonic epithelium. Only a few reports are available on the renewal of mucous cells in the small intestine of rodents (Leblond and Messier, 1958; Thrasher and Greulich, 1966; Merzel and Leblond, 1969; Cairnie, 1970), but no similar studies have been carried out in the large intestine.

Whether or not mucous cells undergo division has been a matter of conjecture in the literature. Because of this, different schools of thought have been developed with regard to the origin of mucous cells. According to one school, mucous cells were independent cells capable of self-renewal (Bizzozero, 1889, 1892; Sacerdotti, 1894; Leblond and Messier, 1958; Thrasher and Greulich, 1966). In addition to the morphological changes in the line of differentiation from the lower portion of the

crypt to the surface or villi of the large or small intestine (Bizzozero, 1889, 1892; Moe, 1955), mucous cells were found to be in mitosis (Bizzozero, 1889, 1892; Sacerdotti, 1894; Zipkin, 1903; Leblond and Messier, 1958; MacDonald et al., 1964; Thrasher and Greulich, 1966; Merzel and Leblond, 1969), and in accordance with this, mucous cells were also found labeled at early periods after injection of ^{3}H-thymidine (Leblond and Messier, 1958; Thrasher and Greulich, 1966; Merzel and Leblond, 1969; Katoaka, 1970).

However, many investigators cast doubt on the dividing capacity of mucous cells (Macklin and Macklin, 1932). Thus, by demonstrating cells intermediate between mucous cells and other types of epithelial cells, a transformation of these epithelial cells to mucous cells was postulated. The speculations that mucous cells had their origin in Paneth cells (Bizzozero, 1892) or in enterochromaffin cells (Schofield, 1951, 1952, 1953) were developed in the small intestine, but no similar references were made for the large intestine. However, the relationship between columnar and mucous cells has been a subject of intensive studies (Macklin and Macklin, 1932; Patzelt, 1936; Palay, 1958), because there was a resemblance between columnar cells and "underdeveloped" mucous cells. On morphological grounds, a number of investigators (Paneth, 1888; Nassonov, 1923, 1924; Clara, 1926; Chlopkow, 1928; Florey, 1932; Palay, 1958; Freeman, 1962, 1966; Hollmann, 1963 and others) believed that mucous cells arose from the differentiation of columnar cells. Chlopkow (1928) described in detail various intermediate stages from columnar cells

to fully developed mucous cells. In Clara's opinion (1926), mucous cells might develop from columnar cells all over the intestinal epithelium, and after completion of the secretory cycle they would revert to columnar cells. Recent kinetic studies by Cairnie (1970) using ^{3}H-thymidine also supported the view that mucous cells were a non-proliferative cell population renewed by differentiation from columnar cells in the lower part of the crypt.

On the other hand, Merzel and Leblond (1969) found a particular type of mucous cells designated as oligomucous cells, which were capable of incorporating ^{3}H-thymidine and thus of undergoing division. They would give rise to mucous cells in a typical goblet form, and had their origin in "poorly differentiated mucus-free" columnar cells at the base of the crypt.

Since mucous cells make up approximately 3-5% of the total epithelial cell population in the rodent small intestine (Quastler and Sherman, 1959; Cairnie, 1970), it would be difficult to obtain an adequate number of mucous cells for analysis. In the murine descending colon, mucous cells occupy approximately 15% of the total population of epithelial cells (Chapter I): therefore, it was felt that this segment of the gastrointestinal tract might be a better model system to examine the mode of renewal of mucous cells.

MATERIALS AND METHODS

The materials and methods used in this investigation were the same as those used in the kinetic study of the vacuolated-columnar cell line (Chapter II). Briefly, three experiments were included:
(1) Experiment II, in which a group of adult female mice were sacrificed at various time intervals after a single injection of ^{3}H-thymidine (Chapter I);
(2) Experiment III (Diurnal Variation), in which four groups of five adult mice each were given ^{3}H-thymidine at four different times of day and sacrificed one hour after injection (Chapter I);
(3) Experiment IV (Continuous infusion), in which a group of adult mice were sacrificed at various times after the start of a continuous infusion of ^{3}H-thymidine (Chapter II).

One micron thick Epon sections of paraformaldehyde-fixed tissues of the descending colon were stained with periodic acid-Schiff and iron-hematoxylin and radioautographed. The methods in histology and radioautography as well as in quantitation of epithelial cells were described in detail in Chapter I.

DIRECT AND DERIVED RESULTS

Turnover rates based on proliferative rate and on transit time

Production of mucous cells by mitosis

In the previous studies (Chapter I), it was established that mucous cells located in the lower portion of the crypt incorporate ^{3}H-thymidine and undergo division. Accordingly, attempts were made to analyze quanti-

tatively the production of mucous cells by mitosis.

The proliferative activity of mucous cells as measured by the labeling index one hour after injection of ^{3}H-thymidine varied remarkably during the day: 5.1% at 04:00 h, 7.3% at 10:00 h, 3.0% at 16:00h and 5.1% at 22:00 h (Experiment III). These four values were averaged and yielded a mean labeling index of 5.1%. Since Thrasher and Greulich (1966) obtained the same DNA synthesis time for both columnar and mucous cells in the mouse small intestine, the DNA synthesis time of 8 hours as found by Thrasher (1967a) in the distal colon of the mouse was used to estimate the overall turnover rate of the mucous cell population. The latter was found to be 0.64 (5.1/8) % cells per hour.

Transit time in the non-dividing segments (7-10) of the crypt and the surface

The migrational activity of the mucous cells in the non-dividing segments of the colonic epithelium was assessed by the use of the data of the continuous infusion experiment (Experiment IV) (see Chapter II). The percent of labeled mucous cells for each non-dividing segment was plotted against various times after initiation of continuous ^{3}H-thymidine infusion (Fig. 3-1). The distances between the curves of increment of percent labeled cells of the segments at 50% labeling level were measured to give the transit times from one segment to the next upper; these measured values were listed in Table 3-1.

Some irregularity in the curves of increment of labeled mucous cells was noted in these segments, particularly in the curve of segment 9.

While the irregularity might be accounted for by the fact that the sample size of mucous cells was only one-fifth of that of vacuolated-columnar cells (Table 1-1) in segment 9, it might also have been due to the fact that in this segment the theca of mucous cells was extremely swollen and the nucleus was pushed to the foot of the goblet causing morphological distortion. Whatever the reason was, the curve of segment 9 was ignored in measurements of transit times.

As shown in Table 3-1, the average transit time of mucous cells was 11.5 hours per segment in the non-dividing segments of the crypt, and was 12.0 hours per segment if the time taken to migrate from segment 10 to the surface was also included. It appeared that mucous cells slowed down slightly on migration from segment 10 to the surface.

Comparison of turnover rates based on transit time and on proliferative rate

The turnover rates of mucous cells were estimated according to two different parameters in Table 3-2.

First, the turnover rate was calculated from the transit times estimated for the non-dividing segments of the crypt. Since the turnover time of a non-dividing segment is equal to the transit time of the segment (t), the turnover time of the whole crypt and the surface would be 11t hours (since there are ten segments in the crypt and one equivalent segment on the surface). Therefore, the turnover rate of the whole epithelium would be $\frac{100}{11t}$ percent cells per hour. This method was used to calculate the figures in the upper part of Table 3-2.

Second, the turnover rate calculated from the rate of proliferation was estimated from the labeling frequency of mucous cells one hour after injection of ^{3}H-thymidine, assuming 8 hours to be the DNA synthesis time. The data obtained from three different experiments were listed in the lower part of Table 3-2.

Comparison revealed that the turnover rates calculated from the rate of proliferation in three experiments were substantially lower than those estimated from transit times. This would indicate that there would be an underproduction of mucous cells by mitosis alone in the colonic epithelium; in other words, more cells were turned over as mucous cells in the non-dividing segments of the crypt than were actually produced by mitosis.

Since, for a steady state, there was an over-production of cells in the vacuolated-columnar cell line (Chapter II) and an underproduction of cells in the mucous cell line, the most probable explanation was that some of the cells in the vacuolated-columnar cell line would transform into mucous cells.

Supporting evidence to this concept of cell transformation was found in the data of continuous infusion experiment (Experiment IV). In spite of a great difference between the labeling of two cell lines at one hour after infusion with ^{3}H-thymidine, the rate of increment of labeled cells in two cell lines was similar (Fig. 2-4), indicating that the low labeling of mucous cells at one hour was compensated by a contribution from the vacuolated-columnar cell line to the mucous cell line. The same

rate of increment of labeled cells in both columnar and mucous cells on the upper half of the villus in the small intestine of rat was found by Cairnie (1970).

In conclusion, mucous cells are formed in two ways: by mitosis of mucous cells themselves on the one hand, and by transformation of cells in the vacuolated-columnar cell line to the mucous cell line on the other.

Analysis of the formation and migration of mucous cells in the proliferative segments of the crypt

Location of mucous cell formation

The region of the crypt where mucous cells were produced by mitosis was demonstrated in the distribution of labeled mucous cells one hour after injection of ^{3}H-thymidine (Table 3-3). Presented in column I in Table 3-3 was the mean percentage of mucous cells in a given segment which were labeled. The percent of whole mucous cell population which consisted of labeled mucous cells in a given segment was given in column II in the same table. The birth rate of mucous cells per hour was calculated from the labeling indices assuming the DNA synthesis time of mucous cells to be 8 hours (column III in Table 3-3). The birth rate was highest in segments 1 and 2 and decreased steadily from segment 3 to segment 6. No mucous cells were produced by mitosis in segment 7 and up.

On the other hand, the area of the crypt in which columnar cells transformed into mucous cells was assumed to be in the lower portion of the crypt in the small intestine of rodents (Merzel and Leblond, 1969; Cairnie, 1970). In the descending

colon of the mouse, the frequency of occurrence of mucous cells increased from segment 1 (12.7%) to segment 4 (19.0%), whereas the relative number of vacuolated cells decreased from segment 1 (83.3%) to segment 4 (80.0%) (Table 1-1). According to the labeling indices of mucous cells (9.9-16.1%) and vacuolated cells (20.1-22.4%) in these lower four segments of the crypt, less mucous cells would be produced than vacuolated cells. Therefore, in order to account for the relative increase in the number of mucous cells from segment 1 to segment 4, transformation of vacuolated cells to mucous cells must have taken place in this region of the crypt.

Frequency of transformation of cells from the vacuolated-columnar cell line to the mucous cell line

The frequency of transformation of cells from the vacuolated-columnar cell line to the mucous cell line was assessed from the relative percentages of numbers of vacuolated (V) and mucous (M) cells in segments 1 through 4 (Table 1-1), assuming that transformed cells were recognized as mucous cells in the next upper segment. If they were recognized as mucous cells in the same segment, they were already included in the mucous cell population. (This statement might not be applied to segment 1, because the transit-time of this segment was longer as will be shown.)

Suppose a fraction ($q_{n,\, n+1}$) of vacuolated cells in segment n would transform into mucous cells between segment n and segment n+1 (suppose also that enterochromaffin cells change independently, if not, the effect would be quantitatively negligible), the increase in mucous cells being

$q_{n,\,n+1} \times V_n$ in 100 cells. Then, between segment n and segment n + 1, the following equation can be set (the formula devised by N.J. Nadler):

$$\frac{M_{n+1}}{V_{n+1}} = \frac{M_n + q_{n,\,n+1} \times V_n}{V_n - q_{n,\,n+1} \times V_n}$$

Hence,
$$q_{n,\,n+1} = \frac{\frac{M_{n+1}}{V_{n+1}} - \frac{M_n}{V_n}}{1 + \frac{M_{n+1}}{V_{n+1}}}$$

The ratios $\frac{M_n}{V_n}$ for n = 1, 2, 3 and 4, were obtained from Table 1-1. Based on this equation, the fraction of vacuolated cells in segment 1 which would transform into mucous cells between segment 1 and segment 2 ($q_{1,2}$) was calculated to be 0.028. Similarly, $q_{2,3} = 0.031$ and $q_{3,4} = 0.012$. In other words, if vacuolated cells in passing from segment n to segment n + 1 were to transform into mucous cells at the rate $q_{n,\,n+1}$, this would account for the change in steady state ratio $\frac{M_n}{V_n}$ to $\frac{M_{n+1}}{V_{n+1}}$. Therefore, the rate of transformation ($tr_{n,\,n+1}$) of vacuolated cells into mucous cells between segment n and segment n+1, in terms of percentage increase in mucous cells, can be obtained from the following formula (the formula devised by N.J. Nadler):

$$tr_{n,\,n+1} = {}_v(K_{in})_{n+1} \times q_{n,\,n+1} \times \frac{V_n}{M_n}$$

where ${}_v(K_{in})_{n,\,n+1}$ was the influx of vacuolated cells in segment n+1 (passing from segment n) (column IV in Table 2-1). Thus, the rate of

transformation of vacuolated cells to mucous cells between segment 1 and segment 2 ($tr_{1,2}$) was estimated to be 0.05 cells per 100 mucous cells per hour. Similarly, $tr_{2,3}$ = 0.08% cells per hour, and $tr_{3,4}$ = 0.04% cells per hour. They were listed in column V in Table 3-3.

Cell fluxes

Fluxes of mucous cells in the dividing segments of the crypt were calculated by the same method and on the same assumptions used in the vacuolated-columnar cell line (Chapter II). However, the calculation of mucous cell fluxes was a little more complex than that of vacuolated-columnar cell fluxes, because of cell transformation.

In segment 1, there is a distinct possibility that some of the vacuolated cells would transform into mucous cells and enter the mitotic cycle as mucous cells in the same segment, but there was no possible way to estimate this fraction; and this fraction had to be ignored. In other words, in the calculations, there was no influx to the mucous cell population in segment 1. Hence, the efflux of segment 1 was taken to be equal to the birth rate in this segment (0.18% cells per hour).

The influx of mucous cells in segment 2 had two sources: (1) the efflux of segment 1 (0.18% cells per hour), and (2) the transformation of vacuolated cells between segment 1 and segment 2 ($tr_{1,2}$ = 0.05% cells per hour); thus, the total influx of mucous cells in segment 2 was 0.18 + 0.05 = 0.23% cells per hour. Since 0.18% cells were produced by mitosis every hour in segment 2, the efflux of segment 2 would be 0.23 + 0.18 = 0.41% cells per hour. Similarly, both influx and efflux of segments 3 and 4 could be calculated. In segments 5 and 6, cell transformation was

not considered; thus, cell fluxes were simply estimated according to the relationship that efflux was equal to influx plus birth rate. These calculated fluxes of cells were listed in columns IV-VII in Table 3-3.

Since no new cells were formed in segments 7-10 and in the surface epithelium, the influx or efflux of mucous cells in these segments would be equal to the efflux of segment 6, which was 0.84% cells per hour.

Mean number of mitotic cycles in each segment of the crypt and mean cell cycle time of mucous cells

The mean number of mitotic cycles of mucous cells for each segment from segment 2 to segment 6 was estimated using the formula demonstrated by Cleaver (1967) (see Chapter II), the results were listed in column VIII in Table 3-3.

For the estimation of mucous cell cycle time and the mean number of mitotic cycles in segment 1, a method devised by N.J. Nadler was used (Chapter II). Since the labeling index of mucous cells in segment 1 was 15.5%, the turnover time would be $\frac{100}{15.5} \times 8 = 51.6$ hours. From this and the transit time of mucous cells in the non-dividing segments of the crypt (11.5 hours per segment), and the mean numbers of mitotic cycles in segments 2-6, the cell cycle time was calculated to be 22.2 hours (cf. 29.7 hours in the vacuolated-columnar cell line). Therefore, the mean number of mitotic cycles of mucous cells in segment 1 was 0.43.

The total number of mitotic cycles that a mucous cell at the base of the crypt and its progeny would go through on ascending the cryptal wall would be 1.94, which was obtained by adding up all the values of the mitotic cycles from segment 1 to segment 6.

Velocity and transit time of mucous cells in the dividing segments (1-6) of the crypt

When cells in the vacuolated-columnar cell line transform into cells in the mucous cell line, no increase in total number of cells occurs; accordingly, transformation of cells does not contribute to any increase in velocity of cell migration; only mitosis of cells does. According to the method devised by N.J. Nadler, the velocity increment in a given segment in the proliferative region of the crypt is equal to the number of mitotic cycles divided by the cell cycle time, and the velocity in a particular segment is obtained as the cumulative velocity increment up to that segment. Thus, transit time is calculated as an inverse of velocity (Chapter II). From the mean number of mitotic cycles in each segment (Table 3-3) and the cell cycle time of 22.2 hours, the velocity and transit time of each dividing segment were computed and listed in Table 3-4. As in the case of vacuolated-columnar cells, mucous cells migrated much more slowly at the base of the crypt, faster as they came to the middle portion of the crypt, and gained a constant speed as they entered the non-dividing segments of the crypt.

The estimated transit time in segment 1 (used interchangeably with turnover time in this segment) was much longer than expected. This could be explained by the fact that the influx of mucous cells in segment 1 from vacuolated cells was ignored in calculation because of the lack of an adequate method to estimate this fraction.

DISCUSSION

Formation of new mucous cells in the colonic epithelium of the mouse

The classic views on the origin of mucous cells in the intestine of adult mammals were divided mainly into two schools depending on whether or not mucous cells were considered to possess the capacity to divide. One school maintained that mucous cells were independent cells capable of self-renewal (Bizzozero, 1889, 1892; Sacerdotti, 1894; Leblond and Messier, 1958; Thrasher and Greulich, 1966), while the other school claimed that mucous cells did not undergo mitosis and originated in columnar cells (Paneth, 1888; Nassonov, 1923, 1924; Clara, 1926; Chlopkow, 1928; Florey, 1932; Cairnie, 1970). The present investigations have demonstrated that mucous cells incorporated ^{3}H-thymidine and underwent division; yet, more cells were turned over as mucous cells in the non-dividing segments of the crypt than were produced only by mitotis. Therefore, it is concluded, in accordance with the observations of Merzel and Leblond (1969) in the mouse small intestine, that mucous cells do arise from cells in the vacuolated-columnar cell line in the lower portion of the crypt, but they are also still capable of undergoing division before they become non-dividing mucous cells in a typical goblet form.

Although the transformation of columnar cells to mucous cells was assumed to occur in the lower portion of the crypt in the small intestine of rodents (Merzel and Leblond, 1969; Cairnie, 1970), the present study presented evidence that such transformation actually takes place in the lower four segments of the crypt in the descending colon of

the mouse.

The number of mitotic cycles (or generations) of mucous cells

The estimation of the mean number of mitotic cycles of mucous cells was complicated by the transformation of vacuolated cells to mucous cells in segments 1 through 4. The present analysis reveals that a mucous cell in segment 1 and its progeny would go through about two mitotic cycles on the average on ascending the cryptal wall before changing to non-dividing mucous cells. The evidence of two mitotic cycles means the existence of three generations of mucous cells.

Of two mitotic cycles, the first cycle takes place in segments 1 and 2, whereas the second cycle spreads over segments 2 through 6. In the second and last mitotic cycle, dividing mucous cells change over to non-dividing ones, in accordance with the slow cut-off model of Cairnie et al. (1965b).

On the other hand, the fate of transformed mucous cells would probably depend on where the cell transformation takes place. If vacuolated cells transform into mucous cells in segments 1 and 2, they would have gone through one mitotic cycle as vacuolated cells (see Fig. 2-5). After transformation, they would probably undergo two more cycles as mucous cells (Table 3-3). If cell transformation occurs between segment 2 and segment 4, these cells have had two mitotic cycles as vacuolated cells before transformation (Fig. 2-5). After transformation, they would likely have one more cycle as mucous cells (Table 3-3). In either case, each cell would go through on the average three mitotic cycles from its birth

to death. Since cells in the vacuolated-columnar cell line also go through three mitotic cycles on the average (Fig. 2-5), each epithelial cell (excluding enterochromaffin cells) in the descending colon of the mouse would experience three mitotic divisions from birth at the base of the crypt to death from the surface no matter upon which cell line it would end.

The present data indicate that mucous cells have an overall shorter cell cycle time than vacuolated-columnar cells (22.2 hours vs. 29.7 hours), contrary to the conclusions of Thrasher and Greulich (1966) that the cell cycle times for columnar and mucous cells were the same in the mouse small intestine. However, the present data are in line with the observations of Cairnie et al. (1965a), who showed in the rat small intestine that the cell cycle time shortens as cells migrated up the cryptal wall and became more differentiated, indicating that among proliferative cells, more differentiated cells had a shorter cell cycle time. Since mucous cells originate in vacuolated cells, it is conceivable that mucous cells may have a shorter cell cycle time. Moreover, the probablilty of a mucous cell being pushed out of the top of a segment will depend on the production of any new cell in that segment, vacuolated-columnar or mucous. Since there are approximately five times more vacuolated-columnar than mucous cells, this would produce a tendency for mucous cells to become depleted from that segment. This tendency would be offset by the transformation of vacuolated-columnar cells to mucous cells. In addition, it is conceivable that the steady state balance of mucous cell production

would also be maintained by a shorter cell cycle time.

Migration of mucous cells

Soon after giving ^{3}H-thymidine, labeled mucous cells were seen in segments 1 through 6. With time, labeled mucous cells made their appearance in the higher segments of the crypt and by 72 hours or later on the surface. They were eventually extruded from the surface as demonstrated in Chapter I. Some irregularity was noted in the migrational velocity of mucous cells from animal to animal, partly due to the variation in the size of mucous cell population among animals (Appendix 1-2).

A tendency is that mucous cells seemed to lag one segment behind vacuolated-columnar cells in migration. This might simply be due to the fact that labeled cells at one hour after injection of ^{3}H-thymidine were found one segment lower in the mucous cell line than in the vacuolated-columnar cell line (cf. Table 2-1 and Table 3-3).

The transit time estimated from the distances between the curves of increment of percent labeled cells of the non-dividing segments at 50% labeling level was slightly greater for mucous cells (11.5 hours per segment) than for vacuolated-columnar cells (9.6 hours per segment). Because of relatively low precision in estimating the migrational velocity in mucous cells, it is not known whether or not such a difference was a true one. Since all the epithelial cells in the intestinal epithelium are joined together by junctional complexes, they are supposed to move as an escalator. Thus, it would be expected that the migrational velocity of both vacuolated-columnar cells and mucous cells ought to be the same.

On the other hand, if the simple escalator model were valid, then theoretically, the cell which occupies position 1 in the bottom segment, by mitosis and migration of daughter cells, ought eventually to replace all other cells, and only cells in this line would occupy the whole of the crypt and all other cells would be depleted. Obviously, this is not the case. Many possibilities exist to modify this model. Pertaining to the argument above about the tendency to mucous cell depletion, it was stated that the tendency would be offset by transformation of vacuolated-columnar cells to mucous cells and by a shorter cell cycle time of mucous cells. Furthermore, if contrary to theoretical expectation on a morphological basis, mucous cells were to migrate for some reasons more slowly than columnar cells (that is, columnar cells would pass over mucous cells in transit), this too would tend to maintain a steady state balance of mucous cell population. With increase in precision in estimating kinetic parameters, this problem will be clarified.

SUMMARY

The production and migration of mucous cells were studied in the descending colonic epithelium of adult female mice given a single injection or a continuous infusion of ^{3}H-thymidine with sacrifice at various times from one hour to 12 days. This investigation was carried out using one micron thick Epon sections which were radioautographed after staining with the periodic acid-Schiff technique and iron-hematoxylin.

Mucous cells located in segments 1 through 6 of the crypt were capable of incorporating ^{3}H-thymidine and thus capable of undergoing mitosis. However, the rate of turnover of mucous cells based on proliferative rate was found to be much lower than the rate of turnover of mucous cells based on the transit time in the non-dividing segments of the crypt. Since there was a concomitant overproduction of cells in the vacuolated-columnar cell line and some morphological similarity between vacuolated cells and newly formed mucous cells in the lower portion of the crypt, it was concluded that some vacuolated cells would give rise to mucous cells. This transformation occurs in the lower four segments of the crypt.

Mucous cells which were formed by transformation would migrate upward along the cryptal wall and accumulate more mucus in the theca; in doing so, they would undergo one or two divisions depending on the site of transformation before they become non-dividing mucous cells. The latter moved up to the surface, where they were lost into the colonic lumen. The migrational velocity of mucous cells might be the same or slightly slower than that of vacuolated-columnar cells.

Table 3-1. Time taken for mucous cells to migrate from segment to segment in the crypt as measured from the distance between curves at 50% labeling level in Fig. 3-1. (Experiment IV).

Segments considered	Transit time (in hours)	Mean Transit time per segment (in hours)
7 to 8	10.0	
8 to 10	24.6	(12.3)
10 to S	13.4	
7 to 10	34.6	11.5
7 to S	48.0	12.0

Table 3-2. Turnover rates in the mucous cell line.

(A) Calculated from transit times of non-proliferative segments:

Experiment	Transit time (hour per segment)	Turnover rate of whole epithelium (% per hour)
IV	11.5[a]	0.79
	12.0[b]	0.76

(B) Calculated from labeling frequency of proliferative segments:

Experiment	% cells labeled at 1 hour	Turnover rate of whole epithelium (% per hour)
II	3.1[c]	0.39
III	5.1[d]	0.64
IV	5.2[e]	0.65

Note:

a and b = Estimated from the distance between curves in Fig. 3-1 at 50% labeling level. a for the mean from segment 7 to segment 10; b for the mean from segment 7 to the surface.

c = animals sacrificed at 13:00 h.

d = mean labeling index from four groups of animals sacrificed at four different times of day.

e = animals sacrificed at 12:00 h.

Table 3-3. The proliferative activity* and related parameters in each segment of the crypt in the mucous cell line.

Segment	For 100 mucous cells in each segment: % cells labeled at 1 hour (I)	For 100 cells in the total mucous cell population: % cells labeled at 1 hour (II)	Birth rate per hour (III)	Influx of cells per hour: By mitosis in lower segment (IV)	By transformation from lower segment (V)	Total (VI)	Efflux of cells per hour (VII)	Mean Number of mitotic cycles (VIII)
10	-	-	-	0.84	-	0.84	0.84	-
9	-	-	-	0.84	-	0.84	0.84	-
8	-	-	-	0.84	-	0.84	0.84	-
7	-	-	-	0.84	-	0.84	0.84	-
6	0.8	0.07	0.01	0.83	-	0.83	0.84	0.01
5	3.6	0.33	0.04	0.79	-	0.79	0.83	0.07
4	9.9	0.90	0.11	0.64	0.04	0.68	0.79	0.21
3	13.1	1.19	0.15	0.41	0.08	0.49	0.64	0.39
2	16.1	1.46	0.18	0.18	0.05	0.23	0.41	0.83
1	15.5	1.41	0.18	-	-	-	0.18	(0.43)

* See Results for details.

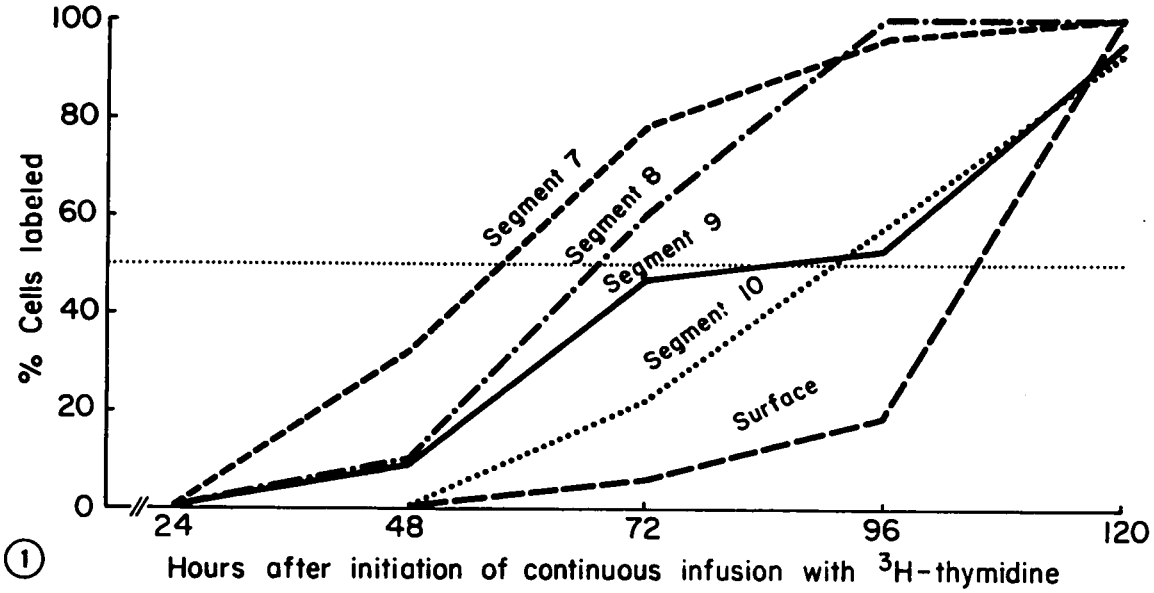
Table 3-4. Transit time of mucous cells in each segment of the crypt.*

Segment in crypt	Mean number of mitotic cycles	Velocity increment in segment	Velocity in segment		Transit time (in hours)	
10	-			0.087		11.5
9	-			0.087		11.5
8	-			0.087		11.5
7	-			0.087		11.5
6	0.01	0.01/22.2	1.94/22.2	0.087	22.2/1.94	11.5
5	0.07	0.07/22.2	1.93/22.2	0.087	22.2/1.93	11.5
4	0.21	0.21/22.2	1.86/22.2	0.084	22.2/1.86	11.9
3	0.39	0.39/22.2	1.65/22.2	0.074	22.2/1.65	13.5
2	0.83	0.83/22.2	1.26/22.2	0.057	22.2/1.26	17.6
1	0.43	0.43/22.2	0.43/22.2	0.019	22.2/0.43	51.6

* See Results for details.

Fig. 3-1. The increment of percent labeled mucous cells in the non-dividing segments of the crypt with various times after initiation of continuous infusion with ^{3}H-thymidine.

The mucous cell line



CHAPTER IV

RENEWAL OF ENTEROCHROMAFFIN CELLS IN THE DESCENDING COLON OF THE MOUSE

INTRODUCTION

Enterochromaffin cells were first described in the stomach by Heidenhain (1870) and in the intestine by Grützner (1879). They were later identified in the epithelium of the entire gastrointestinal tract of most vertebrates (Patzelt, 1936).

The origin of enterochromaffin cells has been the object of much speculation. According to Danisch (1924), they would be derived from the nerve cells of sympathetic ganglions and migrate to the epithelium during the embryonal development. Dias-Amado (1925a, 1925b) considered that they came from wandering cells which entered the epithelium and settled there. In a study of chick embryos, Kull (1925) claimed that enterochromaffin cells arose in the connective tissue of the lamina propria and entered the intestinal epithelium on the 15-16th hatching day; the granules of these cells would be acidophilic at first and become chromaffin on the 17-18th day of incubation.

In contrast, Simard and van Campenhout (1932) found that enterochromaffin cells originated in the intestinal epithelium at the end of the 11th incubation day in chick embryos, but migrated out into the submucosa at later times. The epithelial origin of these cells was widely

accepted (Masson, 1914, 1928; Parat, 1924; Cordier, 1926; Tehver, 1930; Patzelt, 1931; Clara, 1934, Friedmann, 1934, and others), but not their emigration to the submucosa. It was generally assumed that enterochromaffin cells remained indefinitely within the epithelium, although an opinion was also expressed that they could arise from epithelial cells in adults (Patzelt, 1936).

Classically, it was believed that enterochromaffin cells were not capable of division (Patzelt, 1936). And indeed, a few hours after injection of ^{3}H-thymidine, none of these cells were labeled, a further indication that they were not capable of division (Leblond and Messier, 1958; Ferreira, 1970). However, Kull (1925) assumed that enterochromaffin cells could divide, but only when in an "empty" condition, that is, after release of cytoplasmic granules. Deschner and Lipkin (1966), using the Diazo method (Pearse, 1960) to identify enterochromaffin cells, did observe six cells in mitosis out of 15,977 enterochromaffin cells counted and also found 1.2% of enterochromaffin cells labeled one hour after injection of ^{3}H-thymidine. They concluded that these cells were able to proliferate at a slow rate.

On the other hand, Masson (1914) and Parat (1924) believed that enterochromaffin cells originated from amitosis of intestinal epithelial cells by a particular type of differentiation. Friedmann (1934) extended this view and stated that amitosis was indicated by the occasional presence of two enterochromaffin cells side by side and also by observations of enterochromaffin cells with two or three nuclei.

Today, amitosis is not believed to play a role in mammals, except under pathological conditions. Since the existence of mitosis of enterochromaffin cells was in doubt, it was decided to explore their mode of origin and renewal. Although they have been observed both in crypts and on the surface of the colon (Patzelt, 1936), it was not known at all whether they migrated like the other epithelial cells of the intestine.

In the course of a systematic investigation of cell kinetics in the epithelium of the descending colon of the mouse, relevant information was obtained on the renewal behavior of enterochromaffin cells. However, the number of enterochromaffin cells analyzed was relatively small, and therefore, the information obtained may be considered to be preliminary.

MATERIALS AND METHODS

Three experiments which were used to analyze the kinetic behavior of vacuolated-columnar and mucous cells were also considered in this analysis of enterochromaffin cell renewal:

(1) Experiment II, in which a group of adult mice were sacrificed at various times after a single injection of ^{3}H-thymidine (Chapter I);

(2) Experiment III (Diurnal variation), in which four groups of five adult mice each were sacrificed one hour after giving ^{3}H-thymidine but at four different times of day (Chapter I); and

(3) Experiment IV (Continuous infusion), in which a group of adult mice were sacrificed at various times following the initiation of a continuous

infusion of ^{3}H-thymidine (Chapter II).

The descending colon was fixed in 4% paraformaldehyde in 0.1 M Sörensen's buffer. One-micron thick Epon sections were stained with PA - Schiff and iron-hematoxylin and radioautographed. The detail of processing and the methods used in the quantitation of epithelial cells were reported in Chapter I.

Electronmicroscopy of enterochromaffin cells was also performed using materials from Experiment I; both the experiment and electron-microscopic techniques were described in Chapter I.

RESULTS

Enterochromaffin cells were recognized in the descending colon of the mouse by the presence of groups of very fine iron-hematoxylin stained granules in an otherwise pale cytoplasm, and by the appearance of a light, finely granulated, roundish nucleus. These cells were found both in the crypt and on the surface. However, their frequency of occurrence was only 1.5% of the total epithelial cell population. They appeared quite frequently in segments 1 and 2 of the crypt, in decreasing numbers towards the mouth of the crypt, and rarely in segment 10 and in the surface epithelium (Table 1-1; Fig. 4-1).

On proliferation

At one hour after injection of ^{3}H-thymidine, a total of 402 granule-containing enterochromaffin cells were investigated. None was labeled. Furthermore, no mitotic figure was found in cells in which

groups of iron-hematoxylin stained granules were seen in the light microscope. Only in one instance at the electronmicroscopic level, a cell in metaphase was found to contain a very small number of membrane-bound, moderately dense granules identical to typical enterochromaffin granules (Fig. 4-2). However, even if it were assumed that a cell with so few granules could be classified as enterochromaffin, such a cell could not be identified in the light microscope. It was concluded that whenever an enterochromaffin cell contained enough granules to be identified as such, this cell did not undergo mitosis.

On renewal

Although typical enterochromaffin cells were never labeled at one hour after injection of ^{3}H-thymidine, some were by 36 hours and later (Fig. 4-3). In the single injection experiment (Experiment II), the number of labeled enterochromaffin cells was too small and too irregular to allow an adequate investigation of their mode of renewal. But relevant results were obtained in the animals receiving a continuous infusion of ^{3}H-thymidine. Labeling was lacking in the animals sacrificed either one hour or one day after the beginning of the infusion. However, labeling was present in 5 out of 43 (12%) enterochromaffin cells at 2 days (Fig. 4-3), 9 out of 48 (19%) at 3 days (Fig. 4-4), 14 out of 40 (35%) at 4 days (Fig. 4-5), and 8 out of 17 (47%) at 5 days. Between the first and the fifth day, the increment of labeled enterochromaffin cells appeared to be linear at a rate of 0.51% cells per hour (Fig. 4-7).

However, the increase did not continue at later times in spite of the continuation of the infusion. Thus, 31 out of 58 (53%) cells were labeled at eight days (Fig. 4-6), and 38 out of 77 (49%) cells at 12 days after initiation of the infusion (Fig. 4-7).

On migration

In the experiment of continuous infusion with ^{3}H-thymidine (Experiment IV), enterochromaffin cells labeled at two days were found mainly in segments 1 and 2 of the crypt (Fig. 4-3) and rarely up to segment 4. As the infusion continued, labeled enterochromaffin cells were located higher in the cryptal walls. The highest positions of labeled enterochromaffin cells were segment 5 at three days, segment 6 at four and five days, segment 9 at eight days and segment 10 at twelve days after the start of infusion. These observations indicated that enterochromaffin cells migrated upward along the cryptal walls as other epithelial cells did, but at a much slower rate. On the surface of the colon, labeled enterochromaffin cells were not observed.

DISCUSSION

Do enterochromaffin cells divide?

The classic opinion that enterochromaffin cells do not divide (Patzelt, 1936) was challenged by Deschner and Lipkin (1966) who claimed that such mitosis might be found in the human rectum. However, the photograph which they published was not convincing. In accord with the classical opinion, the present light microscopic work with Epon

sections showed granule-containing enterochromaffin cells which revealed neither mitosis nor labeling at one hour after ^{3}H-thymidine injection.

In the electron microscope, however, one metaphase was observed which contained a very small number of granules resembling enterochromaffin cell granules (Fig. 4-2). Ferreira (unpublished) working here on the small intestine of mice also observed the odd metaphase with a very small number of enterochromaffin granules, but no cell with a fair number of granules was in mitosis or showed label at one hour after ^{3}H-thymidine injection. It is possible that dividing cells with a very small number of granules are precursors of enterochromaffin cells. The differentiation of a precursor cell would imply acquiring enough granules to be recognized in the light microscope but at the same time lose the ability to divide. It may be concluded that typical enterochromaffin cells do not divide, whereas the precursors of these cells may do so. Since two identical enterochromaffin cells have rarely been observed side by side (Fig. 4-8), it is even possible that two daughter cells of the division of a precursor cell may be enterochromaffin.

Where do enterochromaffin cells (or their precursors) come from?

The extra-epithelial origin of enterochromaffin cells was postulated by some classical histologists (Danisch, 1924; Dias-Amado, 1925a, 1925b; Kull, 1925), because preparations stained with silver to detect enterochromaffin cells might also show cells of the lamina propria

which contained silver-stained granules. However, such speculations did not gain support because of the relative non-specificity of the silver reaction. On the other hand, Andrew (1957, 1965) speculated that migrating lymphocytes transformed into epithelial cells. These lymphocytes showed some similarity to enterochromaffin cells since cytoplasm was pale and the nuclei of some of them showed loose chromatin. However, the examination of these cells in serial sections revealed not only the absence of enterochromaffin granules, but in some cases the presence of cytoplasmic processes extending in various directions (whereas the single process seen in some enterochromaffin cells abuts on the basement membrane and extends towards the base of the crypt). In addition, enterochromaffin cells were connected by junctional complexes with adjacent epithelial cells (Schofield and Silva, 1968), whereas lymphocytes were not. Finally, at early times after ^{3}H-thymidine, labeled lymphocytes were found at any level of the crypt as well as on the surface, whereas labeled enterochromaffin cells were restricted to the base of the crypt. For all these reasons, the lymphocytic origin of enterochromaffin cells may be dismissed.

A number of investigators (Masson, 1914, 1928; Parat, 1924; Cordier, 1926; Tahver, 1930; Patzelt, 1931; Clara, 1934; Friedmann, 1934 and others), agreed that enterochromaffin cells originated in the entoderm, while in the adult, they would be static or would arise by an unknown mechanism from intestinal epithelial cells (Patzelt, 1936). The suggestion was that enterochromaffin cells differentiated from a

precursor cell. After injection of ^{3}H-thymidine, it would take more than 24 hours for the unidentified precursor cell to divide and transform into a granule-containing enterochromaffin cell. Perhaps the precursor itself came from a poorly differentiated vacuolated cell at the base of the crypt.

How do enterochromaffin cells renew?

Although the origin of enterochromaffin cells was not yet clear, the present study indicated that they did renew and migrate along the cryptal wall toward the surface. Approximately 0.51% cells were renewed per hour between the first and the fifth day of continuous infusion; thereafter their number did not seem to change up to 12 days (Fig. 4-7). And yet upward migration of labeled cells continued.

The first explanation that came to mind was that the enterochromaffin cell population consisted of heterogeneous groups of cells (Hamperl, 1932; Singh, 1964; Schofield et al., 1967; Carvalheira et al., 1968; Forssmann et al., 1969; Capella et al., 1969; Vassallo et al., 1969, among others) which would renew at different rates. In the light microscopic studies, two types of enterochromaffin cells were identified (Chapter I): the usual one with relatively abundant darkly stained granules and the type with fewer granules presumed to be the multivesicular cell of Silva (1967). Although no quantitative assessment was attempted, a fraction of both types appeared to be labeled on continuous infusion of ^{3}H-thymidine. However, the cells with abundant granules developed a

long foot process on migration (Fig. 1-12), which on morphological grounds, might indicate a much slower migrational activity; therefore a possibility that the renewal rate was different in both types of cells could not be entirely excluded. On the other hand, at the electron-microscopic level, two types of enterochromaffin cells were identified in the mouse colon: one with homogeneously spherical granules and the other with irregularly discoid and spherical granules (Fig. 4-9). The unpublished work of Ferreira alluded to the above and indicated that the rate of renewal of the two types in the small intestine was the same.

Assuming that all enterochromaffin cells behaved in a similar manner, another explanation must be sought. The problem is to explain how, in spite of continuous infusion and presumably continuous addition of labeled cells at the rate of 0.51% per hour, their overall percentage did not increase between the fifth and the twelfth day. Presumably, there must be a cell loss balancing the cell addition. Since the highest labeled cells were only in segment 6 at five days, the loss might take place around this region. (Whether the loss is due to cell death, extrusion to the lumen or emigration to the lamina propria is not known.) If this explanation were the only one, the labeling of enterochromaffin cells would not stop increasing, since both the labeled and unlabeled cells which were lost would be replaced by labeled cells and thus the proportion of labeled cells would rise. Perhaps, then, some of the enterochromaffin cells remain static in depressions of the basement membrane (Fig. 4-8) and thus stop migrating. Such cells would be

preserved from being labeled.

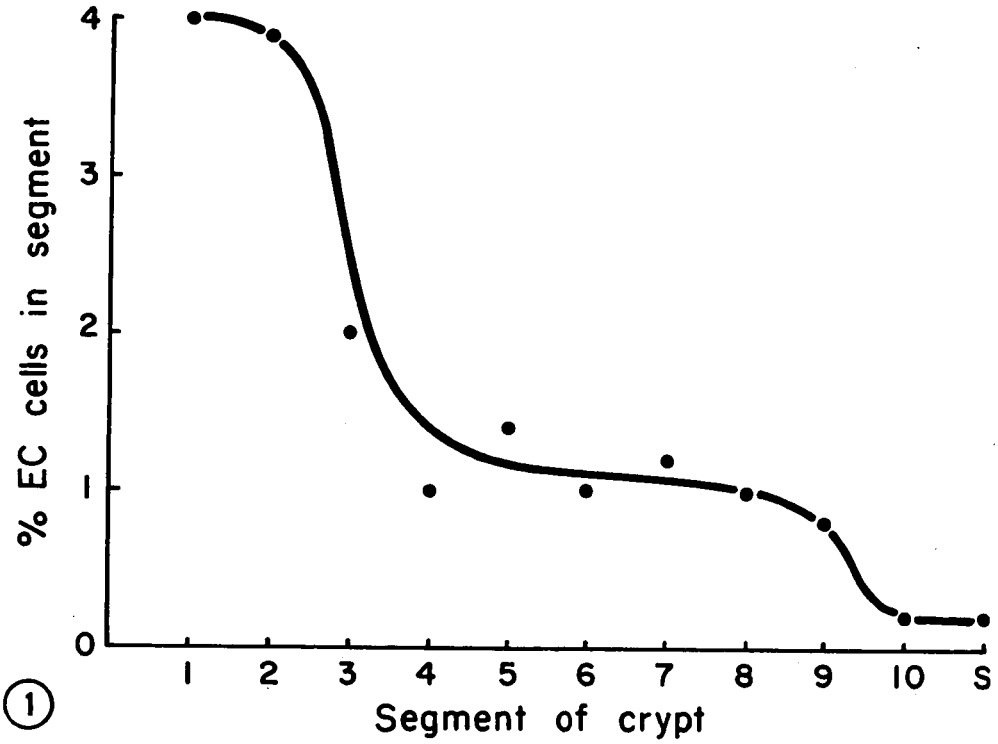
SUMMARY

The labeling and overall behavior of enterochromaffin cells in the descending colon of the mouse was examined by radioautography of semi-thin Epon sections after a single injection or continuous infusion of ^{3}H-thymidine.

Typical enterochromaffin cells do not show mitosis nor do they take up label one hour after injection of ^{3}H-thymidine. Therefore, these cells do not undergo mitosis. Nevertheless, by 36 hours and later after the beginning of continuous infusion of ^{3}H-thymidine, labeled enterochromaffin cells are present. The labeled cells increase in number at the rate of 0.51% per hour between the first and the fifth day; later their number no longer increases, presumably because cells are lost at the same rate as cells are produced. In any case, the labeled enterochromaffin cells migrate upward along the cryptal wall as columnar or mucous cells do.

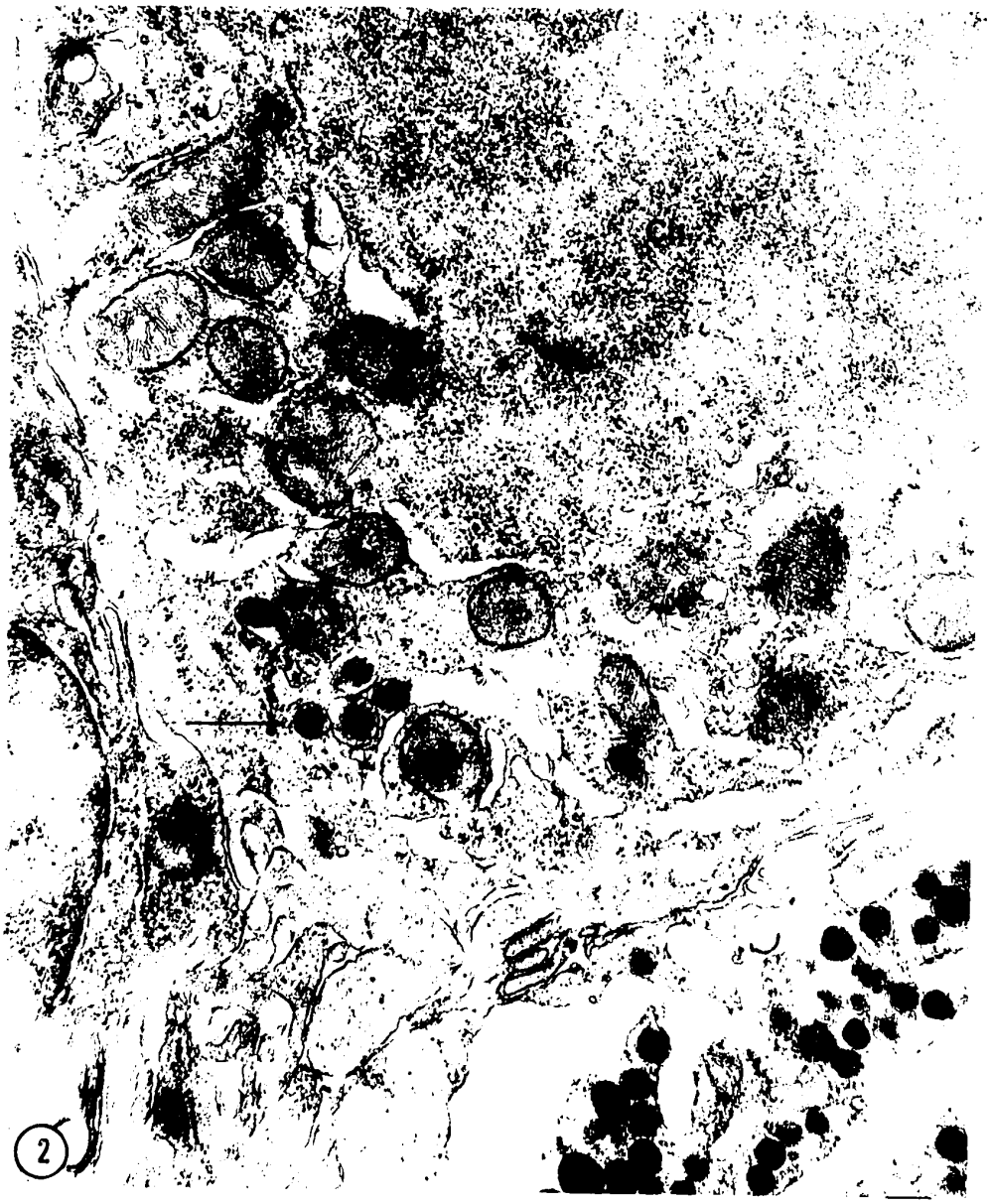
Fig. 4-1. Distribution of enterochromaffin cells in the segments of the crypt and the surface.

Distribution of Enterochromaffin (EC) Cells



(1)

Fig. 4-2. An electronmicrograph of a mitotic cell (metaphase) with slightly dense granular chromosomes (Ch) seen in the right upper portion of the picture. Among mitochondria and cisterna of endoplasmic reticulum located at the periphery of the cell are a few electron dense, membrane-bound granules (arrow) resembling the granules of an enterochromaffin cell at the right lower portion of the picture. x 30, 600.
This and other electronmicrographs are taken from sections stained with both uranyl acetate and lead citrate.


2

Figures 4-3 to 4-6. Light microscopic radioautographs of labeled enterochromaffin cells prepared from animals given continuous infusion of ^{3}H-thymidine.

Fig. 4-3. Two-day interval. Two triangular enterochromaffin cells with pale cytoplasm and light, finely granulated nucleus are seen at the base of the crypt. The one on the right is overlaid with three silver grains (arrow), whereas the one on the left is not labeled. Many other vacuolated and mucous cells are also labeled. x 1300.

Fig. 4-4. Three-day interval. At the base of the crypt is an enterochromaffin cell with pale cytoplasm and light ovoid nucleus (arrow). Cytoplasmic granules are not visible. This cell is labeled as shown by the presence of seven silver grains over the nucleus. Most of other epithelial cells in the crypt were labeled at this period. However, in this radioautograph, few epithelial cells are seen with few silver grains or none, but on examination of adjacent sections, most of them are labeled. x 1300.

Fig. 4-5. Four-day interval. At the base of the crypt is a triangular enterochromaffin cell with granules barely visible at the basal portion of the cell (arrow). The nucleus of this cell is overlaid with six silver grains. All other epithelial cells are labeled. x 1300.

Fig. 4-6. Eight-day interval. In this radioautograph, all the epithelial cells in the lower portion of the crypt are overlaid with a number of silver grains, including a triangular enterochromaffin cell with pale cytoplasm and round nucleus (arrow). x 1300.

3
4
5
6

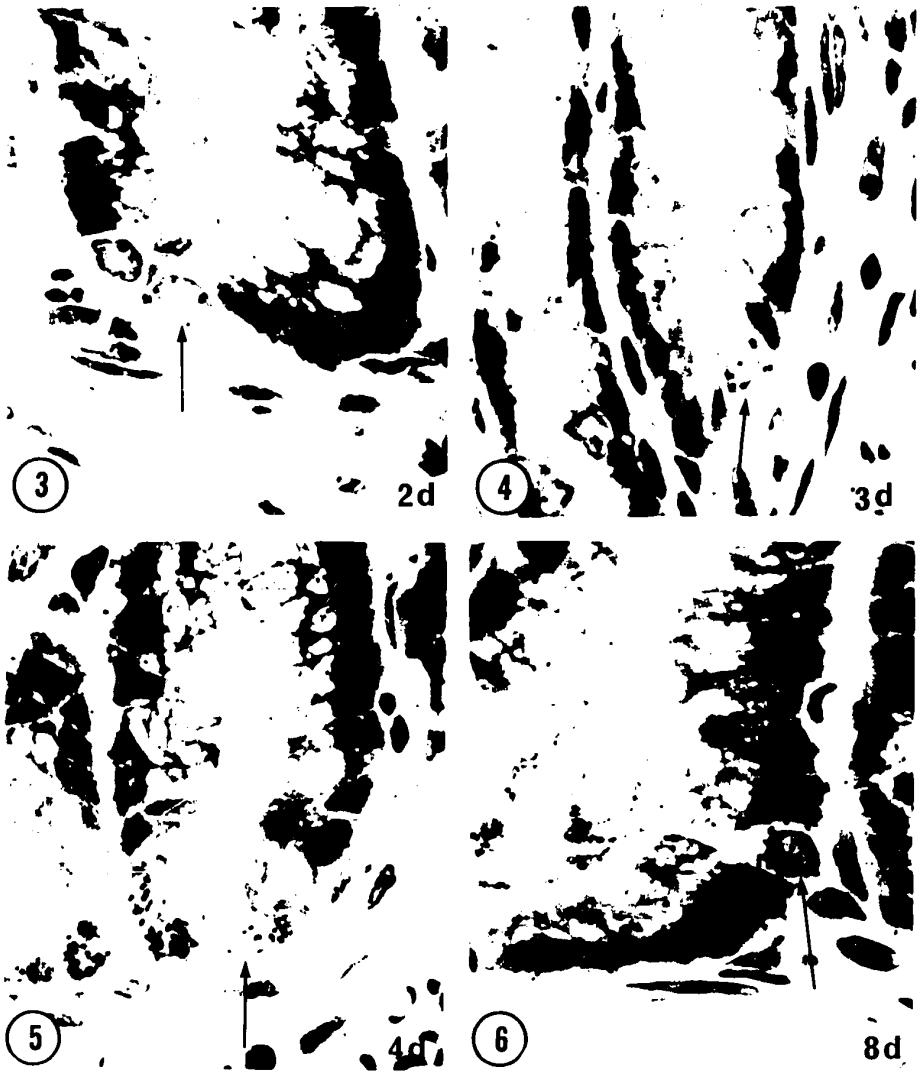
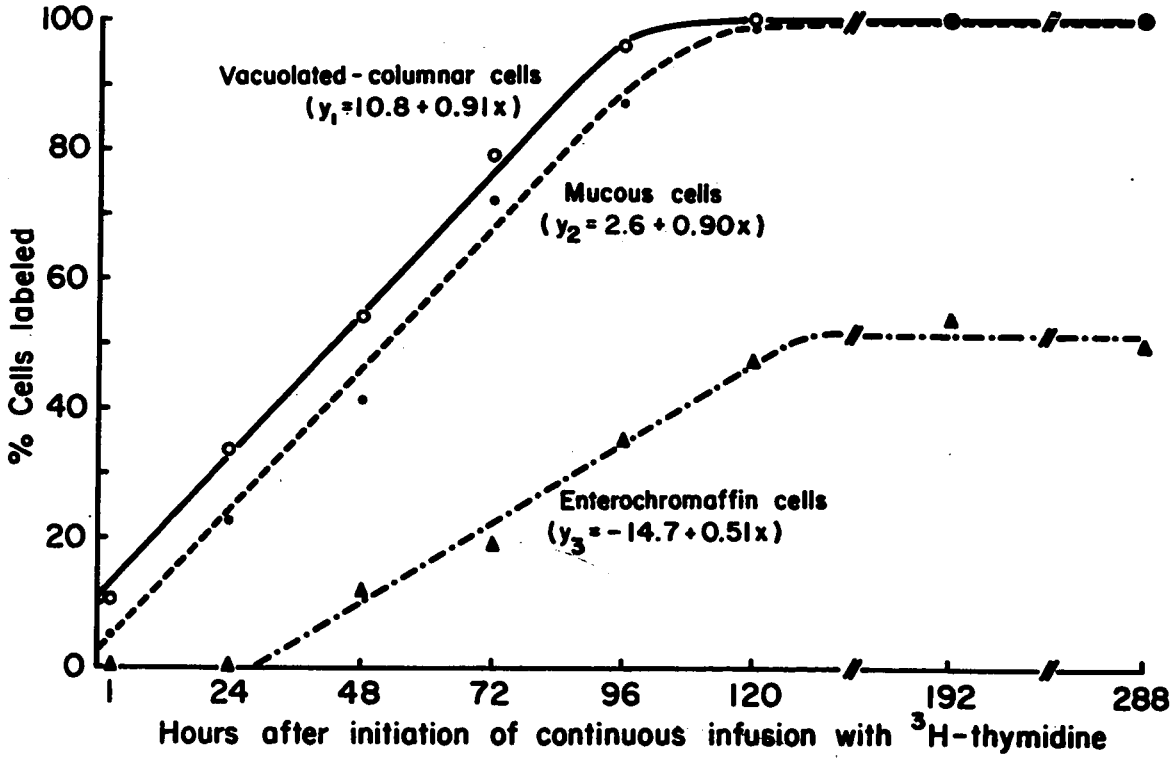

3
2d
4
3d
5
4d
6
8d

Fig. 4-7. The increment of percent labeled cells in the vacuolated-columnar, mucous and entero-chromaffin cell lines at various times after initiation of continuous infusion of ^{3}H-thymidine.


100
80
60
40
20
0
% Cells labeled
Vacuolated - columnar cells
(y_1 = 10.8 + 0.91x)
Mucous cells
(y_2 = 2.6 + 0.90x)
Enterochromaffin cells
(y_3 = -14.7 + 0.51x)
1
24
48
72
96
120
192
288
Hours after initiation of continuous infusion with ^{3}H-thymidine

(7)

Fig. 4-8. Two enterochromaffin cells characterized by the presence of electron dense granules in the cytoplasm are seen side by side at the periphery of a crypt but inside the basement membrane. The lower cell has a short foot process extending toward the base of the crypt and containing a number of granules. Because of the nuclear and cytoplasmic similarities, these two cells may have derived from the same precursor cell. x 11,600.

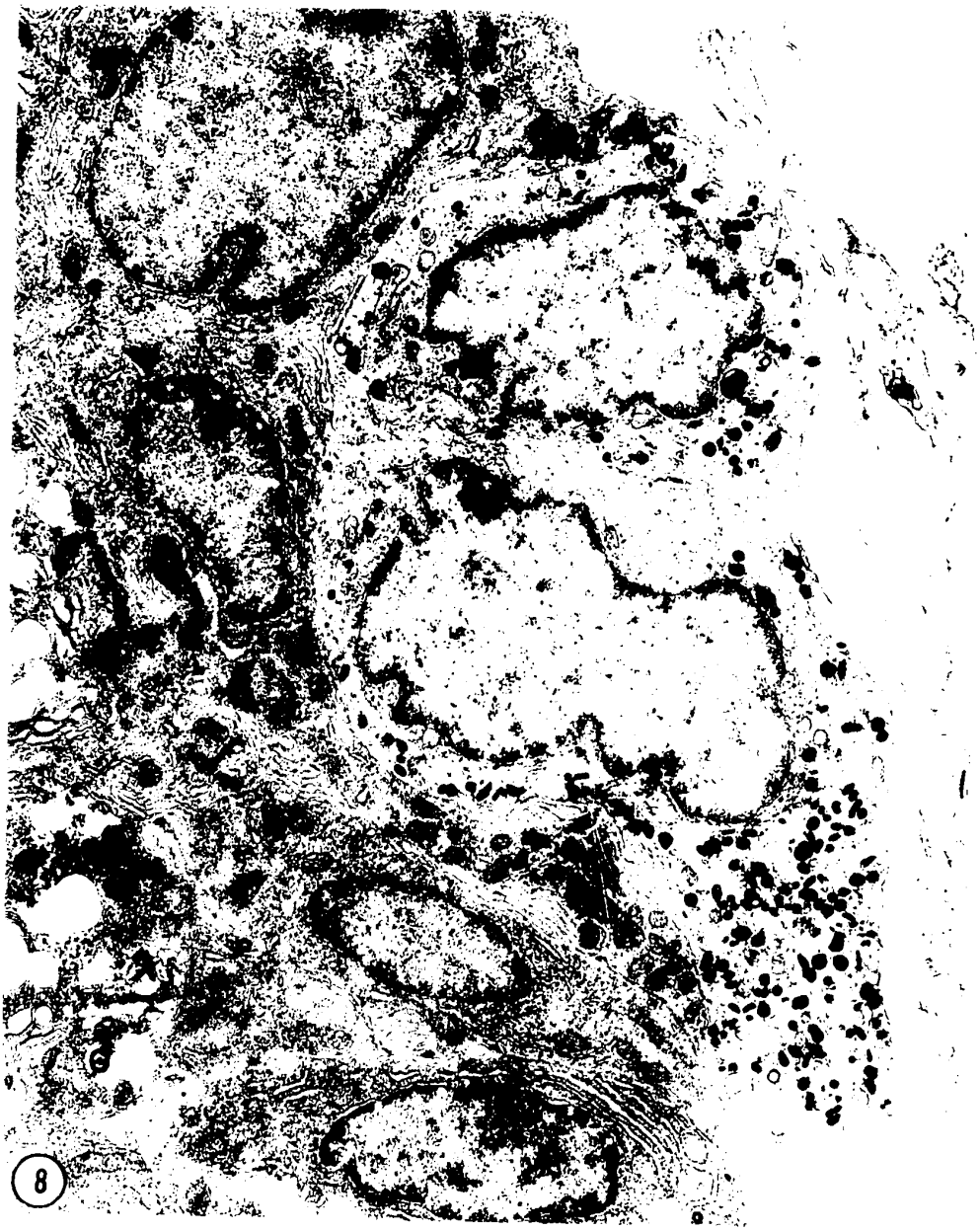
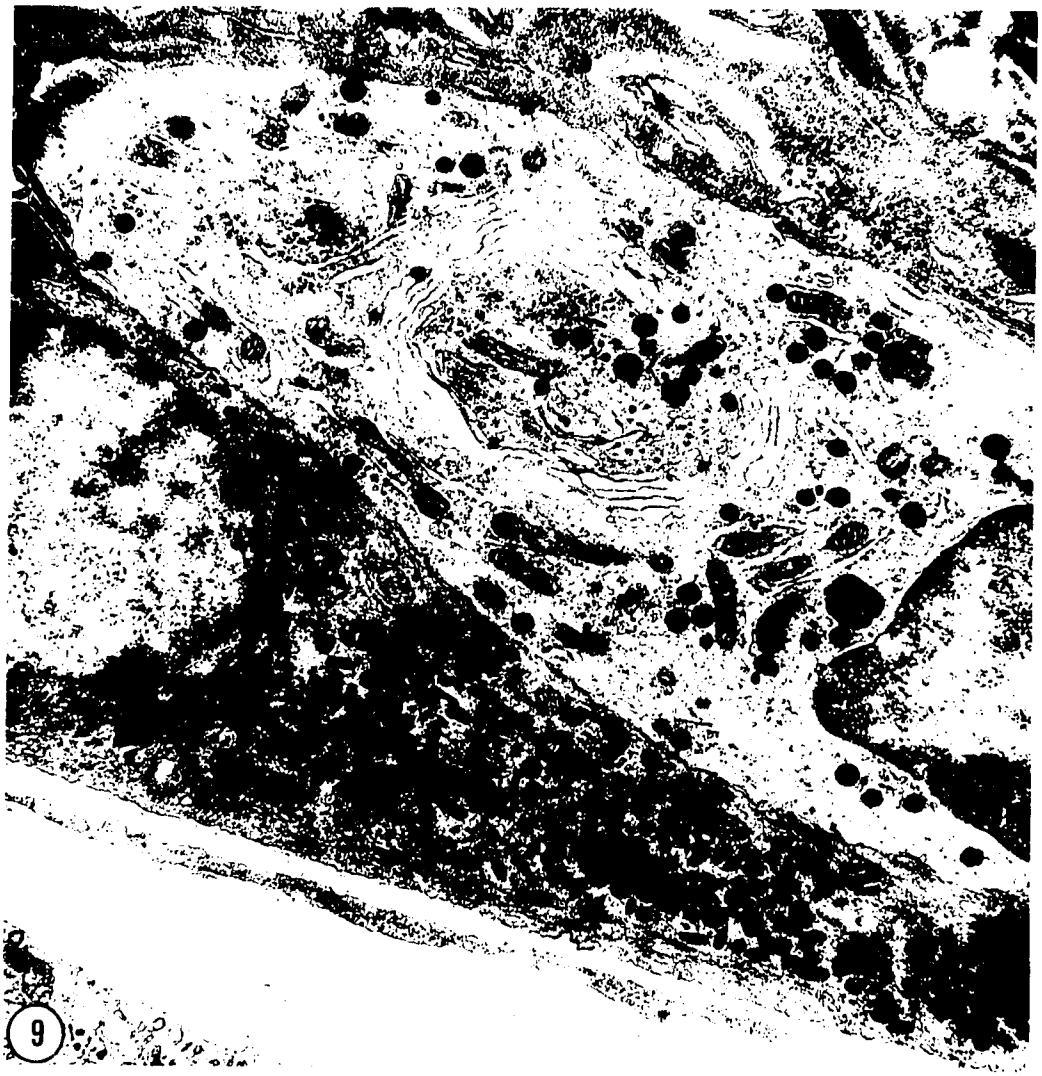

8

Fig. 4-9. Electronmicrograph of two enterochromaffin cells. The lower cell contains two kinds of granules: some are spherical and most of the others are discoid. The cell above it has a lighter cytoplasm, a prominent Golgi apparatus and only a few, mostly spherical granules. x 18,400.



9

CHAPTER V

DIURNAL VARIATION OF THE PROLIFERATIVE ACTIVITY IN THE EPITHELIAL CELLS IN THE DESCENDING COLON OF THE MOUSE

INTRODUCTION

The number of cells undergoing mitosis or DNA synthesis in adult renewing systems varied from time to time during the day (Fortuin-van Leyden, 1926; Carleton, 1934; Blumenfeld, 1939; Cooper and Franklin, 1940; Bullough, 1948; Halberg, 1953; Messier and Leblond, 1960; Pilgrim et al., 1963; Evensen, 1963; Frankfurt, 1967; Brown and Berry, 1968). However, in the small intestinal epithelium of mammals, attempts to demonstrate such a diurnal fluctuation in the rate of cell proliferation have not yielded consistent results. Earlier workers (Fortuin-van Leyden, 1926; Klein and Geisel, 1947; Bullough, 1948; Alov, 1963) found in rodent small intestine either one or two maxima and minima in the number of mitotic figures during the day, but at different times of day. Pilgrim et al. (1963, 1965) counted cells in mitosis and in DNA synthesis in the epithelium of murine small intestine but failed to detect variation at four times of day. However, recent work by Sigdestad et al. (1969) showed a daily fluctuation of the number of cells in mitosis and in DNA synthesis, but found only one maximum and one minimum, with the peak and nadir of the number of mitotic figures per crypt being at 03:00 and 15:00 h respectively.

Such studies of diurnal variation have not been carried out in the colonic epithelium of mammals. In the present investigation, an attempt was made not only to find out if the rate of cell proliferation varied at different times of day and which cell types were involved, but also to relate variations to the size of the various cell populations. This work was done in the descending colon of the mouse.

MATERIALS AND METHODS

This experiment, which has already been described in detail in Chapter I (Experiment III, or Diurnal Variation Experiment), included four groups of five female adult mice each. When they were on the first day of diestrus as judged by daily examination of vaginal smears, each group of animals was given a single subcutaneous injection of 1 μCi of ^{3}H-thymidine (New England Nuclear, specific activity 16.1 Ci/mM) per gram body weight at 03:00, 09:00, 15:00 and 21:00 h respectively; one hour later, a segment of the descending colon within one cm above the pelvic rim was removed under ether anesthesia and fixed in a 4% paraformaldehyde solution in 0.1 M Sörensen's buffer (pH 7.2-7.3). The colonic tissues were embedded in Epon, stained with both the periodic acid-Schiff technique and iron-hematoxylin, and then processed for radioautography. The technical details as well as the method for cell quantitation have been given in Chapter I.

RESULTS

In the descending colon of the mouse, there are three lines of epithelial cells: vacuolated-columnar, mucous, and enterochromaffin cells (Chapter I). The cells in the vacuolated-columnar and mucous cell lines usually produced cells of their own type by mitosis (Chapters I-III), whereas the cells in the enterochromaffin cell line were never seen in mitosis, nor were they ever labeled one hour after injection of ^{3}H-thymidine (Chapter IV). Only the vacuolated-columnar and mucous cell lines will be considered in examining the rates of proliferation.

Diurnal variation in the rate of proliferation

In the vacuolated-columnar cell line (Table 5-1; Fig. 5-1), cells were seen in mitosis at all times of day, with a peak at 10:00 h, but no definite minimum. The labeling index also showed a peak at 10:00 h, but in addition, there was a nadir at 16:00 h.

In the mucous cell line (Table 5-2; Fig. 5-2), mitoses were also present at all times. The peak was at 10:00 h again, but a definite nadir appeared at 22:00 h, with the difference between them being greater than that in the vacuolated-columnar cell line. With regard to labeling, the peak was at 10:00 h and the nadir at 16:00 h.

When individual segments were considered, the labeling index of vacuolated-columnar cells (Table 5-3) varied in general like that for the whole population. The decrease from 10:00 to 16:00 h was smallest in segment 1 and greater as the segments were located higher (Fig. 5-3).

In mucous cells (Table 5-4), the decrease from 10:00 to 16:00 h was prominent in all segments (Fig. 5-3).

Analysis of the epithelial cell populations at various times of day

(1) The crypt sizes:

The crypt size, as expressed by the total number of epithelial cells per cryptal column (Table 5-5), significantly increased from 04:00 to 10:00 h and decreased from 16:00 to 22:00 h.

(2) The population sizes of the surface epithelium:

The number of epithelial cells present between the crypt-surface junction and the site of cell extrusion (and referred to in Table 5-5 as "number of cells per surface epithelium") also varied significantly with a peak at 04:00 h, and a gradual decrease from 04:00 to 16:00 h.

(3) The relative size of the three epithelial cell populations:

The percentages of vacuolated-columnar and mucous cells remained statistically constant throughout the day, but in the enterochromaffin cell line, there were some differences between the higher values at 04:00 and 10:00 h and the lower value at 22:00 h (Table 5-6).

(4) The relative number of vacuolated, transitional and columnar cells in the vacuolated-columnar cell line:

Examination of the percentages of individual sub-cell types in the vacuolated-columnar cell line in the whole population as well as in each segment of the crypt (Table 5-7) revealed that there was a decrease in the relative number of vacuolated cells and concomitant increase in that of columnar cells in the 04:00 h group, as compared with the three

other groups, in which the relative numbers of these sub-cell types were fairly similar to one another.

DISCUSSION

Diurnal variation in the rate of proliferation of the epithelium in the descending colon of the mouse

In the colonic epithelium of the mouse, both the mitotic activity and the frequency of labeling at one hour after ^{3}H-thymidine injection varied with the times of day. This was true of both vacuolated-columnar and mucous cell lines, which both showed a similar diurnal variation of the two parameters.

The peak of mitotic activity was observed at 10:00 h in both cell lines. The nadir was not distinct in the vacuolated-columnar cell line but occurred at 22:00 h in the mucous cell line. There was no information in the literature on diurnal variation in the large intestine, while in the small intestine the reports varied considerably. Of the many observations in the mouse, some indicated no diurnal variation (Pilgrim et al., 1963, 1965), but others showed a distinct one, with either one or two maxima and minima (Klein and Geisel, 1947; Bullough, 1948; Alov, 1963). Perhaps, the most useful observations were those of Sigdestad et al. (1969) working at 3-hour intervals throughout the day. They provided a rather convincing demonstration that there was only one maximum and one minimum in the mitotic activity of the cryptal cells in the mouse small intestine. Under the conditions these authors

used, the peak and nadir of mitotic activity occurred earlier than in the experiment, that is, at 03:00 and 15:00 h respectively.

In the hope of improving the precision of the location of peaks and nadir of mitosis in the actual mitosis curve (solid lines in Fig. 5-4), some consideration may be given to the labeling values. Since in the mouse colon, the mean duration of DNA synthesis is 8 hours and that of the post-synthetic phase and mitosis is 1-2 hours (Thrasher, 1967), it may be assumed that the labeling should precede mitosis by an average of $8/2 = 4$, plus about one hour. By displacing the labeling curve to the right by 5 hours, it would be possible to obtain an approximation to the mitotic curve. While this curve was not drawn in Fig. 5-4, a compromise curve combining its features with those of the actual mitotic counts was drawn as a dotted line. It may be seen that the peak of mitotic activity should fall at the right of that obtained by direct mitotic counts, perhaps around 12:00-13:00 h for both the vacuolated-columnar and mucous cell lines. Using a similar type of reasoning, the nadir is probably around 20:00-22:00 h for the two cell lines.

In the diurnal variation of cell production in the small intestine of the mouse, an importance of feeding was emphasized by Alov (1963), who showed that access to food either in the day or at night could stimulate mitotic activity. In the murine colon, however, feeding does not seem to share such a direct role, because the maximal proliferative activity was observed when the animals were asleep.

On the other hand, the light-dark cycle has been found to play an important role in regulation of mitosis in liver parenchyma and epidermis (Halberg et al., 1958), and the bodily activity on the mitotic rhythm of epidermis (Bullough, 1948). It is quite possible that these factors might also be important in the daily regulation of cell proliferation in the colon, since the mitotic activity was higher when the nocturnal animals were asleep in the daytime. Closely related to these factors is the serum glucocorticoid level, which was found to fluctuate rhythmically during the day in rodents (Halberg et al., 1959), in accordance with a daily rhythm of corticotrophin-releasing factor in the hypothalamus (David-Nelson and Brodish, 1969). If the results were compared with this rhythm of serum glucocorticoid level, a surprisingly good fit was observed in a reverse relationship. At 16:00 h when the serum glucocorticoid level was highest (Halberg et al., 1959), the number of cells in the DNA synthesis phase was minimal, whereas between 04:00 and 10:00 h when the serum glucocorticoid level was lower (Halberg et al., 1959), the labeling of cells was maximal. It is possible, therefore, that glucocorticoid might affect some regulatory mechanism associated with the initiation of DNA synthesis in the late presynthetic phase (Frankfurt, 1968; Kollmorgen and Griffin, 1969) probably by inhibiting thymidine kinase (Kaneko and LePage, 1970). This might result in partial synchronization of cells in the proliferative cycle (Pilgrim et al., 1963, 1965; Brown and Berry, 1968) and cause the diurnal variation in the rate of cell proliferation.

More differentiated cells are more susceptible to diurnal variation

In the vacuolated-columnar cell population, the cells of segment 1 are less differentiated and show less diurnal variation than those in the upper segments (Table 5-3). Furthermore, the mucous cells which were shown to come from vacuolated cells in the lowest segments (Chapter III) and might therefore be considered as being themselves differentiated were subject to pronounced diurnal variation, even in segment 1 (Table 5-4). It may, therefore, be concluded that, among proliferative cells, the more differentiated cells are more susceptible to diurnal variation.

The crypt as a dynamic structure

As shown in Fig. 5-4, there was a close relationship between the mitotic activity of vacuolated-columnar and mucous cells and the number of cells lining the cryptal column (cryptal size) during the day. The slight discrepancy at 10:00 h may be due to the fact that the mitotic activity of the two cell lines probably had a peak around 12:00-13:00 h, instead of 10:00 h, as discussed previously.

Thus, the number of cells lining the cryptal column varied slightly but significantly according to the degree of cell production during the day. This is in line with the fact that the crypt size is maintained by the balance between the rate of cell production in the crypt and that of cell migration out of the crypt; in other words, the crypt is in a dynamic equilibrium under physiological conditions. This dynamic equilibrium may be modified by changes in the rate of cell

production, or in the rate of cell migration, or both. Stevens-Hooper and Blair (1958) showed that the number of epithelial cells lining one side of the cryptal walls and of the villi in the small intestine was significantly reduced in starved rats mainly due to reduced cell production. The crypt sizes were smaller in germ-free animals than in conventional ones, similarly due to the decrease in the rate of cell production in germ-free animals (Abrams et al., 1963; Altmann, 1967). Of interest is the demonstration in this study that the cryptal size varied in accordance with cell production in the crypt even under physiological conditions (Fig. 5-4).

Information on some kinetic parameters of cell populations

Based on the labeling indices and the cryptal sizes at four times of day, it is possible to obtain a rough estimate of the number of cells migrating out of the crypt per cryptal column per unit time, provided that cell migration along the cryptal walls is uni-directional toward the surface and the crypt does not increase its girdle under physiological conditions. As shown in Table 5-8, the number of cells migrating out of crypt per six hours also varied from time to time during the day, and appeared to be a function of both cell production in the crypt and the number of cells lining the cryptal column. The latter suggestion is given support from the study of Abrams et al. (1963), who demonstrated that the migrational velocity of cells along the villi of the small intestine was slower in germ-free animals than in conventional ones,

and at the same time, the crypts were smaller and cell production was less in germ-free animals.

Also based on the number of cells present in the surface epithelium between the crypt-surface junction and the site of cell extrusion (Table 5-5) and the estimated rate of migration of cells from crypt to surface at four times of day (Table 5-8), the rate of cell extrusion may be estimated (Table 5-9). According to this estimation, the rate of cell extrusion varied at different times of day in a manner parallel to crypt size.

On the other hand, in the 04:00 h group in which the number of epithelial cells in the surface epithelium attained maximum, there was a decrease in the relative number of vacuolated cells and concomitant increase in that of columnar cells (Table 5-7). This is in contrast to the remaining three groups in which the relative number of vacuolated cells and columnar cells was essentially similar regardless of their cryptal sizes. This may be taken to indicate that the transformation of vacuolated cells to columnar cells was accelerated between 22:00 and 04:00 h.

The same line of observation was made in the enterochromaffin cell population. In spite of being devoid of dividing capacity, enterochromaffin cells increased in their relative number between 22:00 and 04:00 h (Table 5-6). This was interpreted as vacuolated cells being transformed into enterochromaffin cells during this period (also see Chapter IV).

Information on these parameters of cell population kinetics derived from the data obtained in the present study should be taken as preliminary unless these estimations are confirmed by further experimental evidence. This is particularly true when the three-dimensional structures were analyzed two-dimensionally.

SUMMARY

The rate of proliferation of epithelial cells in the colon was investigated in four groups of adult mice sacrificed one hour after injection of ^{3}H-thymidine respectively at 04:00, 10:00, 16:00 and 22:00 h of day. Semi-thin (1 μ) Epon sections of the colon were radioautographed after staining with periodic acid-Schiff and iron-hematoxylin.

In the descending colon of the mouse, there was a diurnal variation in the rate of proliferation of epithelial cells. This variation was noted in both vacuolated cells and mucous cells. The peak and nadir of mitotic activity in both types of cells appeared to be around 12:00-13:00 h and 20:00-22:00 h respectively, according to the estimation based on the observed mitotic indices and labeling indices at four times of day.

From the analysis of labeling index in each segment of the crypt, it was found that, in the vacuolated-columnar cell line, the poorly differentiated vacuolated cells at the base of the crypt were little affected by the diurnal variation, while more differentiated cells above them were deeply affected. In the mucous cell line (which also originated in the poorly differentiated vacuolated cells in the lower portion of the crypt),

all mucous cells were deeply affected by the diurnal variation. Therefore, in general, among proliferative cells, the more differentiated ones are more susceptible to the diurnal variation.

The number of epithelial cells lining the cryptal column varied according to the degree of cell production in the crypt during the day. A slight daily variation was also noted in the mean number of cells in the surface epithelium. Although there was no statistical difference in the populations of the vacuolated-columnar and mucous cell lines at four times of day, some variation was found in the enterochromaffin cell population during the day.

Table 5-1. Diurnal variation in the rate of proliferation in the vacuolated-columnar cell line. (Five animals per group; the data from the 04:00 h group are repeated at the beginning and end of the Table).

Time of sacrifice	Labeling index	P*** value	Mitotic index	P*** value
04:00	9.5 ± 2.1*		1.1 ± 0.4*	
		P > 0.1		0.01 < P < 0.025
10:00	10.6 ± 2.5		1.9 ± 0.5	
		P < 0.005		0.025 < P < 0.05
16:00	6.0 ± 1.6		1.3 ± 0.4	
		0.05 < P < 0.1		P > 0.1
22:00	8.9 ± 3.3		1.2 ± 0.4	
		P > 0.1		P > 0.1
04:00	9.5 ± 2.1		1.1 ± 0.4	
average	8.8 ± 2.4**		1.4 ± 0.4**	

* Mean ± Standard deviation

** Grand mean ± Weighted standard deviation

*** Determined by the Student's t test

Table 5-2. Diurnal variation in the rate of proliferation in the mucous cell line. (Five animals per group.)

Time of sacrifice	Labeling index	P*** value	Mitotic index	P*** value
04:00	5.1 ± 1.7*		0.6 ± 0.6*	
		0.025< P<0.05		0.01<P< 0.025
10:00	7.3 ± 1.5		2.1 ± 1.1	
		P<0.005		0.05< P<0.1
16:00	3.0 ± 2.0		1.3 ± 0.5	
		0.025<P<0.05		P<0.005
22:00	5.1 ± 1.4		0.3 ± 0.3	
		—		P>0.1
04:00	5.1 ± 1.7		0.6 ± 0.6	
average	5.1 ± 1.7**		1.1 ± 0.6**	

* Mean ± Standard deviation

** Grand mean ± Weighted standard deviation

*** Determined by the Student's t test

Table 5-3. Distribution of the percentages of the labeled cells in the vacuolated-columnar cell line in the segments of the crypt and on the surface at various times of day.

Time of sacrifice:	04:00	10:00	16:00	22:00	average
Surface	-	-	-	-	-
Crypt segment 10	-	-	-	-	-
9	-	-	-	-	-
8	-	-	-	-	-
7	0.4	1.9	0.4	0.6	0.8
6	4.5	7.6	2.4	6.0	5.1
5	15.5	13.9	6.5	11.6	11.9
4	20.6	24.1	13.8	21.9	20.1
3	26.2	26.1	13.1	23.1	22.1
2	24.7	24.7	14.5	18.1	20.5
1	22.5	23.2	18.6	25.1	22.4

Table 5-4. Distribution of the percentages of the labeled cells in the mucous cell line in the segments of the crypt and on the surface at various times of day.

Time of sacrifice:	04:00	10:00	16:00	22:00	average
Surface	-	-	-	-	-
Crypt segment 10	-	-	-	-	-
9	-	-	-	-	-
8	-	-	-	-	-
7	-	-	-	-	-
6	-	2.0	-	1.2	0.8
5	2.0	6.6	1.9	3.8	3.6
4	12.7	9.9	5.4	11.6	9.9
3	10.9	21.4	9.8	10.4	13.1
2	20.0	24.7	7.3	12.5	16.1
1	14.9	21.4	9.9	15.6	15.5

Table 5-5. The sizes of the cryptal columns and the corresponding surface epithelia at various times of day. (Five animals per group.)

Time of sacrifice	No. of cells per cryptal column mean ± S.E.*	P***	No. of cells per surface epithelium mean ± S.E.*	P***
04:00	28.4 ± 0.48		3.8 ± 0.24	
		P < 0.005		0.01 < P < 0.025
10:00	29.6 ± 0.52		3.4 ± 0.24	
		P > 0.1		0.025 < P < 0.05
16:00	29.7 ± 0.49		3.1 ± 0.20	
		P < 0.005		P > 0.1
22:00	27.9 ± 0.43		3.3 ± 0.25	
		0.05 < P < 0.1		0.005 < P < 0.01
04:00	28.4 ± 0.48		3.8 ± 0.24	
average	28.9 ± 0.48**		3.4 ± 0.23**	

* Standard error

** Grand mean ± Weighted standard error

*** Determined by the Student's t test

Table 5-6 Percentages of the various types of epithelial cells in the total epithelial cell population at various times of day. (Five animals per group.)

Time of sacrifice	Vacuolated-Columnar Cells		Mucous Cells		Enterochromaffin Cells	
	Mean ± S.D.*	P***	Mean ± S.D.*	P***	Mean ± S.D.*	P***
04:00	82.3 ± 1.41		16.0 ± 1.54		1.7 ± 0.33	
		P > 0.1		P > 0.1		P = 0
10:00	82.9 ± 1.84		15.4 ± 2.04		1.7 ± 0.43	
		P > 0.1		P > 0.1		0.05 < P < 0.1
16:00	82.3 ± 2.70		16.4 ± 2.46		1.3 ± 0.46	
		P > 0.1		P > 0.1		P > 0.1
22:00	83.8 ± 3.92		15.1 ± 3.98		1.1 ± 0.31	
		P > 0.1		P > 0.1		0.005 < P < 0.01
04:00	82.3 ± 1.41		16.0 ± 1.54		1.7 ± 0.33	
average	82.8 ± 2.47**		15.7 ± 2.51**		1.5 ± 0.38**	

* Standard deviation

** Grand mean ± Weighted standard deviation

*** Determined by the Student's t test

Table 5-7. Percentages of the three sub-cell types in the vacuolated-columnar cell line at various times of day.

Time of sacrifice:	04:00			10:00			16:00			22:00		
Sub-cell types	V	T	C	V	T	C	V	T	C	V	T	C
Surface	-	-	89.9	-	-	90.8	-	-	86.9	-	-	89.8
Crypt segment 10	-	-	85.2	-	-	83.8	-	-	82.8	-	-	86.1
9	-	5.3	74.5	0.5	12.6	69.8	-	11.0	71.5	-	12.2	73.4
8	7.9	49.5	24.4	17.9	50.9	13.5	17.0	54.8	12.8	19.3	55.2	8.9
7	52.7	28.4	0.8	65.9	16.3	0.2	71.9	10.4	-	74.1	8.6	-
6	80.5	1.7	-	79.9	0.5	-	85.5	-	-	83.6	0.2	-
5	81.4	-	-	81.3	-	-	82.3	-	-	80.9	-	-
4	79.4	-	-	79.4	-	-	79.5	-	-	81.6	-	-
3	79.9	-	-	80.7	-	-	79.2	-	-	80.7	-	-
2	78.8	-	-	81.8	-	-	80.1	-	-	83.7	-	-
1	82.4	-	-	85.7	-	-	81.8	-	-	83.1	-	-
In the whole epithelial cell population	49.4	7.7	25.0	52.1	7.3	23.5	52.5	6.9	23.1	53.4	6.9	23.5
	82.1			82.9			82.5			83.8		

V = vacuolated cells. T = transitional cells. C = columnar cells.

Table 5-8. Estimation of the rate of cell migration out of the crypt during the day.

Time of sacrifice	% cell increase as calculated from labeling indices of two cell types (A)	Crypt size (B)	No. of cells increased for the given crypt size (C)	Crypt size 6 hours later (D)	No. of cells migrating out of the crypt over the next 6 hours (E)
04:00	8.6	28.4	2.4	29.6	1.2
10:00	9.9	29.6	2.9	29.7	2.8
16:00	5.4	29.7	1.6	27.9	3.4
22:00	8.2	27.9	2.3	28.4	1.8

Note: (A) Percent cell increase was calculated using the formula:

$$\left(\frac{L_{VC}}{100} \times \frac{N_{VC}}{100} + \frac{L_M}{100} \times \frac{N_M}{100} \right) \times 100$$

where L_{VC} and L_M are the labeling indices of vacuolated-columnar and mucous cells respectively (from Tables 5-1 and 5-2), and N_{VC} and N_M are the percentages of vacuolated-columnar and mucous cells respectively in the total epithelial cell population (from Table 5-6).

(B) From Table 5-5.

(C) $C = \frac{A \times B}{100}$

(D) Obtained from (B).

(E) $E = B + C - D$.

Table 5-9. Estimation of the rate of cell extrusion from the surface during the day.

Time of sacrifice	No. of cells migrating to the surface over the next 6 hours (A)	No. of cells in the surface epithelium (B)	No. of cells in the surface epithelium 6 hours later (C)	No. of cells being extruded over the next 6 hours (D)	Cryp size (E)
04:00	1.2	3.8	3.4	1.6	28.4
10:00	2.8	3.4	3.1	3.1	29.6
16:00	3.4	3.1	3.3	3.2	29.7
22:00	1.8	3.3	3.8	1.3	27.9

Note: (A) Data from Column E in Table 5-8.

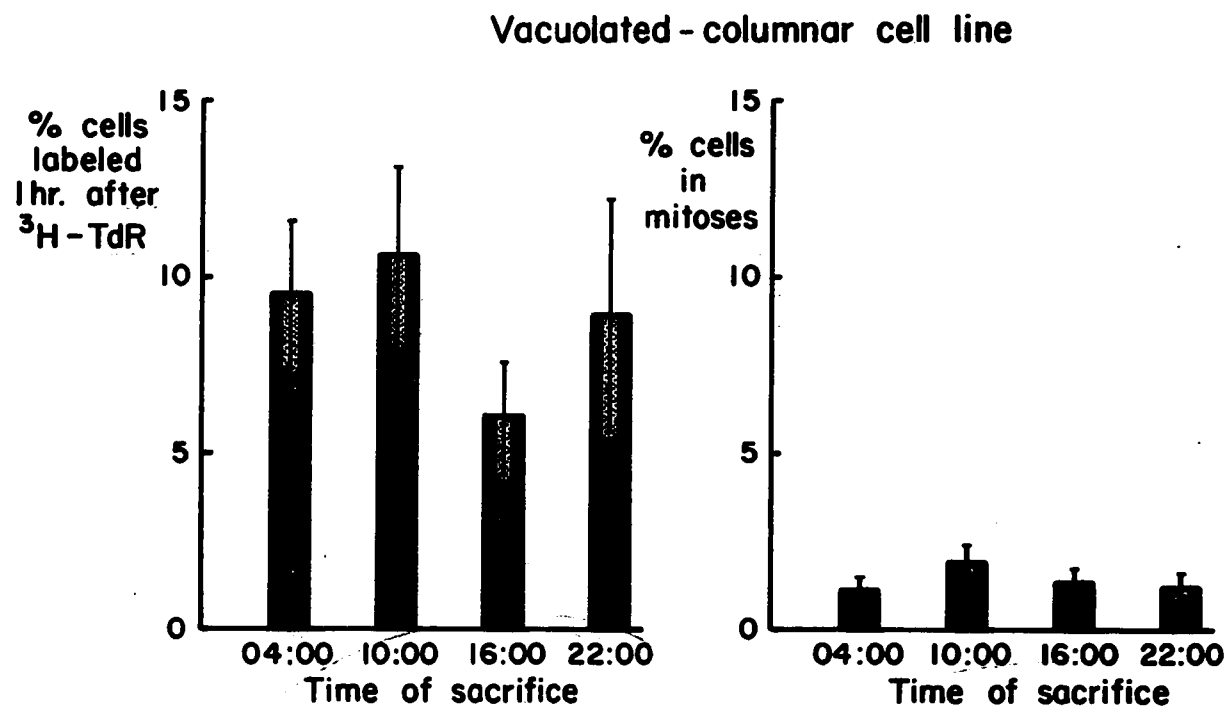
(B) Data from Table 5-5.

(C) Data from (B).

(D) D = A + B - C

(E) Data from Table 5-5.

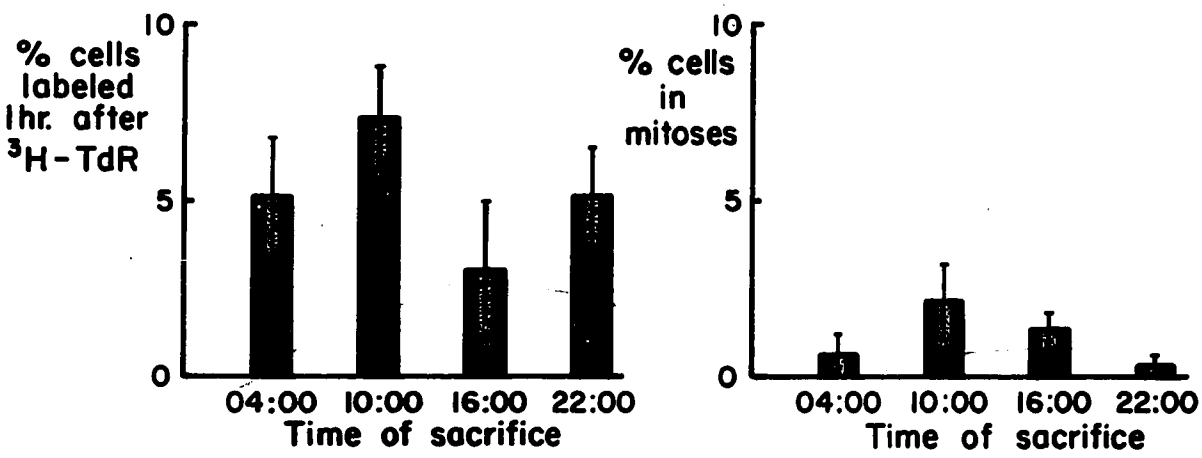
Fig. 5-1. The percentage of cells synthesizing DNA and in mitosis in the vacuolated-columnar cell line at four times of day.


Vacuolated - columnar cell line
% cells labeled 1 hr. after ^{3}H - TdR
15
10
5
0
04:00 10:00 16:00 22:00
Time of sacrifice
% cells in mitoses
15
10
5
0
04:00 10:00 16:00 22:00
Time of sacrifice

(1)

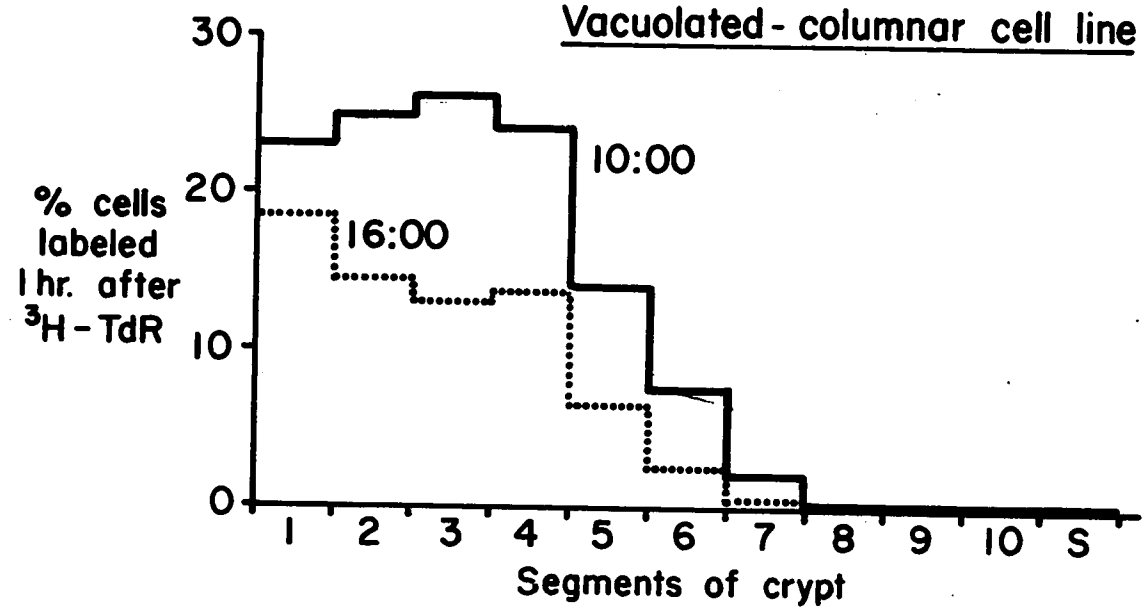
Fig. 5-2. The percentage of cells synthesizing DNA and in mitosis in the mucous cell line at four times of day.

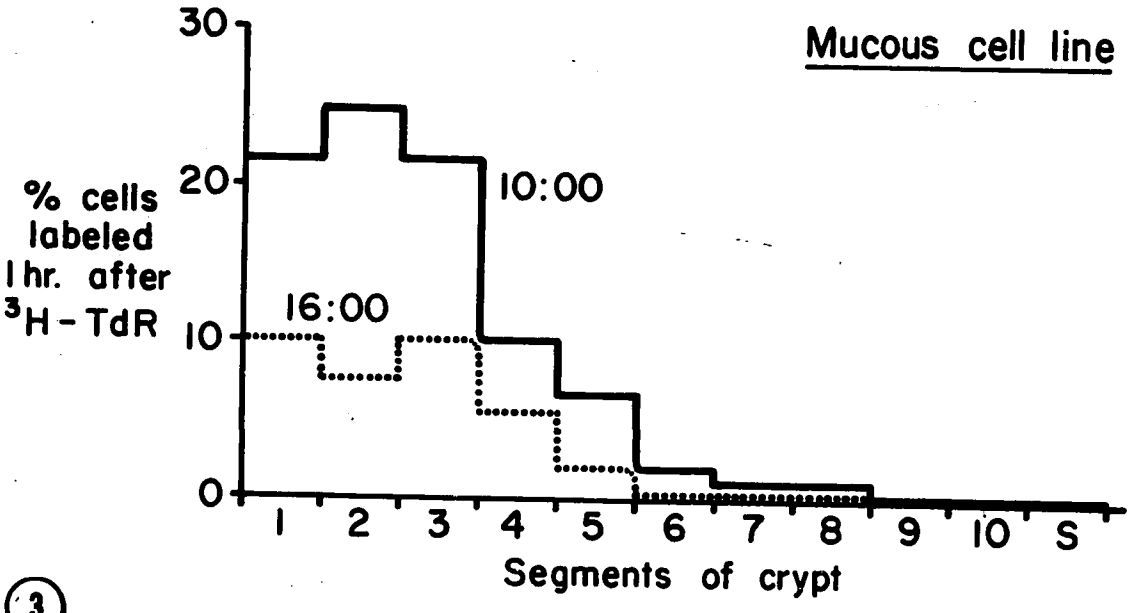
Mucous cell line



(2)

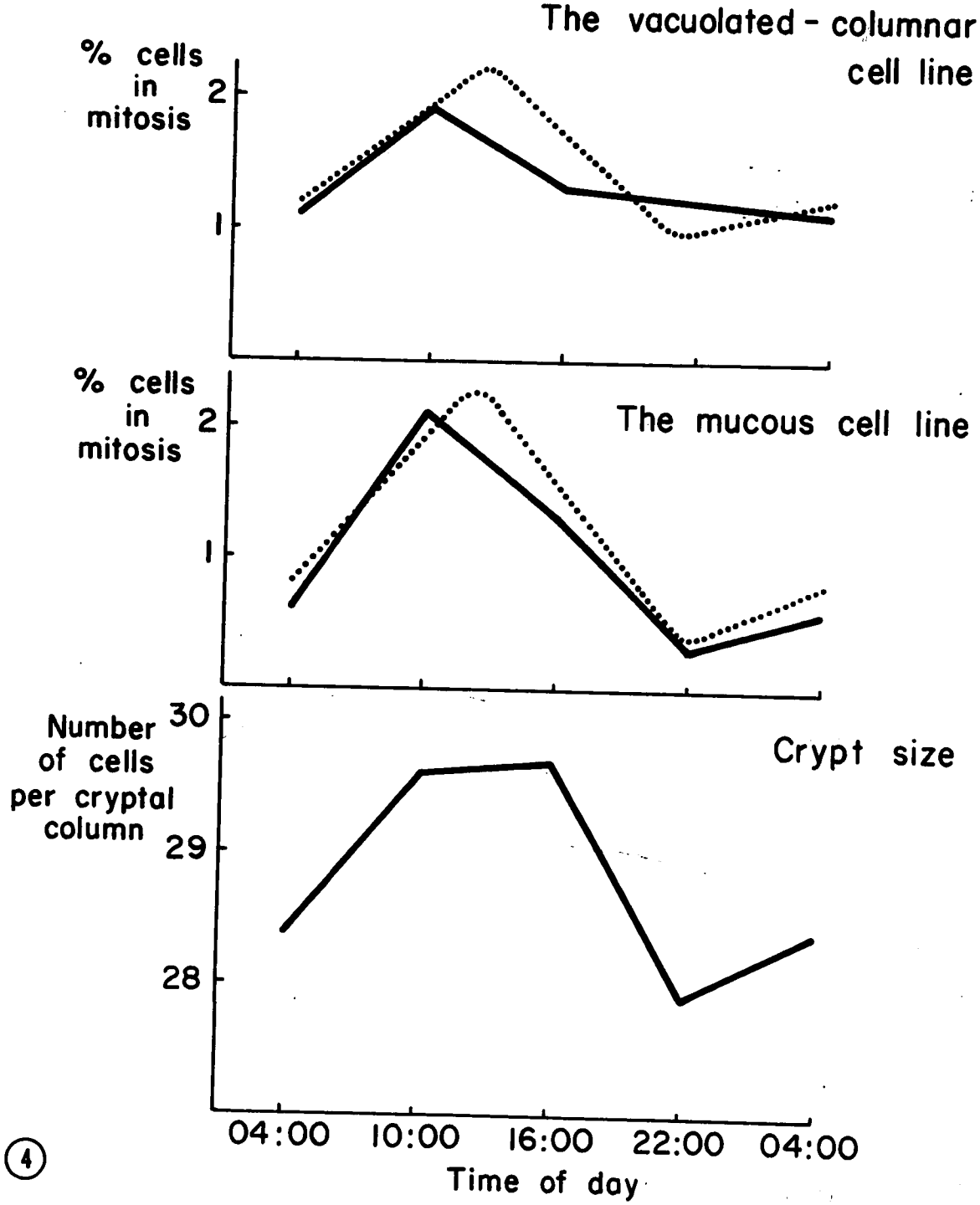
Fig. 5-3. Distribution of percent labeled vacuolated-columnar and mucous cells in the segments of the crypt one hour after ^{3}H-thymidine injection in two groups of animals sacrificed at 10:00 and 16:00 h.


Vacuolated - columnar cell line
30
20
10
0
% cells labeled 1 hr. after ³H - TdR
10:00
16:00
1 2 3 4 5 6 7 8 9 10 S
Segments of crypt


Mucous cell line
30
20
10
0
% cells labeled 1 hr. after ³H - TdR
10:00
16:00
1 2 3 4 5 6 7 8 9 10 S
Segments of crypt

(3)

Fig. 5-4. Mitotic curves of the vacuolated-columnar and mucous cell lines and the cryptal sizes at four times of day.


The vacuolated - columnar cell line
% cells in mitosis
2
1
% cells in mitosis
2
1
The mucous cell line
Number of cells per cryptal column
30
29
28
Crypt size
04:00
10:00
16:00
22:00
04:00
Time of day

(4)

SUMMARY AND CONCLUSIONS

The intestinal epithelium of adult mammals is a renewing cell population. It has been established by previous workers that epithelial cells of the intestine are formed in the lower half of the crypt and migrate upward along the cryptal wall to the surface of the large intestine or villi of the small intestine where the cells are extruded. However, all previous studies either did not distinguish the various types of epithelial cells present in the intestinal epithelium, or else analyzed only the most populous cell type without considering the other cell types. The present investigation was aimed at clarifying the renewal behavior of each of the various types of epithelial cells in the descending colon of the mouse, in the hope of elucidating the relationship, if any, among these cell types.

Concomitant analysis of the different cell lines in the intestinal epithelium was made possible by two technical improvements. First, for routine light microscopic work, serial, semi-thin Epon sections were used, which were stained with periodic acid-Schiff and iron-hematoxylin before radioautographic processing. In the radioautographs thus prepared, every individual cell in the crypt could be identified with great precision as to its cell type and its phase of the mitotic cycle. Second, the cryptal columns in the longitudinally sectioned crypts were divided into ten equal segments, a procedure which turned out to be most useful in investigating the renewal behavior of cells in terms of their relative positions in the crypt.

Four types of epithelial cells are recognized in the descending colon of the mouse: (1) vacuolated cells with numerous periodic acid-Schiff negative vacuoles; (2) ordinary columnar cells with prominent brush border; (3) mucous cells with periodic acid-Schiff positive mucus; and (4) enterochromaffin cells with iron-hematoxylin stained granules in an otherwise pale cytoplasm. It was shown on radioautographs prepared from mice given ^{3}H-thymidine that vacuolated cells migrate up the crypt, and in doing so, transform into columnar cells. Hence, three cell lines exist in the colonic epithelium: (1) the vacuolated-columnar cell line, in which vacuolated cells form the dividing compartment and columnar cells the non-dividing compartment, (2) the mucous cell line, and (3) the enterochromaffin cell line.

In the murine colonic epithelium, the proliferating cells are the vacuolated and the mucous cells, both of which undergo diurnal variation in the rate of proliferation. The peak and nadir of the mitotic activity in both cell types appear to be around 12:00-13:00 and 20:00-22:00 h respectively. Among the proliferating cells, the more differentiated are the cells, the more susceptible they are to diurnal variation.

The vacuolated-columnar cell line includes 80-85% of the total epithelial cell population in the descending colon of the mouse. Vacuolated cells are seen in segments 1-8 of the crypt, and columnar cells in segments 8-10 and also in the surface epithelium. In this cell line, production of cells as vacuolated cells takes place in segments 1-7 of the crypt, with the maximal proliferation in segments 1-4, and in decreasing numbers

toward segment 7. From the rate of proliferation, cell fluxes were calculated, from which the mean number of mitotic cycles in each dividing segment was estimated. A vacuolated cell at the cryptal base and its progeny go through three mitotic cycles on the average before becoming non-dividing columnar cells. The first mitotic cycle occurs in segments 1 and 2 ("stem cell level"), the second one takes place in segments 2 and 3, and the third one may spread over segments 3-7. In the third and last cycle, dividing cells change over to non-dividing cells in accordance with the slow cut-off model. From the curves of increment of labeled cells with time, the transit time of the cells in the non-dividing segments was measured, which was 9.6 hours per segment. From this value, the turnover rate of the total vacuolated-columnar cell population was calculated to be 0.95% cells per hour. Hence, the overall turnover time of this population would be 105 hours. Based on the assumption that cells ascend the cryptal wall as a result of population pressure created by the formation of new cells below, the velocity and transit time of these cells in the dividing segments of the crypt were calculated. The transit time of the vacuolated-columnar cells in segment 1 was 35.7 hours, in segment 2 it was 16.8 hours; and, because the cells gained velocity as they ascended the cryptal wall, reaching a constant speed in the non-dividing segments, the transit time decreased to 9.6 hours per segment in the non-dividing segments. Finally, from these data, the mean cell cycle time of vacuolated-columnar cells was estimated to be 29.7 hours.

Approximately 15% of the total epithelial cell population consists of mucous cells, being present throughout the crypt and also on the surface. The theca is generally small in mucous cells located in segments 1-4, becomes gradually swollen as they are situated higher in the cryptal wall, attains maximal size in segments 8-9, and becomes narrow on the surface. Cryptal mucous cells with small theca multiply by mitosis,which is observed mainly in segments 1-3, in decreasing numbers toward segment 6, but not in segment 7 or above. Comparison was made for both the vacuolated-columnar and mucous cell lines of turnover rates calculated from the transit times in the non-dividing segments and from the proliferative rates of these populations. The results indicated that on the basis of a steady state, there was an over-production of cells in the vacuolated-columnar cell line and an under-production in the mucous cell line. From these and other data, it is concluded that mucous cells arise from vacuolated cells in segments 1-4 as well as from mitoses in segments 1-6. Based on the calculated rate of transformation of cells from the vacuolated-columnar to the mucous cell line and the rate of proliferation of mucous cells in the various segments, the mean number of mitotic cycles of mucous cells was estimated. The data indicate that mucous cells may undergo one or two mitotic cycles depending on where the transformation occurs before changing over to non-dividing mucous cells. The velocity and transit time of mucous cells in both dividing and non-dividing segments were analyzed as in the case of the vacuolated-columnar cell line. The transit time of mucous cells

in segment 1 was 51.6 hours, which was apparently overestimated because of the lack of an adequate method to estimate the influx of mucous cells from vacuolated cells in this segment. The transit time of segment 2 was 17.6 hours, which decreased steadily through the dividing segments to 11.5 hours, the transit time of mucous cells in the non-dividing segments. From the transit time of 11.5 hours per segment in the non-dividing segments, the turnover rate of the total mucous cell population was calculated to be 0.79% cells per hour. Accordingly, the turnover time of the total mucous cell population would be 127 hours. Finally, from these data, the mean cell cycle time was estimated to be 22.2 hours.

The enterochromaffin cell line comprises 1-2% of the total epithelial cell population. Although these cells may be seen in the crypt as well as on the surface, they are concentrated in segments 1-2. Enterochromaffin cells constitute a non-proliferating cell population, which appears to renew by differentiation of precursor cells found among poorly differentiated vacuolated cells at the base of the crypt. Evidence has been presented that enterochromaffin cells migrate upward along the cryptal wall as other epithelial cells do, but at a much slower rate.

Hence, the three cell lines in the descending colon of the mouse are not independent of each other. Both mucous cells and enterochromaffin cells arise from vacuolated cells in the vacuolated-columnar cell line, the predominant cell line in the colonic epithelium; but mucous cells would undergo one or two mitotic cycles after transformation from vacuolated cells.

ORIGINAL CONTRIBUTIONS

In the previous studies on the renewal of intestinal epithelium of mammals, no distinction was made among various types of epithelial cells present in the intestine. In a few investigations, a population of a single cell type was analyzed ignoring the populations of the other cell types present. Therefore, the present work was carried out to find out the renewal behavior of each of the different types of epithelial cells present in the descending colon of the mouse and also to clarify the relationship among these various types. This new approach was made possible by certain technical improvements, and the results thus obtained are essentially new and original, as summarized in the previous section.

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