THE EFFECT OF ESTROGEN ON CELL PROLIFERATION AND DIFFERENTIATION IN COLONIC EPITHELIUM OF THE MOUSE, *MUS MUSCULUS* L.

by

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A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1978

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

August 17, 1978
date

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date

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Abstract

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Martin Benjamin Hoff

Advisor: Dr. William W. L. Chang

The effects of estrogen on the processes of cell proliferation and differentiation were investigated biochemically and autoradiographically in the descending colon of the mouse, a non-target organ of estrogen.

In ovariectomized mice given various doses and regimens of 17β-estradiol, the uterine tubes became engorged and enlarged. The increase in their wet and dry weights was greater after multiple injections than after only one. In the descending colon, estrogen treatments caused no significant change in the RNA, protein and DNA contents, but decreased incorporatin of ^{3}H-thymidine into DNA of colonic mucosa at 4 hours after the final injection. The single high dose (10 ng per gram body weight) regimen induced and maintained the maximal inhibitory effect for the longest period (24 hours). The degree and duration of inhibition of ^{3}H-thymidine incorporation became less after repeated injections of estrogen, suggestive of a tachyphylactic effect. The inhibitory effect of 17β-estradiol was also observed in colonic epithelium of male mice as well as in epithelium induced to proliferate by refeeding fasted mice. Neither the colonic mucosa of mice treated with continuous infusion of estrogen for up to 4 days nor the colonic ex-

plants of a rabbit cultured for 18 hours in estrogenized medium seemed to be affected by the treatment.

For the cell population kinetic studies, autoradiographs were prepared from one micron thick Epon-embedded cross sections of colon taken from ovariectomized mice given ^{3}H-thymidine one hour before sacrifice. Sections were prestained with periodic acid-Schiff reaction and iron hematoxylin. In accord with the biochemical data, the autoradiographic studies revealed that at 4 hours after either a single estrogen injection or the last estrogen injection of the multiple injection regimen, the number of ^{3}H-thymidine-labeled cells per colonic crypt column was decreased, but the number of silver grains per labeled cell did not appear to change as compared to ovariectomized controls. The analysis of these data based on what is known in the literature indicates that the decrease in ^{3}H-thymidine incorporation into the colonic epithelium by estrogen was less likely due to the direct inhibition of DNA synthesis, and most likely due to the alteration of the cell cycle of proliferating cells by promoting passage through the S phase while creating either a G_1- S block or a G_1 prolongation. This type of modulatory action of estrogen on the non-target organ did not involve a net change in the synthesis of RNA, protein or DNA, in contrast to the target organ (e.g. uterus) where a growth response is preceded by the induction of these synthetic processes. It should be noted that after repeated estrogen injections, a modulatory action by estrogen is manifested also in the target organ.

Autoradiographic studies of colonic crypts taken from groups of mice killed in the various stages of the estrous cycle demonstrated a cyclic change in the proliferative activity of vacuolated and mucous

cells and also in the number of vacuolated, columnar and mucous cells per crypt column. The changes observed in the populations of columnar cells (differentiated population) and mucous cells (both immature and differentiated cells) coincided with changes in the progesterone blood level, suggesting that differentiation of colonic epithelial cells was promoted by progesterone. A cyclic change in the cell population kinetics of colonic epithelium during the estrous cycle could be explained by the presumptive actions of estrogen and progesterone as previously described.

Following ovariectomy, a new steady state was created in the colonic crypt, which was characterized by a significant decrease in the crypt size with a relative increase in the vacuolated (proliferative) cell population and a relative increase in the proliferative activity of cells, further evidence that the ovarian hormones are required to maintain the colonic crypt populations in their normal proportions.

THIS THESIS WAS WRITTEN

IN MEMORY OF

MY

FATHER

THIS DISSERTATION IS DEDICATED TO

MY MOTHER

MY SISTER, Shelly

MY NEPHEWS, Neil and Lee

and, of course,

Randi

ACKNOWLEDGEMENTS

I wish to take this opportunity to express my gratitude to the people who lent a hand in some unforgettable way so that this dissertation would be a little bit special.

To my mentor Dr. William Chang, many thanks for your support, guidance and understanding when it really counted the most.

To my good friend Dr. Ki Mak, whose help was deeply appreciated and will not soon be forgotten, thanks.

A special thanks to Der-Yi 'Dolly' Cheng whose friendship and technical instruction made my life in the lab that much more pleasant.

Many thanks to Barbara Park, who was very much more than just a secretary to me and whose skill and ingenuity is very much a part of this thesis.

A much appreciated thanks to Tana Ross, a gem of a person and a great all-around technical person.

To Dr. Harry Smith and Geoffrey Hollander, whose statistical advice and technical assisstance was much appreciated, thanks.

To Jeffrey Smith, a 'blackbelt in photography', who volunteered his time and energy to help out above and beyond the call of duty, thanks.

To Phillip and Sharon Stern, whose technical assistance was deeply appreciated.

To my typists, a big thanks for a job which everyone will always appreciate, especially me.

To my friends in the Department of Anatomy, you know who you are,

a heartfelt thanks for your support, advice and friendship.

And lastly, to my remaining friends at Mount Sinai, who made my stay seem worthwhile, thanks.

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Section 1

HISTORICAL REVIEW

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INTRODUCTION

I. THE CELL CYCLE: PROLIFERATION OR DIFFERENTIATION?

It was the early work of Howard and Pelc (1953) which led to the partitioning of the cell cycle interphase into an S, or DNA synthesis phase, and two 'gap' periods, G_1 and G_2 (fig. 1-1). The G_1 period being the time when RNA's and proteins are being synthesized in preparation for S phase, while G_2 is the preparative time for the M or mitotic phase. Under normal conditions most mammalian cells have relatively constant cell cycle periods with only minor variation (Cameron, 1971). Where significant variations in total cell cycle time exist, the pre-synthetic or G_1 phase is largely accountable (Baserga, 1965; Epifanova, 1971). Differences in cell cycle time may be observed between cells of different tissues or between cells of the same tissue when studied under different environmental conditions.

In 1963 Patt and Quastler discovered that the relatively dormant hepatocyte population could be stimulated into entering the cell cycle. Patt and Quastler modified the Howard and Pelc cell cycle model by including a G_0 or stage of quiescence. The G_0 period is closely associated to G_1 in that cells which are in G_0 when induced to, enter G_1. The existence of G_0 was supported by Lajtha (1964). On the basis of his studies of the liver he, and his colleagues, proposed the existence of two cell subpopulations in a given tissue: 1. a proliferating and differentiating population and 2. a dormant or reserve population. In addition to the G_0 of Patt and Quastler (1963) another G_0 related to G_2 has also been suggested (Epifanova

and Tershikh, 1969; Gelfant, 1963; Fabrikant, 1969; Perry and Schwartz, 1967).

Thus far I have related the cell cycle to the reproduction of cells. Maintaining tissue volume, however, is only one function of a cell population. It is obvious that in order for a tissue or an organ to be functional the cells must become specialized, and so, differentiation becomes an important aspect of the cell's life cycle. In general, a proliferating cell population is, functionally speaking, not specialized, and inversely the mature specialized cells are not mitotically active. Quastler and Sherman (1959) suggested that during a certain period of the cell cycle, which they called the 'critical phase', the choice between proliferation and differentiation is made. The 'critical phase' was thought to occur early in interphase, shortly following mitosis. In support of the 'critical phase', Bullough (1963) introduced his phase of decision, 'dichophase'. Bullough defines this period as the time during which synthetic processes occur which will eventually lead either to division or differentiation. His dichophase occurs between the M and G_1 periods, as did Quastler and Sherman's critical phase. Both, the process of differentiation as well as cell replication have long been appreciated in the gastrointestinal tract.

II. THE DYNAMICS OF THE GASTROINTESTINAL TRACT

As shown in figure 1-2, each segment of the alimentary canal has a discrete zone of actively proliferating cells whose progeny repopulate the epithelium. Although the esophagus and stomach will be omitted from the bulk of the present discussion, suffice it to say, that, the daughter cells of the dividing cell populations of all segments of the alimentary tract differentiate in order to maintain the organs' functionality (Dawson, 1948; Stevens and Leblond, 1953; Leblond and Walker, 1956; Hunt and Hunt, 1962; Bertalanffy, 1962; Lipkin et al., 1963a,b; MacDonald et al., 1964; Baker, 1964; Marques-Pereira and Leblond, 1965; Forssmann et al., 1969; Edwards, 1971).

The structural unit of the small intestinal mucosa consists of the crypt of Lieberkühn and the villus, as seen in figure 1-2c (Macklin and Macklin, 1932; Patzelt, 1936; Toner, 1958). The oneness of the crypt and villus epithelium was not realized until 1888 when the independent efforts of Paneth and Heidenhain came to that conclusion. Unlike the small intestine, the colonic mucosa is flat, for in the adult, it lacks villi. As early as 1882 Patzelt described the production of new cells in the crypt base. He also described the presence of mucous droplets in the apical cytoplasm of some of the columnar cells. Bizzozero, too, noted the numerous mitotic figures in the base of the crypt (1888, 1889, 1892, 1893).

Inherent in the fact that the intestinal mucosa has a rapidly dividing and differentiating epithelium is that the epithelium undergoes a continuous renewing process. The concept of renewal was put forth by Leblond and Stevens (1948). Also, based on their observation and realization, that the size of the epithelium remains fairly constant, Leblond and Stevens (1948) developed the concept of the steady-state system. In other words, the number of cells produced by the proliferative zone, over a given time, equals the number of cells extruded into the lumen, thus, there is no net gain. The steady-state concept is built upon the fact that the daughters of dividing cells migrate up the crypt and are extruded as 'healthy' cells.

The evidence for migratory movement up the crypt came three years before the renewal concept from Friedman (1945). He employed the swelling effect that X-radiation has on the intestinal epithelium to 'label' crypt cells. Later with the advent of autoradiography (Belanger and Leblond, 1946) migration of the crypt cells was confirmed (Leblond et al., 1948; Belanger, 1956; Pelc and Howard, 1956; Leblond et al., 1957; Walker and Leblond, 1958; Chang and Leblond, 1971a; Chang and Nadler, 1975; and others). Besides describing crypt cell migration in the small bowel, Walker and Leblond (1958) also showed crypt cell migration in the colon, which was confirmed by Chang and Leblond (1971a) and Chang and Nadler (1975).

The idea that morphologically 'healthy' cells are continually extruded from the intestinal surface epithelium was borne out by investigators who examined the gut's lumenal contents and found absorptive columnar cells and/or goblet cells (Ramond, 1904; Wright et al., 1940; Stevens Hooper and Blair, 1958; Pink et al., 1970). Another study observed cells being desquamated from villi tips in dog intestine (Creamer et al., 1961), while yet other investigations further substantiated this phenomenon (McMinn, 1954; Bertalanffy and Nagy, 1961; O'Connor, 1966; Imondi and Bird, 1966). It has been estimated that 1.59×10^9 cells are sloughed off of the small intestinal surface epithelium daily in the rat (Enesco and Altmann, 1963).

The dynamics of the renewing cell populations comes into its own with the availability of ^{3}H-thymidine. It has been demonstrated that ^{3}H-thymidine is very specifically incorporporated into newly synthesized deoxyribonucleic acid (Taylor et al., 1957; Amano et al.; 1959). In addition, its stability is excellent for use with many histological techniques (Cleaver, 1967) as well as lending itself well to high resolution autoradiography. Thus, after the introduction of ^{3}H-thymidine, the renewal process of the intestinal epithelium was elegantly substantiated by many investigators (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; MacDonald

et al., 1964; Shorter et al., 1964; Sawicki et al., 1968; Chang and Leblond, 1971a; Chang and Nadler, 1975; and others).

In 1959, Quastler and Sherman employed the concepts of renewal and steady-state status of the intestinal epithelium (Leblond and Stevens, 1948) to further analyze the kinetics of the system. Based on the fact that the small intestinal crypt contains the 'progenitor' cell population whose progeny differentiates to become the mature functional villus epithelium, the epithelial continuum was thus compartmentalized into the proliferating and non-proliferating cell populations. Further analysis was carried out by Quastler (1960, 1963) based upon the compartmental analysis. Chang and Nadler (1975) employed the methods of compartmental analysis in their study of the mouse descending colon, as well.

Assuming that the migration of epithelial cells in the intestinal crypt is a result of the population pressure created by the production of cells at the base, Cairnie et al. (1965a) and Sawicki et al. (1968) estimated that the rate of cell migration in the crypts of the small intestine of the rat and the ascending colon of the guinea pig respectively. Both studies found that the rate of crypt cell migration accelerated in going from the base toward the mouth of the crypt. Using the same assumption as the above studies, but a different approach, Chang and Nadler (1975) came to the same conclusion in the descending colonic crypts of the mouse.

The relationship between cell division and migration has also been investigated in the intestinal crypt. Based on the distribution of ^{3}H-thymidine-labeled cells in the small intestinal crypts of the rat, Cairnie et al. (1965b) proposed the so-called 'slow cut-off' model of cell production along the cryptal wall. According to this proposal, two daughter cells capable of proliferation are produced after a mitotic division in the lower part of the crypt, whereas two daughter cells incapable of replication are formed after a division occurred in the middle to upper part of the crypt. The cell cycle leading to the latter type of mitosis takes place over the wide range of cell positions extending from the middle to upper part of the crypt. By estimating a specific number of mitoses for epithelial cells in their migration along the cryptal wall in the descending colon of the mouse, Chang and Nadler (1975) supported the 'slow cut-off' model of Cairnie et al. (1965b). Recall that the 'critical phase' of Quastler and Sherman (1959) or the 'dichophase' of Bullough (1963) is the time when the cell makes the transition from a reproductive state to a non-reproductive differentiated state. According to the 'slow cut-off' model of Cairnie et al. (1965b), such a transition occurs in the intestinal crypt upon the completion of the terminal division.

III. CELL DIFFERENTIATION IN THE INTESTINAL EPITHELIUM

As the progenitor cells divide and migrate up the crypt, they differentiate into four mature cell types in the small intestine: columnar, or absorptive, mucous, or goblet, enteroendocrine or argentaffin or enterochromaffin, and Paneth (Macklin and Macklin, 1932; Patzelt, 1936; Trier and Rubin, 1965; Trier, 1968; Toner, 1968; Cheng and Leblond, 1974a, 1974b, 1974c; Cheng, 1974a, 1974b). The Paneth cell is not found in the large intestinal epithelium (Chang and Leblond, 1971).

The mucous, or periodic acid-Schiff positive, cells (Chang and Leblond, 1971a) arise from the primitive columnar cell type (Chang and Leblond, 1971a; Cheng, 1974a). The undifferentiated columnar cell in the base of the crypt accumulates mucous droplets in its apical cytoplasm as it becomes increasingly specialized on its migration up the crypt. The intermediate, or maturing, mucous cells have been observed in mitosis (Bizzozero, 1888, 1889, 1892, 1893; Chang and Leblond, 1971a). DNA synthesis in the maturing goblet cell has also been definitively demonstrated by autoradiography (Leblond and Messier, 1958; Thrasher and Greulich, 1966; Chang and Leblond, 1971a). Cairnie (1970), however, failed to observe a mucous cell undergoing division. Mucous cells have a characteristic goblet shape with swollen thecas jutting out toward the lumen of the crypt and a tapered body almost coming to a point at its base which sits on the basal lamina (fig. 1-3). Mature goblet cells are found at the top of the crypt

and in the surface epithelium.

The enteroendocrine cells have traditionally been considered to be incapable of replication (Patzelt, 1936). Experiments using ^{3}H-thymidine seemed to confirm that this was indeed the case (Leblond and Messier, 1958; Ferreira and Leblond, 1971; Chang and Leblond, 1971b). Deschner and Lipkin (1966), however, claimed to have observed enteroendocrine cells in mitosis at a very infrequent rate of about 1 out of each 2,663 enteroendocrine cells which they counted in human rectal epithelium. Employing autoradiography, they determined the labeling index of the enteroendocrine cell population to be 1.2%. Using electron microscopic autoradiography, Cheng and Leblond (1974b) found that enteroendocrine cells which contained a very small number of electron dense granules were labeled occasionally with ^{3}H-thymidine and Chang (1970) found a similar cell in mitosis with electron microscopy. Recently, Leblond and Tsubouchi (1978) described the existence of two subpopulations of enteroendocrine cells in the mouse colon: (1) typical enteroendocrine cells and (2) caveolated cells. They appeared to renew at different rates. In the stomach and small intestine, several subtypes of enteroendocrine cells were also observed (Forssman et al., 1969; Ferriera, 1970; Moxey and Trier, 1978), but whether or not these subtypes of enteroendocrine cells renew at different rates has not been established.

Paneth cells are unique to the small bowel epithelium and are

mainly seen in the base of the crypts (Cheng, 1974b). Patzelt (1936) claimed to have observed Paneth cells in mitosis. It has also been claimed that Paneth cells incorporated ^{3}H-thymidine shortly after a pulse injection (Thrasher and Greulich, 1966; Deschner, 1967). To the contrary, however, other studies have failed to observe Paneth cells in mitosis (Hampton, 1968; Leblond et al., 1968; Troughton and Trier, 1969; Cheng et al., 1969; Cairnie, 1970), although some of the very same investigations did in fact note ^{3}H-thymidine-labeled Paneth cells 1-3 days after label administration (Troughton and Trier, 1969; Cheng et al., 1969; Cairnie, 1970). The evidence thus suggests that the precursor of the Paneth cell may indeed be the undifferentiated columnar cell (Hampton, 1968; Cheng et al., 1969), though Cairnie (1970) suggested that the progenitor cell type of the Paneth cell lies outside the epithelium. It may also be concluded that the Paneth cell population is renewed at a very slow rate.

The immature undifferentiated cells at the base of the crypt is generally considered to be the precursor of the other cell types found in the intestine. In the colon of man, mouse, rat and rabbit these cells contain conspicuous vacuoles in their apical cytoplasm (Hollman, 1965; Chang and Leblond, 1971a) (fig. 1-4). The vacuoles are most prominent in these columnar cells located in the intermediate levels of the proliferative zone. This cell type makes up the major cell population of the entire replicating cell population of the colonic crypt (Chang and Leblond, 1971a). The vacuoles are thought to contain acidic carbohydrate material

(Wetzel et al., 1966) and there is evidence that suggests that these vacuoles are secreted into the lumen of the crypt (Chang and Leblond, 1971a). The vacuoles are rather inconspicuous in the epithelial cells basally located in the crypt and become more obvious as these cells migrate upward. At high levels of the proliferative compartment the vacuoles become depleted so that the once unspecialized primitive vacuolated columnar cell differentiates into the functionally and morphologically mature absorptive columnar cell found high in the crypt and on the lumenal surface (fig. 1-3). The mature columnar cell is no longer capable of replication.

IV. THE RELATIONSHIP BETWEEN REPLICATION AND DIFFERENTIATION IN THE INTESTINAL EPITHELIUM

Cell division and cell differentiation are intimately related processes which continually occur in the intestinal epithelium. In the small intestine of mammals, epithelial cells are produced only in the crypt, and differentiate during their migration up the cryptal wall and onto the villus to be extruded only when they reach the villus tip (Leblond and Stevens, 1948; Leblond, Stevens and Bogoroch, 1948). Thus, the small intestinal epithelium can be topographically divided into two compartments: 1. the proliferative compartment in the crypt and 2. the functional compartment on the villus (Quastler and Sherman, 1959). The kinetic aspects of the transition between these two compartments have been analyzed by Quastler and Sherman (1959) and by Cairnie et al. (1965a,b), while the cytological changes associated with epithelial cell renewal and the inter-relationship of the various types of epithelial cells, with respect to proliferation and differentiation, have been investigated by Cheng (1974a,b) and Cheng and Leblond (1974a,b).

The present investigation employed the descending colon as a model to study how estrogen affects this inter-relationship between replication and differentiation. At this point it would be worthwhile to point out some of the special features of the murine colon. In the descending colon of the mouse the progenitor or 'stem' cells found at the base of the crypt are pluripotential (Chang and Leblond, 1971a). In the course of their migration along the cryptal

wall they differentiate into the mature cell types (columnar, mucous and enteroendocrine) (see fig. 1-3). Once a cell is fully mature it no longer divides. Also, as seen in figure 1-3, although four types of cells can be identified in the colonic epithelium, there are only three cell lines: 1. vacuolated-columnar, 2. mucous and 3. entero-endocrine (Chang and Leblond, 1971a). The present study was only concerned with the former two cell lines. As it was eluded to above, the vaculated-columnar and mucous cell lines have a common ancestor or 'stem' cell, namely the primitive vacuolated cell found in the base of the crypt. However, the two cell lines diverge as the daughters of the 'stem' cell begin to differentiate into either the definitive vacuolated cells or the recognizable mucous cells.

As the young mucous cell migrates up the wall of the crypt, the apical portion begins to swell with the formation of increasing amounts of mucous material. As previously mentioned, only the immature mucous cells containing relatively little mucous material can divide. Although this last comment seems to indicate that proliferating and mature mucous cells are distinguishable from each other, it should be emphasized that only a relative difference exists between them. Thus, on a morpholgical basis, mature goblet cells cannot be accurately distinguished from those mucous cells which are capable of replication.

In contrast to the mucous cell population, the vacuolated-columnar cell line can be conveniently compartmentalized into a

proliferative (vacuolated cells) and a differentiated (columnar cells) cell population using both topographical and morphological criteria. Such compartmentalization facilitates cell population kinetic studies in that the vacuolated cell population vis à vis proliferative activity and the columnar cell population vis à vis transformation from vacuolated cells can be analyzed. On the other hand, analysis of the mucous cell population must take into consideration a two-fold effect on cell production: 1. the rate of mucous cell proliferation and 2. the rate of transformation from vacuolated cells into mucous cells (Chang and Nadler, 1975). It can therefore be appreciated that the descending colon of the mouse lends itself well to various studies concerned with the relationship between replication and differentiation.

Cairnie et al. (1965b) have estimated that under normal conditions, in the small intestinal crypt of the rat, at least two mitotic divisions are required for epithelial cell maturation. Chang and Nadler (1975), on the other hand, using the model of the mouse colonic crypt, calculated that an average of three cell divisions take place before the daughters of progenitor cell in the crypt base differentiates into the mature non-dividing cells found in the upper part of the crypt. That is to say, during a cell's migration up the crypt, it undergoes a process of stepwise differentiation which is intimately related to the mitotic cycles. Generally, there seems to be an inverse relationship between a cell's ability to replicate and differentiate. There is evidence,

however, which indicates that the two processes are not absolutely exclusive of each other. Case in point is the fact that cells recognizable as intestinal mucous cells (Bizzozero, 1888, 1889, 1892, 1893; Chang and Leblond, 1971a) as well as definitive vacuolated cells in the upper portion of the proliferative compartment of the colonic crypt (Chang and Leblond, 1971a) have been seen dividing. It appears, however, that there is a critical division beyond which an intestinal crypt cell is unable to divide. This mitosis corresponds to the terminal division of Cairnie et al. (1965a) and Chang and Nadler (1975).

V. EFFECTORS OF CELL DIFFERENTIATION

As stated before, the epithelium of the small intestine can be roughly divided into the proliferative (crypt) and the functional (villus) compartment by their topography. In the descending colon of the mouse, these two compartments can be separated more accurately by their topography and morphology of constituent epithelial cells. Hence, the status of the differentiation of epithelial cells is easily appreciated in the intestines. In spite of this fact, effectors of differentiation have not been studied in the intestines to advantage, although there is evidence that differentiation of epithelial cells is affected in certain pathological clinical conditions such as sprue.

It has been well known that many types of cells will continue proliferating in an appropriate in vitro condition, but differentiation of cells does not easily occur in vitro except under certain specific conditions. In this regard, in vitro studies of the rat mammary gland and chick oviduct are worthy of mention. The former study demonstrated that mammary gland could be induced to synthesize casein in the presence of insulin, hydrocortisone and prolactin, but not if anyone of these hormones was absent (Juergens et al., 1965; Turkington et al., 1965; Stockdale and Topper, 1966; Vonderhaar et al., 1973a). Morphologically, mammary gland acinar development was also induced when all of these three hormones were present, but not if any one of them was absent (Vonderhaar et al., 1973b). In the chick oviduct, estrogen has been shown to induce the pseudostratified epithelium to transform into ciliated columnar epithelium (O'Malley et al., 1969; Palmiter, 1972; Means and O'Malley, 1974), whereas progesterone induced differentiation, causing

oviduct goblet cells to secrete specialized products (Kohler et al., 1969; Oka and Schimke, 1969). In addition, a differentiating effect of these ovarian hormones on the human fallopian tube epithelium has been known for many years (Copenhaver et al., 1978).

Other studies concerning inducers of differentiation dealt with embryonic induction (Niv and Twitty, 1953; Saxen and Toivonen, 1962; Kallman and Grobstein, 1964; Grobstein, 1967; Lehtonen et al., 1975). In addition, other substances have been suspected to induce differentiation in specific organ systems: erythropoietin, thrombopoietin, thymopoietin, thymosin and others (Rutter, 1978). What becomes clear from these studies is that cell differentiation is more dependent on the environment than cell proliferation, and that certain specific environmental factors are required for the differentiation of certain specific cell types. It is possible that these environmental factors act on the critical phase or dichophase of cell cycle as the study of Vonderhaar et al., (1973b) suggests.

VI. EFFECTORS OF CELL PROLIFERATION: GENERAL

It has been well recognized that tissue growth is modulated by a 'hierarchy' of growth regulators (Teir, 1952). That is to say, effectors of cell proliferation are not all equipotent for a given tissue. According to their site of production, effectors may be classified as being local or systemic (distal). Local effectors of cell division are produced by a given tissue and control the mitotic activity of the tissue. Whether physical (e.g., population pressure in the tissue) or chemical diffusible factors (e.g., chalone, see below), local effectors of cell division appear to be tissue- or organ- specific and to have the greatest control over the growth of the tissue or organ. On the other hand, systemic effectors are usually blood-borne compounds and affect the mitotic activity of an organ or organs remote from their site of production. Neural stimulation may be considered as a distal effector, as well.

Several different local effectors of cell proliferation have been documented in the literature. Caspari (1972) introduced the concept of necrohormones in order to explain tissue growth. According to this concept necrotic cells release a substance which induces proliferative activity when present in low amounts, but inhibits mitoses when present in large amounts. In their investigation of lacrimal gland and liver repair, Teir et al. (1967) seemed to concur with the necrohormone theory. Another variation on this theme came from Tumanishvili (1967). He proposed that mitotic control is determined by the nuclear:cytoplasm ratio. He observed that when the nuclear concentration decreased, mitotic

activity increased, and vice versa. Tumanishvili postulated, then, that nuclei synthesize an inhibitor of cell division, whereas a cytoplasmic substance promotes proliferation.

Today it is generally believed that local regulating factors are repressive in nature. According to Bullough (1962), "Opportunity, not stimulus, is all that is needed for cell division..." In other words, when the repressor is removed, proliferation procedes. It has been observed that epidermal extracts can inhibit epidermal mitoses (Bullough and Johnson, 1951; Bullough and Laurence, 1960, 1961). Bullough and Laurence (1960, 1961) also showed that a substance synthesized in the epidermis specifically inhibited epidermal mitoses, while a hypodermic substance specifically inhibited hypodermal cell proliferation. This inhibitor, or chalone, may explain the healing phenomena which was once attributed to a 'wound hormone' (Abercrombie, 1957; Swann, 1958; Johnson and McMinn, 1960). There has been what may seem to be contradictory evidence to the chalone theory, however. Extracts of lacrimal gland and liver were observed to stimulate cell proliferation (Teir, 1951a, 1951b; Teir and Ravanti, 1953), although, these studies did not seem to indicate that the effect was organospecific. The evidence that a chalone exists for many, if not all, tissues and organs is convincing. At present a chalone has been shown to exist for kidney and liver (Saetren, 1963), epidermis (Bullough and Laurence, 1964a, 1964b) as well as other systems (see Houck, 1976).

Systemic effectors of mitotic activity include hormones and growth factors. Several growth factors have been identified and well charac-

terized. Some of these factors are epidermal growth factor (Carpenter and Cohen, 1978), fibroblast growth factor (Gospodarowicz et al., 1978) and nerve growth factor (Berger and Shooter, 1978; Thoenen et al., 1978). They are peptide in nature and each appear to have its specific 'target' tissue.

Hormones are usually always present in the blood, but the secretion of hormones is regulated through feed-back mechanisms which thus maintain the homeostatic control of the body (Zawadowsky, 1941; Drischel, 1956). Hormones usually have their own 'target' tissues and organs upon which they exert a pronounced effect (Villee, 1962; Segal, 1966), although the specificity of and the degree of response in the target tissues may vary. Investigations into the mode of action of hormones have taken predominantly three paths: (1) hormonal influence on enzyme activity (Talalay and Williams-Ashman, 1960; Villee et al., 1960; Villee, 1962; Talalay, 1962), (2) hormonal effects on the genome (Monod et al., 1963; Ui and Mueller, 1963; Talwar et al., 1964; Notides and Gorski, 1966; Jensen and DeSombre, 1973; Chan and O'Malley, 1976; Villee, 1974; Hamilton, 1968; Epifanova, 1971; and others) and (3) hormonal effects on the cell membrane (Hechter, 1957, 1961; Hechter and Lester, 1960; Engel, 1961; Willmer, 1961; Gruenstein and Wynn, 1970; Bar and Hechter, 1969). There are two classes of hormones: 1. polypeptide and 2. steroid. Polypeptide hormone activity is mediated via a membrane receptor complex, whose second messanger cAMP is formed which in turn produces the hormonal effects by stimulating protein and RNA synthesis (Sutherland, et al., 1965). The steroid hormones, most notably estrogen, also stimulates RNA and protein synthesis (Gorski et al., 1965;

Hamilton, 1968; O'Malley et al., 1969; Villee, 1974; Chan and O'Malley, 1976), however, sex steroids bind to a cytosol receptor and the complex translocates into the nucleus (Toft and Gorski, 1966; Gorski et al., 1968; Jensen and DeSombre, 1973; Chan and O'Malley, 1976; and others). There is evidence, though, that specific estrogen receptor sites are also located on 'target' cell membranes but are absent from the 'non-target' cell membranes (Pietras and Szego, 1977).

In speaking of a tissue or an organ as being the 'target' of a hormone, the implication is that the hormone specifically affects that tissue or organ uniquely. However, a hormone may produce responses in many tissues and organs. Indeed, hormonal effects need not be confined to a single organ or a group of organs that have a common function. As Epifanova (1971) pointed out, hormones are not required for cell division but they can alter the cell's life cycle and in that way effect the cell's reproduction in both the target and non-target tissues and organs. Therefore, it appears that the modulatory action of a hormone on target and non-target tissues and organs is only quantitatively different.

There is also evidence which indicates the existence of an inter-relationship between local and systemic effectors of cell proliferation. An example of the interplay between local and systemic effectors of cell proliferation was demonstrated by Bullough and Laurence (1964): the inhibitory effect of the epidermal chalone (local effector) on the mitotic activity of epidermis was enhanced by adrenalin (systemic effector). Moreover, Bullough and Laurence (1964b) speculate that diurnal patterns of proliferative activity may be attributed to the diurnal cycles of

adrenalin blood levels: as adrenalin levels rise, the inhibition of cell division would likewise increase, and vice versa.

VII. EFFECTORS OF CELL PROLIFERATION: THE INTESTINE

Because of a very high proliferative activity and the continuous renewal of the epithelium along with a convenient distinction between the proliferative and the functional compartment, the intestine is one of the organs in which the role of local and systemic effectors of cell proliferation has been extensively investigated.

Before going further, it may be appropriate to mention several known facts concerning the cell cycle of the intestinal epithelium. It has been shown that the duration of the cell cycle periods shows a regional variation. In their pioneer work on the labeled mitoses curve method of analysis, Quastler and Sherman (1959) found that in a given segment of the intestine, the duration of various cell cycle periods remained fairly constant with the G_1 phase demonstrating the most variation. However, even within the same segment of the intestine (mouse colon), Lipkin and Quastler (1962), Thrasher (1967), and Chang and Nadler (1975) found different values for both the duration of cell cycle and the duration of the various cycle phases. Lipkin and his colleagues (Lipkin et al., 1963a, b; Lipkin, 1965a, b) have also indicated that this variability exists in human intestinal epithelium. Proliferative activity also varies from segment to segment in the alimentary canal (Lipkin and Deschner, 1968). Furthermore, Cairnie et al., (1965a) demonstrated variability in the cell cycle time along the wall of the crypt with the G_1 phase reflecting the most variation; a shorter cell cycle being found in cells located higher in the crypt. Their findings were supported by Rowinski and Sawicki (1972) but are somewhat different from those of Thrasher and Greulich (1965), Lipkin and Bell (1968) and Chang and Nadler

(1975).

Many natural and artifical environmental factors have been shown to modify the proliferative activity and/or the cell cycle parameters of epithelial cells in the intestines. Many of these factors are presented and classified in table 1-1.

Table 1-1

NON-HORMONAL FACTORS WHICH FFECT PROLIFERATIVE ACTIVITY IN THE INTESTINAL EPITHELIUM

A. FACTORS WHICH STIMULATE PROLIFERATE ACTIVITY

BACTERIAL FLORA	Abrams et al. (1963); Lesher et al., 1964; Cook and Bird (1973)
BOWEL RESECTION	Weser and Hernandez (1971); Hansen and Osborne (1971); McDermott and Roudnew (1976); Hanson et al. (1977); Obertop et al. (1977); Oscarson et al. (1977); Nundy et al. (1977).
CHOLINERGIC STIMULATION	Tutton (1975b)
NEURAL STIMULATION	Tutton (1975a)
REFEEDING FASTED ANIMALS	McMannus and Isselbacher (1970); Altmann (1972); Aldewachi et al. (1975); Hagemann and Stragand (1977).
VITAMIN D	Birge and Alpers (1973)

B. FACTORS WHICH INHIBIT PROLIFERATIVE ACTIVITY

AGING	Lesher et al. (1961b); Thrasher and Greulich (1965); Thrasher (1967a, b); Cameron (1972); Tutton (1973a).
ALCOHOL INGESTION	Baraona et al. (1974)
CYCLIC AMP	Alpers and Philpott (1975)

Table 1-1 (continued)

B. FACTORS WHICH INHIBIT PROLIFERATIVE ACTIVITY (continued)

FASTING (starvation)	Stevens Hooper and Blair (1958); Brown et al. (1963); McMannus and Isselbacher (1970); Altmann (1972); Heird et al. (1974); Lohrs et al. (1974); Aldewachi et al. (1975); Alpers and Philpott (1975); Lichtenberger et al. (1976a); Mak and Chang (1976); Hagemann and Stragand (1977); Oscarson et al. (1977)
HYDROXYUREA	Al-Dewachi et al. (1977)
INTESTINAL EXTRACT (chalone?)	Tutton (1973b); Sassier and Bergeron (1977)
IRRADIATION	Trier and Browning (1966); Hageman and Lesher (1971); Rijke et al. (1975)
LACK OF LUMENAL CONTENT	Gleeson et al. (1972); Heird et al. (1974); Levine et al. (1974); Johnson et al. (1975a); Keren et al. (1975); Eastwood (1976); Rijke et al (1977); Janne et al. (1977)
RENAL FAILURE (uremia)	Castrup et al. (1970); McDermott et al. (1974a, 1974b)
SYMPATHECTOMY (chemical and surgical)	Tutton and Helme (1974)
THEOPHYLLINE	ibid.

C. OTHER FACTORS WHICH FFECT THE PROLIFERATIVE ACTIVITY

	ACTION DEMONSTRATED	
CALCIUM	stimulates by stimulating gastrin secretion (see table 1-2 for action of gastrin)	Berreras et al. (1967); Reeder et al. (1970); Christiansen et al. (1974, 1975)
DIURNAL RHYTHM	cyclic variation	Fortuin-van Leyden (1926); Bullough (1948); Alov (1963); Sigdestad et al. (1969); Sigdestad and Lesher (1970, 1972);

Table 1-1 (continued)

C. OTHER FACTORS WHICH AFFECT PROLIFERATIVE ACTIVITY (continued)

	ACTION DEMONSTRATED	
		Chang (1971); Al-Dewachi et al. (1976)
HORMONES	(see table 1-2)	
STRESS	none inhibit stimulate	Rasanen (1963) Tutton and Helme (1973) Tutton (1978)
SYMPATHECTOMY (chemical and surgical)	abolishes diurnal rhythm	Tutton and Helme (1974)

From table 1-1, one can readily appreciate the broad spectrum of factors which affect intestinal epithelial cell proliferation. Indeed, intestinal cell proliferation is quite sensitive to changes in its environment.

In recent years, hormonal effects on the intestine have been the subject of a great deal of the gastroenterologic literature. There are two groups of hormones: those produced by endocrine organs, and those synthesized by enteroendocrine cells in the gastrointestinal tract. The intestine-specific hormones have been adequately reviewed by several investigators (Rayford et al., 1976; Johnson, 1976; Grossman, 1977; Enochs and Johnson, 1977). Both groups of hormones modulate the proliferative activity of epithelial cells in the intestine (see table 1-2). However, the mode of modulation of these hormones on the intestine appears to be complex and may involve the interaction with other hormones. Of historical interest is the finding of Dorchester and Haist (1952) that secretin levels in the intestine were influenced by the pituitary. Leblond and Carriere (1955) showed that cell division in the gut was depressed with hypophy-

sectomy and/or thyroidectomy. They further demonstrated that growth hormone increased proliferative activity in the intestinal epithelium of animals that were either hypophysectomized or thyroidectomized, but thyroxine would increase cell proliferation only if the pituitary was intact. Recently growth hormone has been shown to promote gastrin secretion (Enochs and Johnson, 1975, 1976), and gastrin is known to stimulate intestinal cell proliferation (Williams et al., 1972; Pansu, 1974; Johnson and Guthrie, 1974; Johnson et al., 1975; Mak and Chang, 1976). Hence, the regulatory mechanism of hormones on the intestinal cell proliferation indeed, involves the interactions of several hormones of several organs. An example of such complex interplay involves gastrin (as illustrated in figure 8 of Enochs and Johnson (1977)).

Table 1-2

HORMONES SHOWN TO AFFECT PROLIFERATIVE ACTIVITY OF THE INTESTINAL EPITHELIUM (with mode of action)

A. HORMONES PRODUCED BY THE GASTROINTESTINAL TRACT

1. STIMULATORY BY DIRECT (?) ACTION

GASTRIN (pentagastrin)	Williams et al. (1972); Pansu (1974); Johnson and Guthrie (1974); Johnson et al. (1975); Mak and Chang (1976)
SEROTONIN	Tutton (1974)

2. STIMULATORY BY INDIRECT ACTION

BOMBESIN (increases gastrin secretion)	Bertaccini et al. (1974); Fender et al. (1975); Espamer and Melchiorri (1975)
CHOLECYSTOKININ (increases serum gastrin)	Lanciault et al. (1976)

Table 1-2 (continued)

HORMONES SHOWN TO AFFECT PROLIFERATIVE ACTIVITY OF THE INTESTINAL EPITHELIUM (with mode of action) (continued)

3. INHIBITORY BY INDIRECT ACTION

GASTRIC INHIBITORY POLYPEPTIDE (inhibits food-stimulated gastrin secretion)	Rayford et al. (1974); Villar et atl (1975); Walsh and Grossman (1975a, 1975b)
GLUCAGON (inhibits gastrin secretion)	Walsh and Grossman (1975a, 1975b)
SECRETIN (inhibits action of gastrin & pentagastrin)	Pansu et al. (1974); Johnson and Guthrie (1974)
SOMATOSTATIN (inhibits growth hormone & thyrotropin secretion)	Hall et al. (1973)
VASOACTIVE INTESTINAL POLYPEPTIDE (inhibits food-stimulated gastrin secretion)	Villar et al. (1975); Walsh and Grossman (1975a, 1975b)

B. HORMONES PRODUCED BY OTHER ENDOCRINE ORGANS*

1. STIMULATORY (MODE OF ACTION)

ADRENOCORTICOTROPIC HORMONE** (?) (evidence suggests indirect action; see effect of adrenalectomy below)	Tutton (1973c); Rasanen and Teir (1961)
GROWTH HORMONE (prevents hypophysectomy-induced atrophy) (Promotes gastrin secretion)	Enochs and Johnson (1975, 1976)
NORADRENALIN (- adrenergic stimulation)	Tutton and Helme (1974)
TESTOSTERONE (reduces cell cycle time)	Tuohimaa and Niemi (1968); Wright et al. (1972)

Table 1-2 (continued)

HORMONES SHOWN TO AFFECT PROLIFERATIVE ACTIVITY OF THE INTESTINAL EPITHELIUM (mode of action) (continued)

THYROXIN (dependent on pituitary)	Leblond and Carriere (1955)

2. INHIBITORY

ADRENALIN (β-adrenergic stimulation)	Tutton and Helme (1974)
CORTICOSTEROID (?)	Lahtiharju et al. (1964); Lazuchev and Avetisyan (1972)
GLUCOCORTICOID (?)	Rasanen (1963); Lahtiharju (1966)

C. DRUGS AND TREATMENTS WHICH EFFECT THE ENDOCRINE SYSTEM

1. STIMULATORY

ADRENALECTOMY (see c.2 below)	Rasanen and Teir (1961)
PROPRANOLOL AND PRACTOLOL (β-adrenergic blockade)	Tutton and Helme (1974)

2. INHIBITORY

ADRENALECTOMY (see c.1 above)	Tutton (1973c)
CONOVID BIRTH CONTROL PILL; NORETHYNODREL AND MESTRANOL (?) jejunal mucosal atrophy)	Watson and Murray (1966)
HYPOPHYSECTOMY (probably due to lack of growth hormone)	Leblond and Carriere (1955)
ISOPRENALINE (β-adrenergic stimulation	ibid.

Table 1-2 (continued)

HORMONES SHOWN TO EFFECT PROLIFERATIVE ACTIVITY OF THE INTESTINAL EPITHELIUM (with mode of action) (continued)

PHENTOLAMINE (α-adrenergic blockade)	Tutton and Helme (1974)

*Estrogen is omitted from table 1-2 (for estrogenic effects on intestinal cell proliferation see table 1-3).

**Rasanen (1963) found no effect.

VIII. ESTROGEN AS AN EFFECTOR OF CELL PROLIFERATION

Estrogen is a systemic effector of cell proliferation. In its target organs, this hormone induces a growth-related response which involves the synthesis of RNA, protein and DNA (Gorski et al., 1965; Hamilton, 1968; O'Malley et al., 1969; Villee, 1974; Chan and O'Malley, 1976). This response is initiated by the interaction of estrogen with its cytosol receptor (Taft and Gorski, 1966; Gorski et al., 1968; Jensen et al., 1968; Jensen and DeSombre, 1973; Chan and O'Malley, 1976) and is eventually manifested by increase in DNA synthesis, mitoses and growth.

In some of the non-target organs, the intestine, for one, the estrogen cytosol receptor has not been identified. However, such a receptor has been demonstrated in some non-target organs such as pancreas (Sandberg and Rosenthal, 1974), eosinophils (Tchernitchin and Tchernitchin, 1976), heart (Stumpf and Sar, 1977), fibroblasts (Jung-Testas et al., 1976) and brain* (McEwen, 1976). However, the significance of the presence of estrogen cytosol receptors in most of these non-target organs is not known.

On the other hand, Pietras and Szego (1977) have demonstrated specific estrogen binding sites on cell membrane of target organs, which epithelial cells of the intestine lacks. However, neither the signifi-

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*Today some investigators consider brain as a target organ of estrogen. In this regard, it may be mentioned that the physiological action of estrogen metabolites is recently under intense study. Catechol estrogens have been of great interest since they are found in much higher concentrations than the mother compound in the brain (Paul and Axelrod, 1977).

cance of the membrane receptors of estrogen in relation to its specific action in the target cells nor the relationship between the membrane and cytosol receptors of estrogen is clear.

In both the studies of W.S. Bullough (1946) and H.F. Bullough (1946), estrogen has been shown to increase the mitotic index even in non-target organs (including various segments of the alimentary canal) (see table 1-3). Epifanova concurred with this view in her studies of corneal epithelium (Epifanova, 1966). Galand et al. (1967) have demonstrated that the duration of the S phase, and consequently the cell cycle time, became shortened in epithelial cells in four segments of intestine following estrogenization of ovariectomized mice, but they did not report either the ^{3}H-thymidine labeling or mitotic indices after the estrogen treatment.

Contrary to these findings, several investigators (Crafts, 1941; Vollmer and Gordon, 1941; Mirand et al., 1959; Dukes and Goldwasser, 1961; Mirand and Gordon, 1966; Gordon et al., 1968) have found that erythropoiesis in bone marrow was inhibited by estrogen, resulting in anemia and death of some animals. It is also known that general body growth is retarded by estrogen treatment (Paesi and Dejongh, 1954).

In short, estrogen has definitely been shown to stimulate cell proliferation in the target organs, but it is far from being clear that estrogen has the same effect in the non-target organs.

Table 1-3

"NON-TARGET" TISSUES AFFECTED BY ESTROGEN

TISSUE	EFFECT	REFERENCE
general body	retards growth	Paesi and Dejongh, 1954
skin	increases mitotic index	Bullough, H.F., 1946; Talbot et al., 1952
sebaceous gland	increases mitotic index	Bullough, W.S., 1946; Talbot et al., 1952
corneal epithelium	increases mitotic index	Epifanova, 1966
vasculature -intestinal	occludes(?)	Kilpatrick et al., 1968; Ward and Stevenson, 1968
-nasal mucosa	dilates	Talbot et al., 1952
-mesenteric arterioles	increases constrictor response	Altura, 1975
bone marrow	atrophies; inhibits erythropoiesis	Crafts, 1941; Vollmer and Gordon, 1941; Mirand et al., 1959; Dukes and Goldwasser, 1961; Mirand and Gordon, 1966; Gordon et al., 1968
	decreases iron content	Dutta and Mukherjee, 1963
thyroid	inhibits release of thyroid hormones (T_3 and T_4)	Evered, 1976
parathyroid	increases mitotic index	Bullough, W.S., 1946
pituitary	promotes release of growth hormone	Ojeda, 1977, Root, 1972
adrenal cortex	increases mitotic index	Bullough, W.S., 1946
urinary tract epithelium	same	ibid.
pancreatic acini	same	ibid.

Table 1-3 (continued)

TISSUE	EFFECT	REFERENCE
liver	alters ratio of polyploid hepatocytes (?)	Swartz, 1962; Swartz and Sams, 1961; Hoffman and Swartz, 1962; Carriere, 1969
gastrointestinal tract		
-esophageal epithelium	increases mitotic index	Bullough, W.S., 1946
-stomach mucosa	same	ibid.
	decrease acid output; promote ulcer healing	Crean, 1963
-duodenal epithelium	increases mitotic index	Bullough, W.S., 1946
	reduces cell cycle parameters	Galand et al., 1967
-jejunal epithelium	same	ibid.
-ileal epithelium	same	ibid.
	mucosal atrophy	Watson and Murray, 1966
-colonic epithelium	reduces cell cycle parameters	Galand et al., 1967
	increases mitotic index	Bullough, W.S., 1946
-rectal epithelium	increases mitotic index	Bullough, W.S., 1946

IX. PURPOSE OF THE PRESENT INVESTIGATION

The descending colon of the mouse, as described previously, is an excellent model to study factors which regulate the processes of cell proliferation and differentiation. Since there are some uncertainties about the mode of action of estrogen on the non-target organs, I have decided to explore the effect of estrogen on the processes of cell proliferation and differentiation in the mouse colon, with the hope that differential responses of the target and non-target organs to the hormone may be defined.

More specifically, I have addressed myself to the following questions:

1. Does the estrous cycle modulate proliferation, differentiation and maturation of the epithelial cells in the mouse colon? If so, how? Which cell type is primarily affected? Does the size of the crypt and its major cell populations vary during the estrous cycle?

2. Does ovariectomy affect the processes of cell proliferation and differentiation of epithelial cells in the mouse colon? If so, how?

3. Can exogenous estrogen (17β-estradiol) influence cell proliferation in the colonic epithelium in female ovariectomized mice and male mice? Is the effect dose-dependent? What is the time course of the effect? Does the administration of estrogen

in various regimens vary the effect? Can a mechanism for the estrogenic effect in the colon be postulated? Can the effect of estrogen in the colon be related to what has been described in the target organs?

To investigate these questions, two approaches were used.

1. Cell population kinetic studies employing autoradiographs of Epon-embedded colon tissue taken from mice given ^{3}H-thymidine: Parameters studied included crypt size (see Section II), size of the mucous cell population and size of vacuolated (proliferative) and columnar (non-proliferative and functional) cell populations (in the vacuolated-columnar cell line), labeling index of the total crypt epithelium and labeling indices of vacuolated-columnar and mucous cell populations.

2. Biochemical studies determining the concentration of DNA, RNA and protein and specific activity (^{3}H-thymidine incorporation expressed as cpm per microgram of DNA) of the colonic mucosa, as well as specific activity of the acid soluble pool (cpm /µg DNA) of the colonic mucosa.

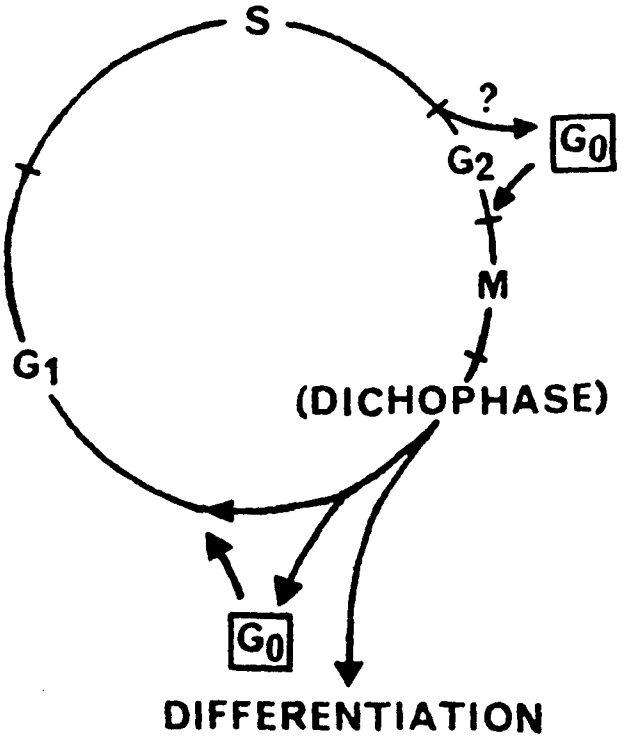


Fig. 1-1 Schematic representation of the cell cycle (see text for detailed description).

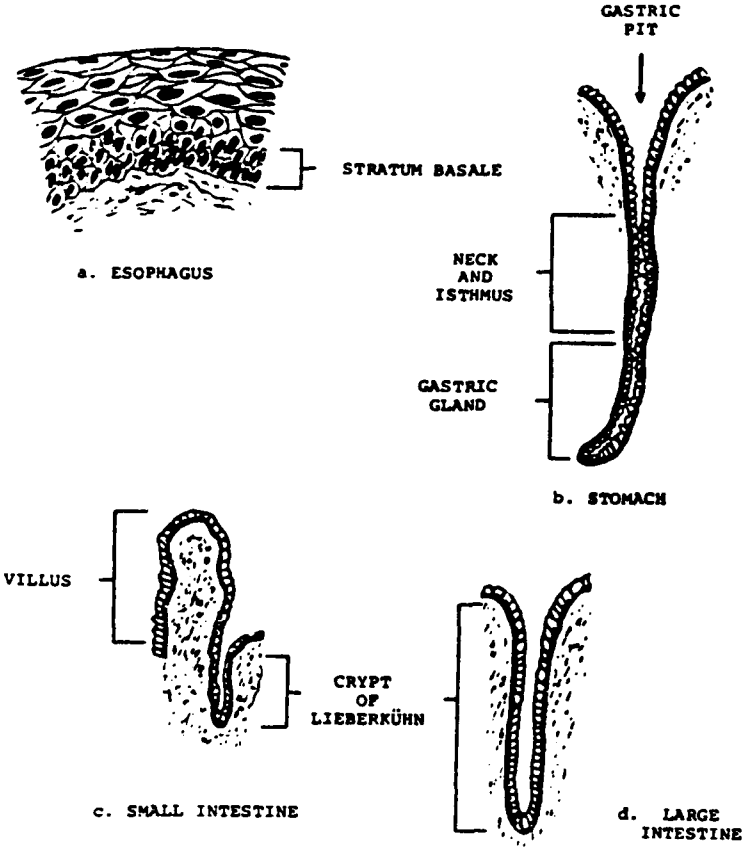


Fig. 1-2 Epithelium of various segments of the alimentary canal showing the proliferative zone of each: a. esophagus (stratum basale), b. stomach (isthmus and neck regions), c. small intestine (crypt), d. large intestine (lower 2/3 of crypt).


M
M

Fig. 1-4 Basal portion of colonic crypt of the mouse containing prominent vacuolated (arrowheads) and immature mucous (m) cells. x 2000.


m
m

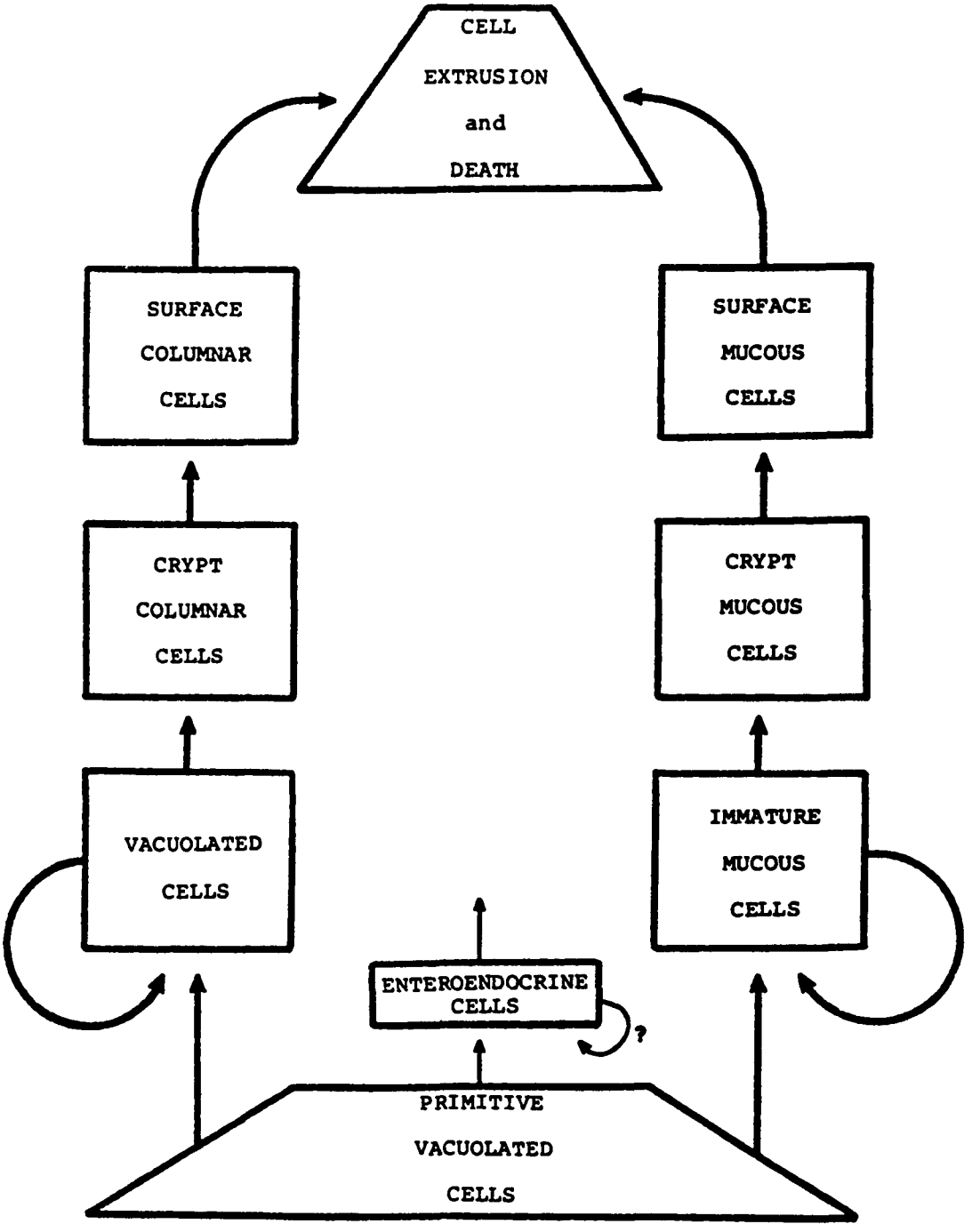


Fig. 1-5 The life histories of the epithelial cell lines of the descending colon of the mouse.

Section 2

EXPERIMENT:

AN AUTORADIOGRAPHIC CELL POPULATION KINETIC STUDY

of

MOUSE COLONIC EPITHELIUM DURING THE ESTROUS CYCLE

MATERIALS AND METHODS

Young adult virgin female CF-1 mice of 12 weeks of age (Charles River Breeding Laboratories, Wilmington, Mass.) were maintained in wire mesh cages, 4 to a cage, in an air-conditioned room with a day-night environment approximately equivalent to nature, and they were given laboratory Purina Chow and water ad libitum. Vaginal lavages were examined daily and only those animals that had two or more regular estrous cycles were used. Over a period of 3-4 weeks groups of 4 mice were killed at early diestrus (D_1), later diestrus (D_2), proestrus (P), estrus (E), and early (M_1) and later (M_2) metestrus using smear staging criteria similar to those of Allen (1922) (table 2-1). In another group of 4 mice, at 12 weeks of age, bilateral ovariectomies were performed using a dorsal approach. Postoperatively, they were caged in an air-conditioned room and given laboratory Purina Chow and water ad libitum for three weeks before killing. All the animals were killed under ether anesthesia between 9:00 and 11:00 h. in order to avoid the effect of diurnal variation on intestinal epithelial cell proliferation. One hour before killing, each mouse received 1 μCi per gram body weight of ^{3}H-thymidine (New England Nuclear, Boston, Mass.; specific activity 20 Ci per mmole). A segment of descending colon 1-2 cm from the anus was excised and cut transversely into ring-like pieces 3-5 mm in length. The latter were placed in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for fixation overnight in the cold. Following dehydration in a series of graded concentrations of acetone, the tissues were embedded in Epon 812 (Ladd Research Industries, Burlington, Vt.). The colonic rings were oriented so that on

sectioning the entire cross section of the colon could be viewed (fig. 2-1). This was accomplished by cutting off the conical end of embedding capsules leaving an open-ended capsule in which the colonic ring was positioned flush against the closed cap. Sectioning was carried out on an LKB-Huxley ultramicrotome. Five or six 1 μm thick serial sections were placed in one row on a glass slide with each oriented identically for easy section-to-section reference. A second serially sectioned row was placed under the first. Ten sections were cut and discarded between the two serially cut rows insuring that each row of sections did not contain cells identical to the other. Two to four slides per animal were prepared in this manner always discarding ten 1 μ thick sections after collecting each row of sections. Prestaining was carried out with the periodic acid-Schiff technique and iron-hematoxylin (Chang and Leblond, 1971a). Slides were dipped in photographic emulsion NTB-2 (Eastman Kodak, Rochester, N.Y.) for autoradiography, exposed for 4 weeks and developed (Kopriwa and Leblond, 1962).

In the autoradiographs, only those crypts that were longitudinally sectioned and lined by a single layer of epithelial cells along the basement membrane were used for analysis. In each crypt column (one side of a longitudinally sectioned crypt from the midpoint of the base to that cell at the point where a 45^{o} angle is made with the surface epithelium) (fig. 2-2) the number of vacuolated cells, columnar cells and mucous cells (for cell types, see Chang and Leblond, 1971a) (see figs. 1-3 and 1-4) as well as the number of their labeled and mitotic cells was recorded (fig. 2-3). Tabulation and statistical

analysis of these data were carried out using the computer system of The City University of New York under the guidance of and with the technical assistance from the Department of Biostatistics (Dr. Harry Smith, Chairman) of our institution. Forty crypt columns were tabulated for each animal used, therefore, 160 crypt columns were recorded for each stage of the sex cycle in addition to 160 crypt columns recorded for the ovariectomized group. The Student's t-test was performed for each cell population studied, with stage-to-stage contrasts. In addition, in order to compare the ovariectomized group to the cycling animals as a whole, a nested analysis of variance was carried out contrasting the mice of all 6 stages of the estrous cycle to the ovariectomized group. The ovariectomized group was weighted to compensate for the difference in sample size.

In analyzing the data the greatest overall variance was found to be among the observations made within each animal, thus, supporting our decision to count a large number of crypt columns per animal in our analysis since the overall variance (s^2) equals:

$$\frac{s^2 \text{ Between columns within animals}}{r} + \frac{s^2 \text{ Between animals within groups}}{(r)(k)} + \frac{s^2 \text{ Between groups}}{(r)(k)(t)}$$

where r = no. of columns, k = no. of animals per group and t = no. of groups.

RESULTS

Effect of estrous cycle on crypt cell populations

In the descending colon of the mouse, the crypt size, defined as the number of epithelial cells lining one side of a longitudinally sectioned crypt (crypt column), was 35 cells on the average, but fluctuated between 34 and 36 cells during the course of the estrous cycle. Peak cellularity per crypt column was exhibited during early diestrus and estrus, whereas nadirs in the crypt size were observed during late diestrus and late metestrus (fig. 2-4). The crypt size during early diestrus (35.7 cells) and during estrus (36.1 cells) was significantly different ($p < 0.05$) from that seen during late diestrus (34.2 cells) and late metestrus (34.5 cells) (see appendices A and B).

In the crypt, there were four types of epithelial cells: vacuolated, columnar, mucous and enteroendocrine (argentaffin), but there were only three cell lines: vacuolated-columnar, mucous and enteroendocrine, because vacuolated cells proliferated in the lower two-thirds of the crypt and on migration to the upper part of the crypt lost their vacuoles and became non-proliferating columnar cells (see Chang and Leblond, 1971a) (see fig. 1-5). Since enteroendocrine cells occupied only about 1% of the total epithelial cell population, they were excluded from the study.

In the vacuolated-columnar cell line (the main cell line in the

colonic crypt), the number of both proliferating vacuolated cells and non-proliferating columnar cells per crypt column varied during the course of the estrous cycle (fig. 2-4). The vacuolated cell population expanded during diestrus reaching its zenith during late diestrus when it comprised an average of 67% of the crypt column. From its peak, the size of the vacuolated cell population declined to lower values reaching the nadir during late metestrus (54.2%) (fig. 2-5). The number of vacuolated cells per crypt column during late diestrus (22.9) or proestrus (21.9) was significantly different ($p < 0.001$) from the number of vacuolated cells present during estrus (19.9), metestrus (19.5) and early diestrus (19.5). The changes in the size of the columnar cell population followed a trend that was just opposite to that of the vacuolated cell population. A trough in the size of the columnar cell population was seen during late diestrus and proestrus, when this population occupied between 15.6% and 17.1% of the crypt column population, whereas a crest was noted during estrus and early metestrus, when the columnar cells totaled between 27% and 29% of the crypt column. Of interest was a sharp decline in the size of columnar cell population during diestrus (from 23.6% to 15.6%) and an equally sharp rise in the transition from proestrus to estrus (from 17.1% to 26.7%). Statistically, the average number of columnar cells observed per crypt column during late diestrus (5.4) or proestrus (6.1) was significantly different ($p < 0.001$) from early diestrus (8.7), estrus (9.8) and metestrus (9.5) (appendix B).

The size of the mucous cell population did not seem to fluctuate during the estrous cycle to the extent that the vacuolated or columnar cell populations did (fig. 2-4). The number of mucous cells per crypt column decreased during diestrus and then remained fairly constant from late diestrus through estrus. This number was smallest during early metestrus, comprising about 13.1% of crypt column population, and became larger during late metestrus and early diestrus, when it made up about 21% of the crypt column population (fig. 2-5). Statistically, the size of mucous cell population during early metestrus (4.6) was significantly smaller ($p < 0.001$) than it was during either late metestrus (7.4) or early diestrus (7.5) (see appendix B).

Effect of estrous cycle on crypt cell proliferation

The overall labeling index, expressed as the percentage of labeled epithelial cells per crypt column (one hour after administration of ^{3}H-thymidine), showed some fluctuations during the course of estrous cycle (fig. 2-6). From late diestrus to estrus, there was a steady increase in the overall labeling index, reaching a peak during estrus (9.6%). This was followed by a decline in the index between estrous and early metestrus. During late metestrus a second peak was seen of about 9.1%. The labeling index during estrus (9.6%) was significantly higher ($p < 0.05$) than during early diestrus (8.3%), late diestrus (8.2%), and early metestrus (7.6%).

The percent of ^{3}H-thymidine-labeled vacuolated cells in the total epithelial cell population or in the vacuolated-columnar cell population per crypt column during the course of estrous cycle essentially followed a pattern similar to the overall labeling index of colonic crypt, the highest labeling being observed during proestrus and estrus (figs. 2-6 and 2-7). In these cases, the nadirs in the proliferative activity were observed during late diestrus and early metestrus, whereas peak labeling was seen as a plateau extending from proestrus through estrus. The increase in the percentage of labeled vacuolated cells in either the total crypt column population or the vacuolated-columnar cell population per crypt column from late diestrus to estrus was quite significant ($p < 0.025$) (see appendix B).

The percent of ^{3}H-thymidine-labeled mucous cells in either the total epithelial cell population or in the mucous cell population per crypt column showed two peaks, during estrus and late metestrus, and nadirs during early metestrus and early diestrus, but in general the variation in the proliferative activity of mucous cells during estrous cycle was less prominent than that of vacuolated cells (figs. 2-6 and 2-7).

Effect of ovariectomy on colonic crypt

Three weeks after bilateral ovariectomy, the colonic crypt size was significantly reduced ($p < 0.001$) to a mean of 27.4 cells per crypt column from the mean value of 35.1 cells in the intact mice

(fig. 2-4). This reduction reflected significant decreases in all three of the cell populations considered: a decrease to 4.9 columnar cells per crypt column in ovariectomized mice from 8.2 cells in the intact mice (40.1% decrease) ($p < 0.01$); a decrease to 18.2 cells in ovariectomized mice from 20.5 cells in intact mice in the vacuolated cell population (11.2% decrease) ($p < 0.05$); and a decrease to 4.3 cells from 6.5 cells in the mucous cell population (33.6% decrease) ($p < 0.05$) (table 2-2). It appeared, therefore, that, although each of colonic epithelial cell populations depended on ovarian hormones for its maintenance of a proper population size, the mature cells were more dependent on these hormones than immature proliferating cells in the colonic crypt. In other words, as a result of ovariectomy, the colonic crypt size decreased and the crypt contained proportionally more undifferentiated proliferating vacuolated cells than were seen in the crypts of the intact animals.

In spite of a smaller crypt size, the crypt in ovariectomized mice had slightly more ^{3}H-thymidine labeled cells per crypt column (3.4) than was observed in intact animals (3.1) (table 2-2). The percent of labeled cells per crypt column was 12.1 in the ovariectomized animals as compared to 8.7 in the intact animals, a significant increase ($p < 0.05$). The increase in ^{3}H-thymidine labeling after ovariectomy was entirely accounted for by a 47.7% ($p < 0.05$) increase in the labeling of vacuolated cells. The percent of labeled vacuolated cells in the vacuolated-columnar cell population was 13.4 in the ovariectomized group as compared to a mean

of 9.3 in the intact mice, a significant increase ($p < 0.025$), whereas the percent of labeled mucous cells in the mucous cell population was 4.9 in the ovariectomized mice as compared to 5.7 in the intact mice (fig. 2-7; table 2-2), a decrease that was not statistically significant.

TABLE 2-1

STAGING OF THE ESTROUS CYCLE (after Allen (1922))

STAGE (Duration in days)	FUNCTIONAL DESCRIPTION	RELATIVE NUMBERS OF CELLS FOUND IN VAGINAL SMEAR		
		EPITHELIAL	CORNIFIED EPITHELIAL	LEUKOCYTES
DIESTRUS (1-3)	-Relative quiescence	+	±	+
PROESTRUS (<1)	-Growth and congestion -Vulva; pink and swollen -Vagina gapes open	+++	–	–
ESTRUS (1)	-Sexual excitement -Swollen vulva -Vaginal Orifice open; dull white in color	±	+++	–
METESTRUS I (1)	-Returning to diestrus -Vulva not swollen -Vaginal orifice open	–	+++ (in clumps)	–
METESTRUS II (1-2)	-Vaginal orifice closed	(–	+++	+) early
		(–	++	+++) late

TABLE 2-2

Cell population changes in crypts of ovariectomized mice as compared to intact mice.*

CELL POPULATION PER CRYPT COLUMN	INTACT (MEAN±S.E.M.)	OVARIECTOMIZED (MEAN±S.E.M.)
TOTAL	35.11±0.18	27.43±0.39[w]
COLUMNAR	8.16±0.14	4.89±0.15[x]
VACUOLATED	20.48±0.14	18.20±0.29[z]
MUCOUS	6.47±0.10	4.34±0.18[z]
% COLUMNAR	22.30±0.30	17.67±0.53
% VACUOLATED	58.90±0.40	66.57±0.64[z]
% MUCOUS	18.40±0.30	17.98±0.24
TOTAL LABELED	3.08±0.07	3.41±0.17
LABELED VACUOLATED	2.70±0.07	3.18±0.17
LABELED MUCOUS	0.38±0.02	0.23±0.04
% TOTAL LABELED	8.70±0.20	12.05±0.55[z]
% LABELED VACUOLATED	7.60±0.20	11.27±0.54[z]
% LABELED MUCOUS	1.10±0.10	0.78±0.14
LABELED VACUOLATED/ VACUOLATED+COLUMNAR**	9.30±0.20	13.41±0.64[y]
LABELED MUCOUS/MUCOUS**	5.70±0.30	4.86±0.93

Note: w= $P < 0.001$
x= $P < 0.010$
y= $P < 0.025$
z= $P < 0.050$

* Contrast by nested analysis of variance.
** Expressed in percent form.

Fig. 2-1 Cross section of the mouse descending colon. x 50.

Fig. 2-2 Boundaries of the crypt column: surface epithelium (S), upper boundary (UB) and midpoint of the crypt base (M).

Fig. 2-3 Autoradiograph showing the basal portion of a crypt of the mouse descending colon with ^{3}H-thymidine-labeled cells (arrows) and a mitotic figure (*). x 2000.

Fig. 2-4 Variations in the number of the various cell types and the total of cells in the mouse colonic crypt during the estrous cycle. In this and the following figures the ovariectomized group is labeled 'ovx' and each point indicates the mean value ± S.E.

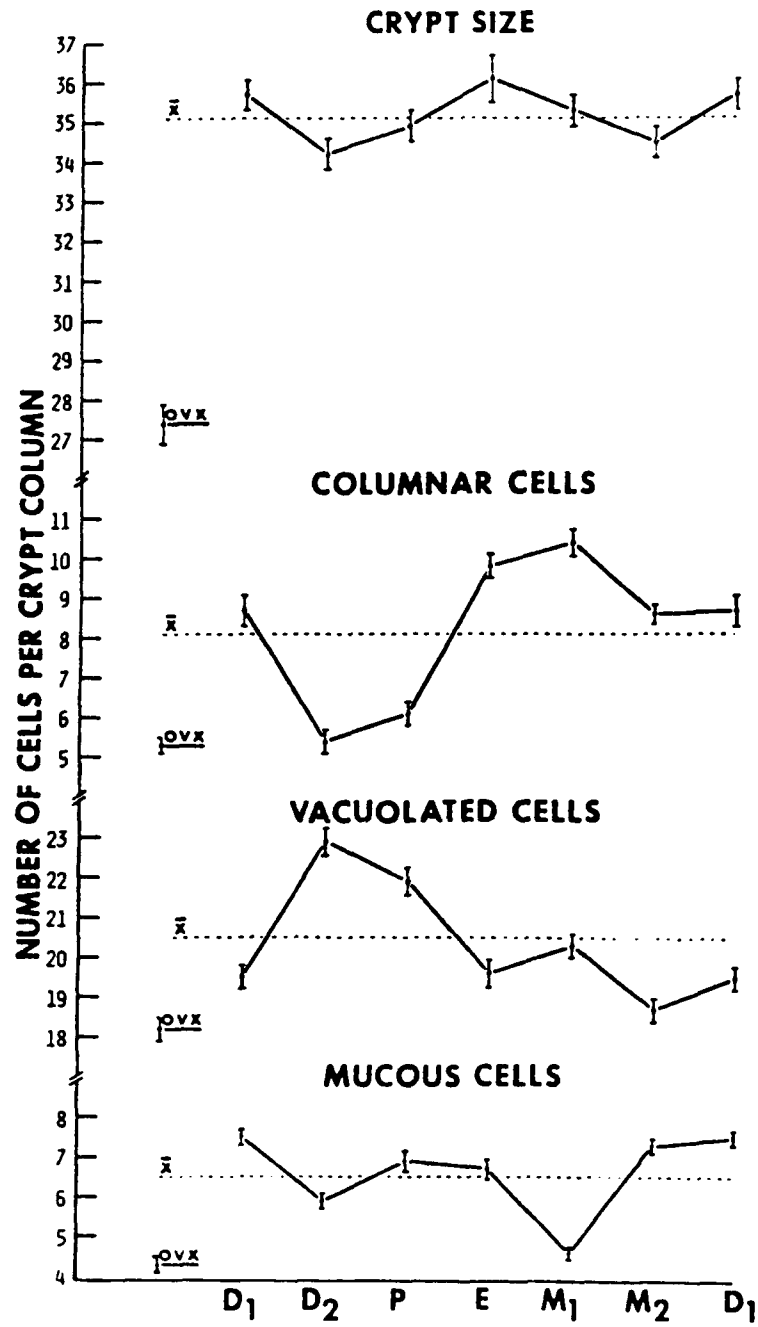

CRYPT SIZE
COLUMNAR CELLS
VACUOLATED CELLS
MUCOUS CELLS
NUMBER OF CELLS PER CRYPT COLUMN
37
36
35
34
33
32
31
30
29
28
27
11
10
9
8
7
6
5
23
22
21
20
19
18
8
7
6
5
4
x̄
OVX
D1
D2
P
E
M1
M2
D1

Fig. 2-5 Variations in the percentage of the various cell types in the mouse colonic crypt during the estrous cycle.

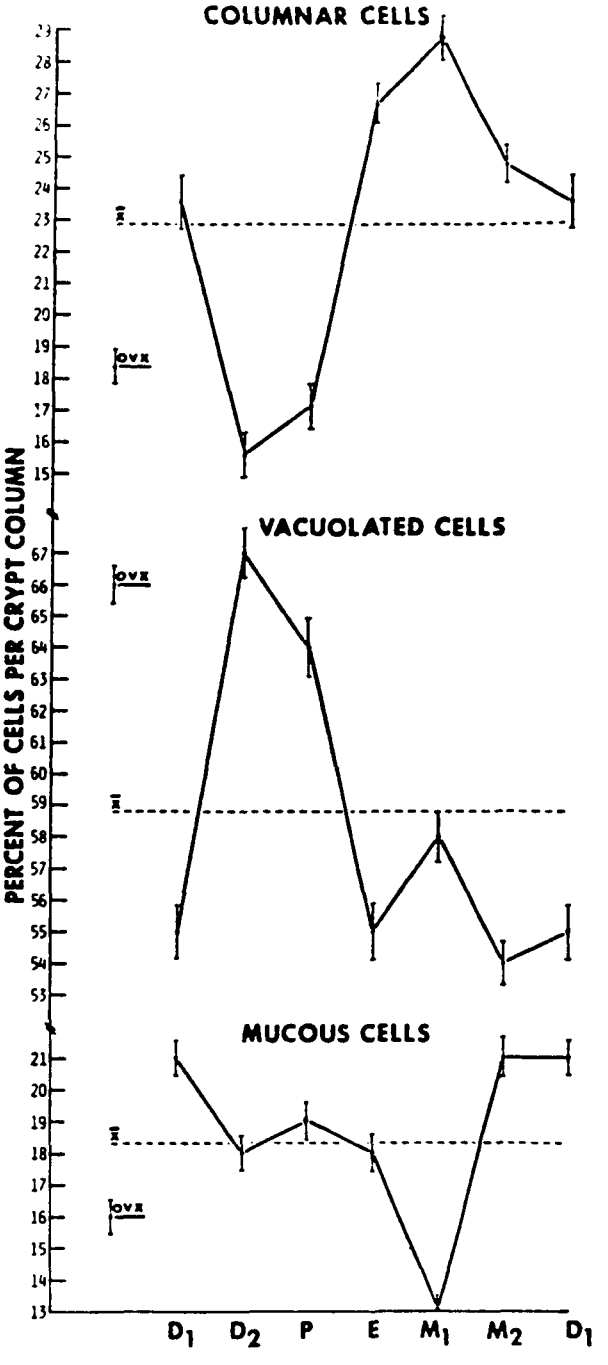

COLUMNAR CELLS
VACUOLATED CELLS
MUCOUS CELLS
PERCENT OF CELLS PER CRYPT COLUMN
OVX
D1 D2 P E M1 M2 D1

Fig. 2-6 Variations in the percentage of total labeled cells, labeled vacuolated cells and labeled musous cells in mouse colonic crypt during the estrous cycle.

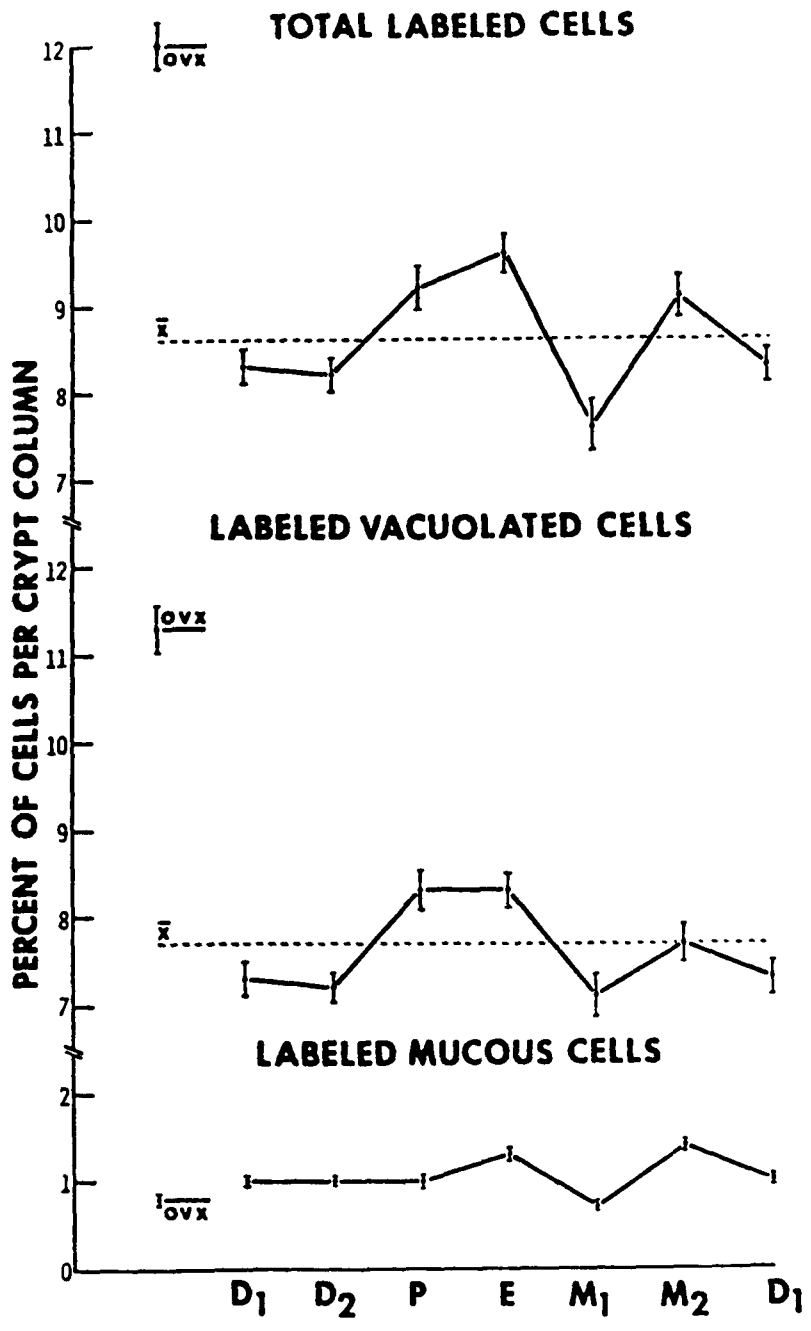
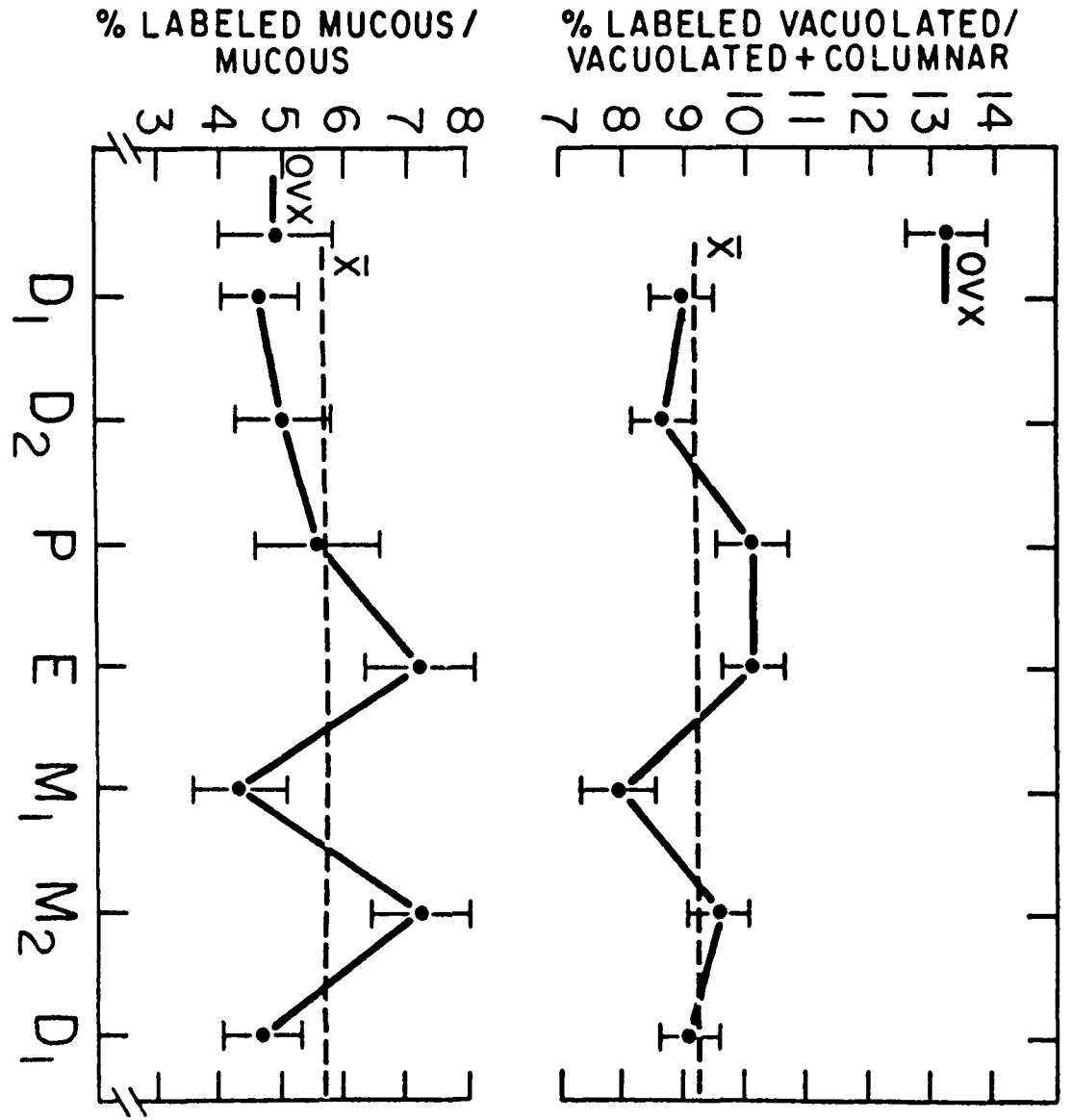

TOTAL LABELED CELLS
LABELED VACUOLATED CELLS
LABELED MUCOUS CELLS
PERCENT OF CELLS PER CRYPT COLUMN
ovx
x̄
0
1
2
7
8
9
10
11
12
D1
D2
P
E
M1
M2
D1

Fig. 2-7 Variations in the percentage of labeled vacuolated cells of the vacuolated-columnar cell population and variations in the percentage of labeled mucous cells of the mucous cell population in the mouse colonic crypt during the estrous cycle.


% LABELED MUCOUS / MUCOUS
% LABELED VACUOLATED / VACUOLATED + COLUMNAR
3
4
5
6
7
8
7
8
9
10
11
12
13
14
ovx
x
Tovx
D1
D2
P
E
M1
M2
D1

Section 3

EXPERIMENT:

BIOCHEMICAL STUDIES OF COLONIC MUCOSA

AFTER ESTROGEN TREATMENT

MATERIALS AND METHODS

Animals

Young adult male (8-10 weeks of age) and virgin female (10-12 weeks of age) CF-1 mice (Charles River Breeding Laboratories, Wilmington, Mass.) were placed in wire cages in groups of four mice of the same sex in an air-conditioned, artificially illuminated room with near natural day-night intervals. Laboratory Purina Chow and water were given ad libitum. Approximately one week after arrival, female mice were ovariectomized bilaterally by a dorsal approach. Postoperatively, vaginal lavage was performed periodically to access the effectiveness of the ovariectomies, and the animals were used in experiments (see below) three or more weeks after the operation.

Estrogen

Estrogen used was 17β-estradiol (17β-E_2) (Steraloids, Inc., Wilton, N.H.). The crystalline 17β-E_2 was dissolved and maintained in 100% ethanol in the refrigerator as a stock solution. The stock solution was diluted with bacteriostatic saline immediately before use. Doses administered were either 1, 10 or 50 ng of 17β-E_2 per gram body weight. Dilutions were such that 0.01 ml of the final solution per gram body weight was injected. Control mice received the equivalent volume of alcohol-saline solution. Injections were given subcutaneously either in the lateral abdominal wall or the nape of the neck.

When constant infusion of 17β-E_2 was used, silastic capsules packed with crystalline 17β-E_2 were prepared from silastic tubing (0.058 inches (0.23 mm) I.D., 0.077 inches (0.30 mm) O.D.) (Dow Corning Corp., Midland, Michigan) in a manner similar to Legan et al. (1975). After plugging one end of a short length of tubing (~10 mm) with a piece of wooden applicator, the overlapping collar of tubing was sealed with Silastic Medical Adhesive: Silicone Type A (Dow Corning Corp., Midland, Michigan). Then, crystalline 17β-E_2 was introduced into the tubing with the aid of the tip of a Pasteur pipet (as a funnel) and a straightened paper clip (as a ram-rod). The tubing was tightly packed with the crystals against the wooden plug without distorting the tube. When a 5 mm length of the tubing was packed, another wooden plug was inserted into the open end, maintaining the undistorted 5 mm length, and the tubing was sealed as before. Mock capsules were made as above without the estrogen. The capsules were bathed in normal sterile saline at room temperature during the 24 hours preceding implantation.

Experiments

A. Ovariectomized mice treated with estrogen.

Four regimens were investigated:

1. Single injection experiments.

A single injection of 10 ng of 17β-E_2 per gram body weight was given to each of 48 mice. They were killed in groups of 12 mice, 4, 8, 16 and 24 hours after injection. The control group consisted

of 12 mice that were sacrificed 16 hours after injection of the veh vehicle. The injections were adjusted in such a way that all the mice were killed between 9:00 and 11:00 h.

2. Multiple injection experiments.

In the first experiment, one low dose injection of 1 ng of 17β-E_2 per gram body weight was administered to each of 100 mice between 17:00 and 19:00 h on three consecutive days. A fourth and final injection was given either 4 (24 mice), 12 (8 mice), 16 (46 mice) 20 (14 mice) or 28 (8 mice) hours preceding sacrifice between 9:00 and 11:00 h (fig. 3-1). For each period, there was a control group of 16 mice, which received only the vehicle and were otherwise treated similarly to the experimental groups.

In the second experiment, 12 mice were given one high dose injection of 10 ng of 17β-E_2 per gram body weight between 17:00 and 19:00 h on three consecutive days. A fourth and final injection was administered on day 4 of the experiment 4 hours before sacrificing between 9:00 and 11:00 h. A group of 12 mice, constituting the control, was treated similarly with the vehicle.

In the third experiment, 14 mice were treated similarly as in the second experiment, but with a very high dose of 50 ng of 17β-E_2 per gram body weight per injection. The control group also consisted of 14 mice.

3. Priming experiments.

In the first experiment, one low dose injection of 1 ng of 17β-E_2 per gram body weight was given to each of 14 mice between 17:00 and 19:00 h on three consecutive days. Thirty-six hours after the third injection, a fourth and final injection was administered 4 hours prior to killing between 9:00 and 11:00 h. Sixteen mice, similarly treated with the vehicle, comprised the control group.

In the second experiment, 50 mice received one high dose injection of 10 ng of 17β-E_2 per gram body weight between 17:00 and 19:00 h on three consecutive days. A fourth injection was given 36 hours before a fifth and final injection, which was administered to each of four groups of mice, either 4 (12 mice), 8 (12 mice), 16 (13 mice) or 24 (13 mice) hours prior to sacrificing between 9:00 and 11:00 h (fig. 3-2). Six mice treated similarly with the vehicle comprised the control group.

4. Continuous infusion experiments.

Under ether anesthesia, saline-bathed silastic capsules packed with 17β-E_2 were implanted subcutaneously into the lateral body wall of 22 mice. Postoperatively, the animals were kept in wire cages and were killed 1 (8 mice), 2 (7 mice), 4 (7 mice) and 8 (7 mice) days after implantation. Twenty-one mice were implanted with mock capsules, being used as controls, and sacrificed 1 (8 mice), 2 (6 mice) and 4 (7 mice) days after implantation.

In another experiment, 6 mice each received one injection of 1 ng of 17β-E_2 per gram body weight between 17:00 and 19:00 h on three consecutive days. On day 4, an estrogen capsule was implanted between 17:00 and 19:00 h. At 16 hours after implantation the animals were sacrificed. Seven mice were given three injections of the vehicle and had a mock capsule implanted at times corresponding to the estrogen-treated group; they served as controls.

B. Intact male mice treated with estrogen.

Seven male mice were injected once with 1 ng of 17β-E_2 per gram body weight between 17:00 and 19:00 h on four consecutive days. The animals were sacrificed 16 hours after the fourth and final injection. The control group, which consisted of seven mice, was injected with vehicle only at the same times as the experimental group.

In a separate experiment, 6 male mice received implants of silastic capsules packed with 17β-E_2 and were sacrificed 2 days later. Mock capsules were implanted into 6 control mice.

C. Fasting-refeeding experiment.

Twenty-nine ovariectomized mice were used. Seven mice were given both food and water ad libitum. Twenty-two mice were fasted for 48 hours but water was given ad libitum. At the end of 48 hours, 7 of the fasted mice were sacrificed, while the remaining 15 mice had their food returned. At the same time, 8 out of the 15 refed mice received a single injection of 10 ng of 17β-E_2 per gram body

weight. At 16 hours after the onset of refeeding, all of the 15 refed mice were sacrificed. The seven non-fasted mice were killed during the course of experiment at the same time of day as the other mice.

In these experiments, all mice were killed between 9:00 and 11:00 h in order to avoid the effect of diurnal variation on intestinal epithelial cell proliferation. One hour prior to killing, all mice received 1 μCi per gram of ^{3}H-thymidine (New England Nuclear, Boston, Mass.; specific activity 20 Ci per mmole). Under ether anesthesia, mice were bled by severing the heart. Both the uterine tubes and the whole descending colon were immediately removed. The uterine tubes were excised from the ligatures (applied at the time of ovariectomy) to the vagina in order to determine the effectiveness of estrogenization by gross observation and wet and dry weights. Immediately upon excision of the colon, from the junction of the transverse and descending colon to the rectum, distally, a 3-5 mm segment was cut from the rectal end and placed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight in the cold for autoradiography (see Part 3). The remaining portion of the colon was longitudinally slit open and rinsed in cold saline to remove fecal material. The colon was laid flat on a glass plate over ice with its serosal surface down. Using glass coverslips, the mucosa and submucosa were scraped off of the muscularis externa. The scraped mucosae were placed into pre-weighed Beem embedding capsules and immediately frozen over dry ice. Weighing of the mucosal scrapings was done with rapidity so as to avoid

thawing. Specimens were stored in a freezer until assays were carried out, but storage never exceeded one week.

Biochemical methods

A 5% homogenate of each colonic mucosal scraping was made in either cold distilled water or saline using a constant drive motor and tissue grinder (A. H. Thomas Co., Philadelphia, Pa.). To avoid cross-sample contamination, a clean vessel and pestle were used for each homogenization. Two alliquots were taken from each homogenate for determination of protein content by the method of Lowry et al. (1951).

Two additional alliquots were taken for assaying DNA content. DNA was separated using the method of Schmidt and Thannhauser (1945) and then quantitated colorimetrically, using Beckman DB-G Grating spectrophotometer, according to Burton (1956). From the same supernatant used to quantitate DNA content, an alliquot was taken for scintillation counting using the Beckman LS-150 Liquid Scintillation System to determine the amount of ^{3}H-thymidine incorporated into the DNA. The cocktail used for scintillation counting was Aquasol (New England Nuclear, Boston, Mass.). The specific activity of the mucosa was expressed in counts per minute (cpm) per µg DNA. Alliquots were also obtained from the pooled supernatants of the 5 and 10 percent tricholoroacetic acid washings and were used to determine the radioactivity of the acid soluble components of the mucosa, i.e.,

the amount of ^{3}H-thymidine that was available but was not incorporated into DNA. RNA content was determined using the method of Lin and Schjeide(1969). In assaying protein, DNA and RNA, each of two alliquots was determined independently and the mean of the two determinations was employed in the final statistical analysis.

Organ Culture

The descending colon was excised from an immature (4 month old) female New Zealand white rabbit (Perfection Breeders, Inc., Douglassville, Pa.) under ether anesthesia. The excised segment of bowel was flushed through with normal saline to remove fecal matter. Using the Multipurpose Biopsy Tube, Model 4.7 mm (Quinton Instruments, Seattle, Wash.), forty biopsies were excised placing each in total culture medium as it was taken. The culture medium used consisted of 88.5% RPMI Medium with Hepes buffer (Grand Island Biological Co., Grand Island, N.Y.), 10% fetal calf serum (pre-incubated at 56^{0} for one hour for enzyme inactivation) (GIBCO), 1% penicillin-streptomycin-fungizone (GIBCO), and 0.5% gentamicin (Shering Diagnostics, Port Reading, N.J.). Colonic explants were placed two on a stainless steel grid (screen) in organ culture dishes (Falcon Plastics, Oxnard, Calif.) and cultured according to Trier (1976) (fig. 3-3). A humid environment was maintained in each dish by using a paper ring (blotter) saturated with sterile distilled water. Explants were initially cultured for 6 hours in an atmosphere of 95% oxygen and 5% carbon dioxide at $37^{0}C$. At the end of 6 hours, the 40 explants were divided equally into 5 groups: one

control group being placed in fresh medium without estrogen and four experimental groups each placed in fresh medium containing one of four different concentrations of 17β-E_2 (1 µg, 1 ng, 10 ng or 100 ng per ml of complete medium). In addition, each culture dish contained 2 µCi of ^{3}H-thymidine per ml of the fresh medium. Cultures were re-gassed and incubated for an additional 18 hours. At the end of incubation, all cultures were harvested and immediately frozen over dry ice.

From each explant, the DNA content and the relative amount of ^{3}H-thymidine incorporated was determined. Each explant was digested overnight in 0.5 ml of 1N NaOH. Using 0.2 ml of the NaOH digest, DNA determination was made according to Ceriotti (1952) and Bonting and Jones (1957). To the remaining digest, 0.7 ml of distilled water, 3 drops of acetic acid and 10 ml of Aquasol 2 (New England Nuclear, Boston, Mass.) were added and scintillation counting was performed as described previously.

All experiments were analyzed by the Student's two-tailed t-test.

RESULTS

Uterine weight of ovariectomized mice with estrogen treatment

In the ovariectomized untreated mice, the uterine tubes formed slender Y-shaped structures which appeared to be atrophic, and weighed 26.4 mg, on the average. Treatment of ovariectomized mice with 17β-E_2 caused engorgement, enlargement and an increase in the wet weight of the uterine tubes, but the degree of this response seemed to depend upon the dose and regimen of estrogen (table 3-1). Uterine dry weight was seen to vary with estrogen dose and regimen as well.

Following a single injection of 10 ng of 17β-E_2 per gram body weight (gbw), there was a steady increase in the wet weight of uterine tubes with time up to 57.0 mg at 24 hours from the control value of 22.3 mg, however, there was no change in dry weight. In the low dose multiple injection regimen, however, the wet weight of uterine tubes was greatest (151.1 mg) at 4 hours after the last injection and decreased steadily thereafter to 91.1 mg at 28 hours, whereas the dry weight was greatest between 12 and 20 hours after the last injection.

The increase in the uterine weight (wet and dry) was greatest when multiple injections of estrogen were given, i.e. the multiple injection regimen or the priming regimen, with the consecutive multiple injection regimen causing a greater increase in uterine weight than the priming regimen. In the multiple injection regimen, the greatest uterine weight (185.0 mg) was obtained with 10 ng of

17β-E_2 per gbw; this increase in uterine weight was greater than obtained with either the low (1 ng per gbw) or the very high (50 ng per gbw) dose of estrogen. Uterine dry weight was more significantly increased with multiple 1 and 10 ng per gbw ($p < 0.001$) injection regimens than with 50 ng per gbw ($p < 0.02$) as compared to the controls.

With continuous infusion of estrogen, using the silastic capsule implantation technique, the uterine wet weight increased to 68.6 mg at 1 day, to 123.4 mg at 4 days and 140.1 mg at 8 days; the average uterine wet weight for all implant control mice was 41.3 mg. Dry weight results yielded an increase to 19.7 mg after 1 day and 31.6 mg after 4 days. Dry uterine weight after 8 days of implantation was 27.4 mg, compared to the overall average uterine dry weight of 16.7 mg for the implant control groups.

Effects of three estrogen injection regimens on colonic mucosa of ovariectomized mice

In ovariectomized mice treated with various doses and regimens of 17β-E_2, incorportation of ^{3}H-thymidine into the colonic mucosa was inhibited at 4 hours after the last estrogen injection (fig. 3-4). The high dose (10 ng per gbw) of estrogen was more effective than the low dose (1 ng per gbw), but the very high dose (50 ng per gbw) of estrogen did not cause further inhibition of ^{3}H-thymidine incorporation. However, in the multiple injection experiments, the inhibitory response on ^{3}H-thymidine incorporation became more uniform as the estrogen dose was increased, as shown by a decreasing standard error of the mean with increasing dose. Among the three injection

regimens, the inhibition of ^{3}H-thymidine incorporation was most remarkable in the single high dose experiment (75% inhibition) and least remarkable in the low dose priming experiment (18% inhibition).

Figure 3-5 compares the effect of the three injection regimens of estrogen of ^{3}H-thymidine incorporation into colonic mucosae at 4 and 16 hours after the last injection. In all regimens, ^{3}H-thymidine incorporation was greater at 16 hours than at 4 hours. Inhibition of DNA synthesis was still obvious at 16 hours with the single or multiple injection regimen. In the priming regimen, the amount of ^{3}H-thymidine incorporated at 16 hours exceeded the control value, but the difference was not statistically significant.

The time course studies revealed that ^{3}H-thymidine incorporation was significantly and continuously suppressed for at least 24 hours following a single high dose of estrogen (fig. 3-6). The low dose multiple regimen (fig. 3-7) yielded a significant suppression of ^{3}H-thymidine incorporation at 4 hours ($p < 0.005$) and 16 hours ($p < 0.02$); animals killed at 12 and 20 hours after the last injection presented specific activities above the control value (33% and 18% respectively), though these values were not statistically different from the control. In the high dose priming regimen (fig. 3-2), ^{3}H-thymidine incorporation into colonic mucosa was suppressed in animals killed at 4 and 8 hours after the last injection ($p < 0.05$) (fig. 3-8). Although the 16-hour group had a mean specific activity of 29% above the control level and the 24-hour group had a

value of 18% below the control, neither of these was significantly different from the control.

Uptake of ^{3}H-thymidine into the acid soluble fraction of the colonic mucosa was determined in the high dose single injection and the high dose priming experiments (table 3-2). In the single injection regimen, ^{3}H-thymidine uptake at 4, 8 and 24 hours after injection was significantly higher ($p < 0.05$) than that of the control, but the uptake at 16 hours was not. The priming experiment presented ^{3}H-thymidine uptake at 4, 8 and 16 hours after the last injection lower than, but not significantly different from, the control value. It was, however, significantly lower ($p < 0.05$) at 24 hours.

The protein or RNA concentration (mg protein or µg RNA per mg wet weight) of colonic mucosa was not affected by any of the estrogen injection regimens (tables 3-2 and 3-3). The DNA concentration (µg DNA per mg wet weight) of colonic mucosa did not appear to be influenced by estrogen in the single injection, the priming or the low dose multiple injection regimen (table 3-3). However, in the multiple injection experiments, employing 10 or 50 ng of 17β-E_2 per gbw, the DNA concentration was significantly increased ($p < 0.005$) as compared to the control.

Effects of continuous infusion of estrogen on colonic mucosa of ovariectomized mice (fig. 3-9)

Following implantation of 17β-E_2-packed silastic capsules into

the subcutaneous tissue of ovariectomized mice, incorporation of ^{3}H-thymidine into colonic mucosal DNA determined at 1, 2 and 4 days after implantation was above the control value: 12% after 1 day, 6% after 2 days and 4% after 4 days. In mice that received 1 ng of 17β-E_2 per gbw daily for 3 days before implantation and sacrificed 16 hours after implantation, the mean specific activity was 15% above the control. None of these groups was significantly different from the control. The DNA and protein concentrations of the experimental mice were similar to the controls.

Effect of estrogen on colonic mucosa of intact male mice (fig. 3-10)

Male mice, that received 4 daily injections of 1 ng of 17β-E_2 per gbw and were killed 16 hours after the last, incorporated significantly less thymidine than control animals ($p < 0.05$).

In the 2 day estrogen implant experiment, ^{3}H-thymidine incorporation by colonic mucosa of male mice was about 15% less than the controls, although statistically this was not significant.

The above responses of colonic mucosa in male mice to estrogen did not seem to differ from those seen in ovariectomized mice. In addition, no change in mucosal DNA or protein content was observed in either male or ovariectomized female mice (table 3-4).

Effect of estrogen on colonic mucosa induced to proliferate by fasting-refeeding in ovariectomized mice (fig. 3-11)

^{3}H-thymidine incorporation into the colonic mucosa in mice fasted for 48 hours was 20% of the control value which was significantly less than that of the control ($p < 0.005$). Following 16 hours of refeeding, ^{3}H-thymidine incorporation increased significantly ($p < 0.001$) to 285% of the control value (=100%). In the estrogenized refed mice, the specific activity was 240% of the control value and was significantly lower ($p < 0.05$) than the refed group without estrogen.

Uptake of ^{3}H-thymidine into the acid soluble fraction of the colonic mucosa was unchanged following 48 hours of fasting but was significantly decreased in both of the refed groups ($p < 0.001$) as compared to the non-fasted control group (table 3-5).

The RNA and protein concentration (mg protein or µg RNA per mg wet weight) was similar for all four groups (table 3-5). The DNA concentration, however, was significantly increased in the fasted group as well as in both of the refed groups as compared to the control ($p < 0.02$). The protein/DNA ratio ($p < 0.02$) and the RNA/DNA ratio ($p < 0.05$) were decreased in all three of the fasted groups as compared to the controls.

Effect of estrogen on rabbit colonic mucosal explants in organ culture (fig. 3-12)

Following 18 hours of culture in the presence of 1 µg, 1 ng or 10 ng 17β-E_2 per ml of medium, the amount of ^{3}H-thymidine incorporated cumulatively for 18 hours into colonic mucosal explants was between 15 and 20% lower than the control level; these differences were not significant. The explants that were cultured with medium containing 100 ng of 17β-E_2 per ml had a mean specific activity 10% above the control value; again not a significant difference.

TABLE 3-1

Comparison of the effects of various doses and regimens of 17β-E_2 on uterine weight gain (mean±S.E.M.)

SINGLE HIGH DOSE		CONTROL	4h	8h	16h	24h	
	WET WEIGHT	22.3±1.9	33.2± 4.7	44.0± 5.0*	46.3±2.6*	57.0± 4.7*	
	DRY WEIGHT	16.0±2.9	13.2± 2.2	15.8± 2.2	12.1±1.8	17.9± 2.7	
SINGLE VERY HIGH DOSE							
	WET WEIGHT	31.7±3.6	36.4± 2.2				
	DRY WEIGHT	11.4±1.7	10.7± 1.6				
MULTIPLE LOW DOSE		CONTROL	4h	12h	16h	20h	28h
	WET WEIGHT	27.0±1.5	151.1±10.4*	118.7±13.3	101.4±6.6*	109.1±10.8*	91.1±6.2
	DRY WEIGHT	12.3±0.6	27.4± 2.2*	34.9± 4.6*	31.0±4.1*	34.4± 4.4*	25.2±3.2
MULTIPLE HIGH DOSE							
	WET WEIGHT	33.3±2.3	185.0±12.7				
	DRY WEIGHT	10.5±1.6	27.0± 4.7*				
MULTIPLE VERY HIGH DOSE							
	WET WEIGHT	31.7±3.6	121.2±14.1*				
	DRY WEIGHT	11.4±1.7	18.9± 2.9*				
LOW DOSE PRIMING		CONTROL	4h	8h	16h	24h	
	WET WEIGHT	33.3±2.3	98.1± 5.2*				
	DRY WEIGHT	10.5±1.6	24.2± 3.5*				
HIGH DOSE PRIMING							
	WET WEIGHT	24.3±3.5		109.7±10.8*	77.5±4.9*	92.7±11.5*	
	DRY WEIGHT	14.3±3.0		22.4± 3.2*	21.6±3.1*	23.5± 3.4*	
ESTROGEN IMPLANTS**		CONTROL	2/3 DAY***	1 DAY	4 DAY	8 DAY	
	WET WEIGHT	41.3±5.9	110.6±14.6*	68.6±14.5	123.4±6.6*	140.1±12.9*	
	DRY WEIGHT	16.7±1.5	21.8± 3.7	19.7± 2.7	31.6±4.0	27.4± 3.6	

Low Dose = 1 ng per gram body weight
High Dose = 10 ng per gram body weight
Very High Dose = 50 ng per gram body weight

* significantly different from control.
** see materials and methods for description.
*** preceded by 1 injection of 1ng/gbw 17β-E_2 daily for 3days

TABLE 3-2

Comparison of tritium content of acid soluble fraction and RNA content of mucosa between estrogen-treated and untreated ovariectomized mice

	$\frac{\text{RNA}(\mu g)}{\text{WET WEIGHT}(mg)}$	$\frac{\text{RNA}(\mu g)}{\text{DNA}(\mu g)}$	$\frac{\text{ACID SOLUBLE CPM}}{\text{DNA}(\mu g)}$
SINGLE HIGH DOSE			
CONTROL	7.32±0.76*	1.48±0.10	27.70±4.77
4h	6.62±0.41	1.82±0.11	47.15±6.27**
8h	7.67±0.44	1.90±0.16	41.85±3.54**
16h	7.42±1.99	1.41±0.16	31.27±2.32
24h	6.68±0.92	1.85±0.24	42.50±3.13**
HIGH DOSE PRIMING			
CONTROL	7.45±1.22	2.08±0.19	63.10±7.90
4h	7.02±0.94	2.20±0.18	55.92±3.40
8h	7.49±0.52	2.18±0.20	58.93±5.46
16h	7.24±0.36	1.87±0.14	53.00±4.33
24h	8.87±0.98	2.01±0.21	41.81±3.47**

Note: Low Dose = 1 ng/gram body weight (gbw)
High Dose = 10 ng/gbw
* Mean±S.E.M.
** Significant at at least a $P<0.05$ level of significance compared to control using Student's t-test.

TABLE 3-3

Comparison of protein and DNA content of mucosa between estrogen-treated and untreated ovariectomized mice.

	PROTEIN(mg) / WET WEIGHT(mg)	DNA(μg) / WET WEIGHT(mg)	PROTEIN(mg) / DNA(μg)
SINGLE HIGH DOSE			
CONTROL	0.104±0.011*	5.00±0.59	21.64±2.51
4h	0.074±0.006**	3.67±0.22	20.10±0.91
8h	0.086±0.005	4.16±0.31	21.05±1.55
16h	0.093±0.011	5.13±0.94	18.04±0.72
24h	0.081±0.006**	3.72±0.27	22.09±2.05
MULTIPLE LOW DOSE			
CONTROL	0.094±0.004	4.10±0.17	23.73±0.81
4h	0.086±0.005	3.71±0.25	24.58±2.54
12h	---	---	25.56±1.35
16h	0.090±0.005	3.87±0.25	23.33±1.37
20h	0.109±0.011	3.68±0.31	29.25±0.98
MULTIPLE HIGH DOSE			
CONTROL	0.093±0.005	2.94±0.25	32.28±1.36
4h	0.106±0.006	5.36±0.27**	20.14±0.64
MULTIPLE VERY HIGH DOSE			
CONTROL	0.093±0.005	2.94±0.25	32.28±1.36
4h	0.095±0.005	5.04±0.33**	19.04±0.53
LOW DOSE PRIMING			
CONTROL	0.093±0.005	2.94±0.25	32.28±1.36
4h	0.100±0.004	2.94±0.14	34.49±1.18
HIGH DOSE PRIMING			
CONTROL	0.092±0.006	3.50±0.25	26.34±1.19
4h	0.088±0.009	3.13±0.26	27.74±0.99
8h	0.101±0.004	3.46±0.09	29.29±1.23
16h	0.102±0.002	3.88±0.11	26.47±1.12
24h	0.118±0.011**	4.34±0.14**	27.34±2.71
ESTROGEN IMPLANTS			
CONTROL	0.094±0.005	4.73±0.14	19.87±0.59
2/3 day***	0.099±0.007	4.39±0.33	22.69±0.69
1 day	0.107±0.010	4.70±0.37	21.90±0.98
2 day	0.102±0.007	4.35±0.43	24.05±1.22
4 day	0.080±0.002	4.91±0.14	16.44±0.54

Note: Low Dose = 1 ng/gram body weight(gbw)
High Dose = 10 ng/gbw
Very High Dose = 50 ng/gbw

* Mean±S.E.M.

** Significant at at least $P<0.05$ level as compared to control using Student's t-test.

***Preceded by one 1ng/gbw injection of 17β-E_2 daily for 3 days.

TABLE 3-4

Comparison of protein and DNA content in mucosa between estrogen-treated male and ovariectomized female mice.

		PROTEIN(mg)/WET WEIGHT(mg)	DNA(μg)/WET WEIGHT(mg)	PROTEIN(mg)/DNA(μg)
LOW DOSE MULTIPLE				
MALE:	CONTROL	0.076±0.005[a]	4.11±0.23	18.62±1.14
	TREATED[b]	0.088±0.009	4.05±0.42	21.85±1.31
FEMALE:	CONTROL	0.094±0.004	4.10±0.17	23.73±0.81
	TREATED[b]	0.090±0.005	3.87±0.25	23.33±1.37
ESTROGEN IMPLANT				
MALE:	CONTROL	0.108±0.009	5.28±0.34	20.51±1.26
	TREATED[c]	0.101±0.008	4.82±0.32	20.93±0.95
FEMALE:	CONTROL	0.094±0.005	4.73±0.14	19.87±0.59
	TREATED[c]	0.102±0.007	4.35±0.43	24.05±1.22

Note:

a: Mean ± S.E.M.

b: Sacrificed 16h after the fourth injection.

c: Sacrificed 2 days after estrogen capsule implantation.

TABLE 3-5

Comparison of various biochemical parameters of the colonic mucosa between groups of the fasting-refeeding experiment.

	PROTEIN(mg) / WET WEIGHT(mg)	DNA(μg) / WET WEIGHT(mg)	PROTEIN(mg) / DNA(μg)	RNA(μg) / WET WEIGHT(mg)	RNA(μg) / DNA(μg)	ACID SOLUBLE CPM / DNA(μg)
CONTROL	0.125±0.011[a]	4.67±0.21	27.12±2.86	9.76±0.26	2.12±0.09	48.11±2.86
48h FASTED	0.108±0.005	5.80±0.29[d]	16.22±1.69[d]	8.64±0.56	1.49±0.11	46.92±3.65
REFED-UNTREATED[b]	0.129±0.012	5.82±0.22[d]	22.24±1.89	9.46±0.56	1.64±0.11	37.50±0.078[d]
REFED-TREATED[c]	0.111±0.005	5.43±0.20[d]	20.50±0.34[d]	9.15±0.45	1.69±0.08	35.50±1.37[d]

Note:

a: Mean ± S.E.M.

b: Fasted for 48h; sacrificed 16h after onset of refeeding

c: As in refed-untreat but given 1 10ng/gram body weight 17β-E_2 injection at time of refeeding.

d: Significant at at least a $P < 0.05$ level of significance as compared to control.

Fig. 3-1

LOW DOSE MULTIPLE INJECTION SCHEDULE

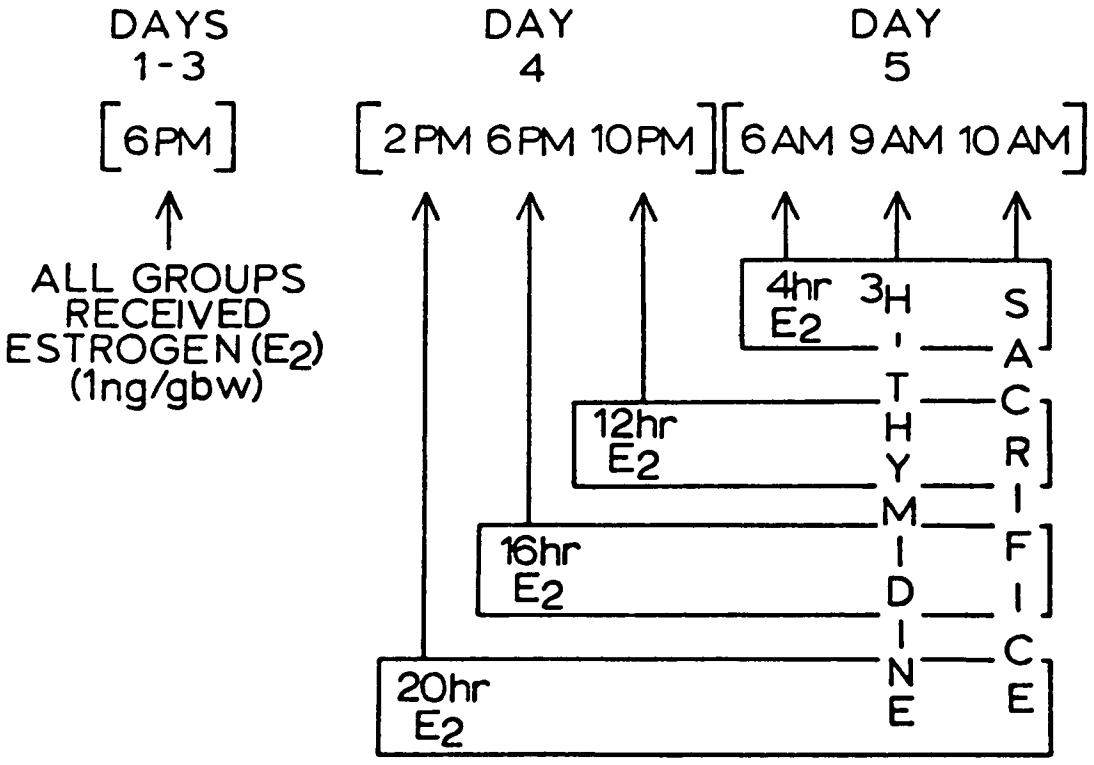
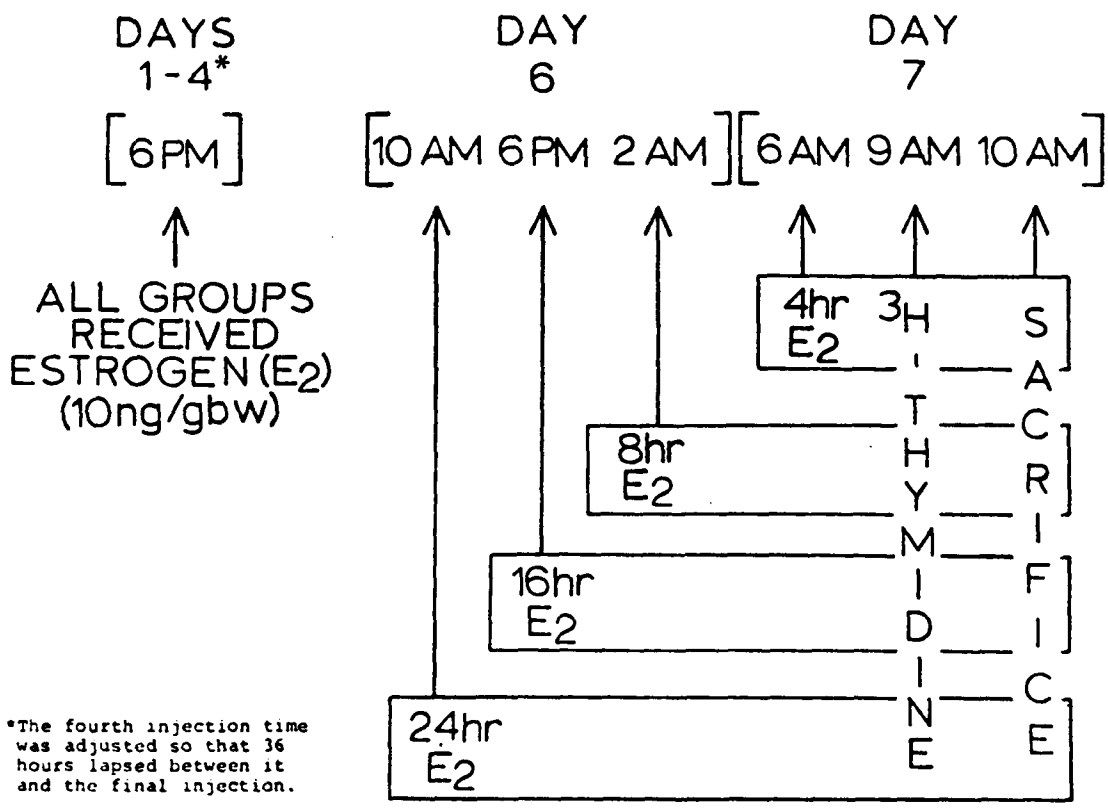


Fig. 3-2

HIGH DOSE PRIMING INJECTION SCHEDULE



*The fourth injection time was adjusted so that 36 hours lapsed between it and the final injection.

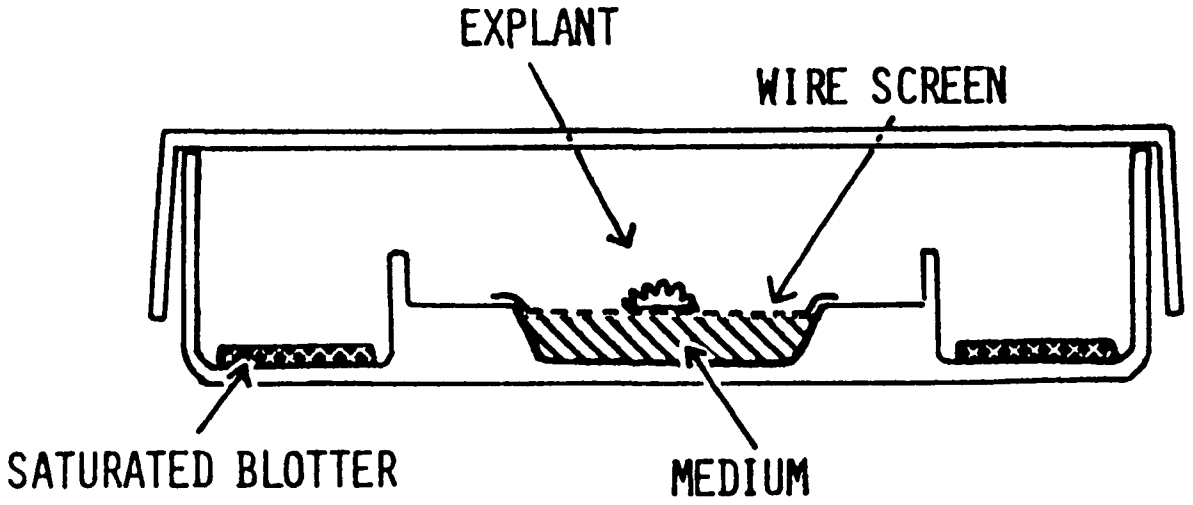
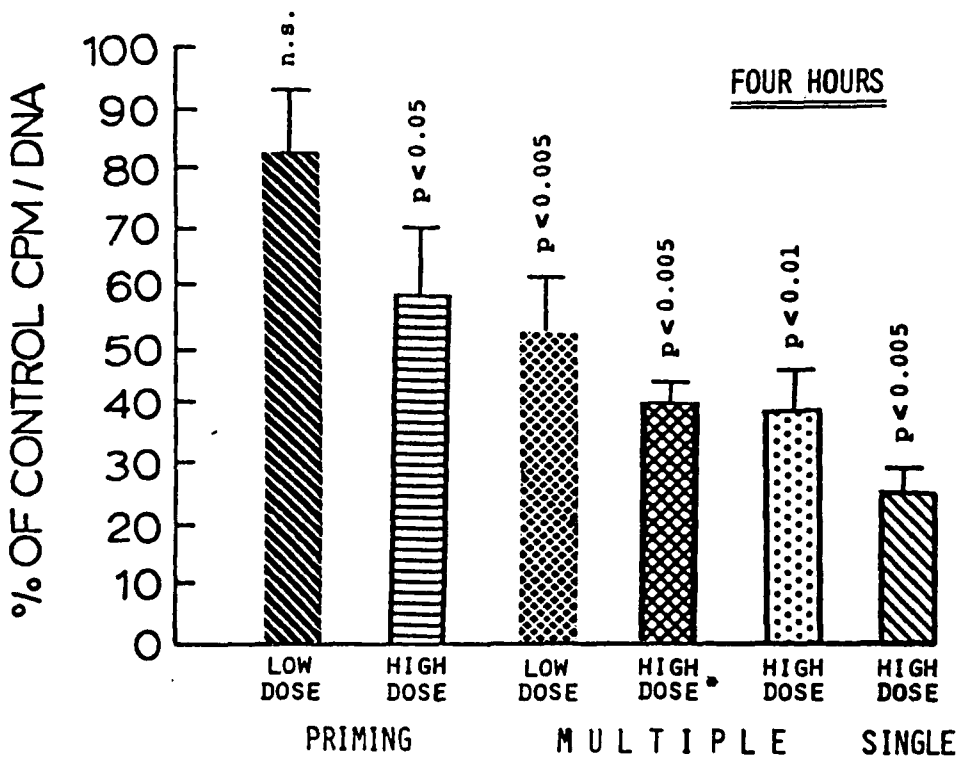


Fig. 3-3 Diagram of organ culture set-up.



LOW DOSE : 1 NG/GBW
HIGH DOSE : 10 NG/GBW
HIGH DOSE* : 50 NG/GBW

Fig. 3-4 Comparison of percent of ^{3}H-thymidine incorporation into mucosa of ovariectomized mice at 4 hours after the last injection of 17β-E_2 as compared to control (100%) between various treatment doses and regimens.

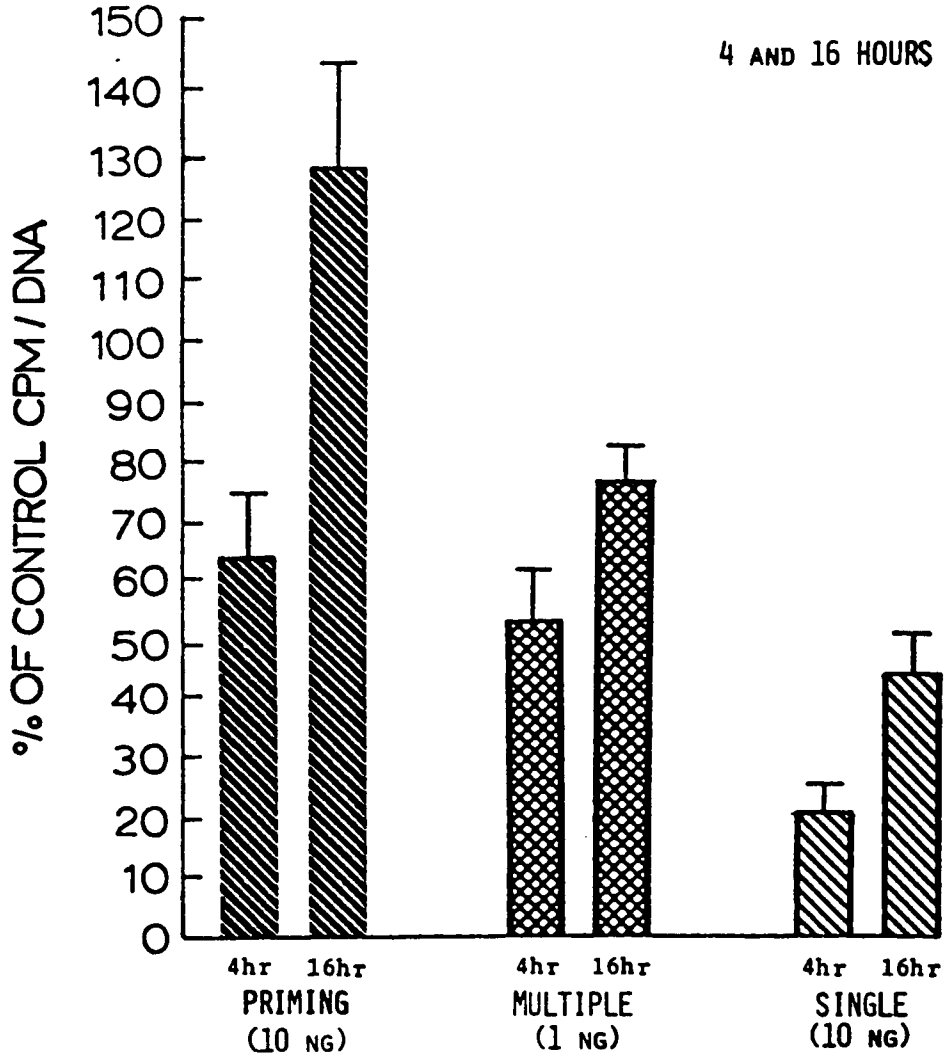


Fig. 3-5 Comparison of percent of ^{3}H-thymidine incorporation into mucosa of ovariectomized mice at 4 and 16 hours after the last 17β-E_2 injection as compared to control (100%) between three regimens.

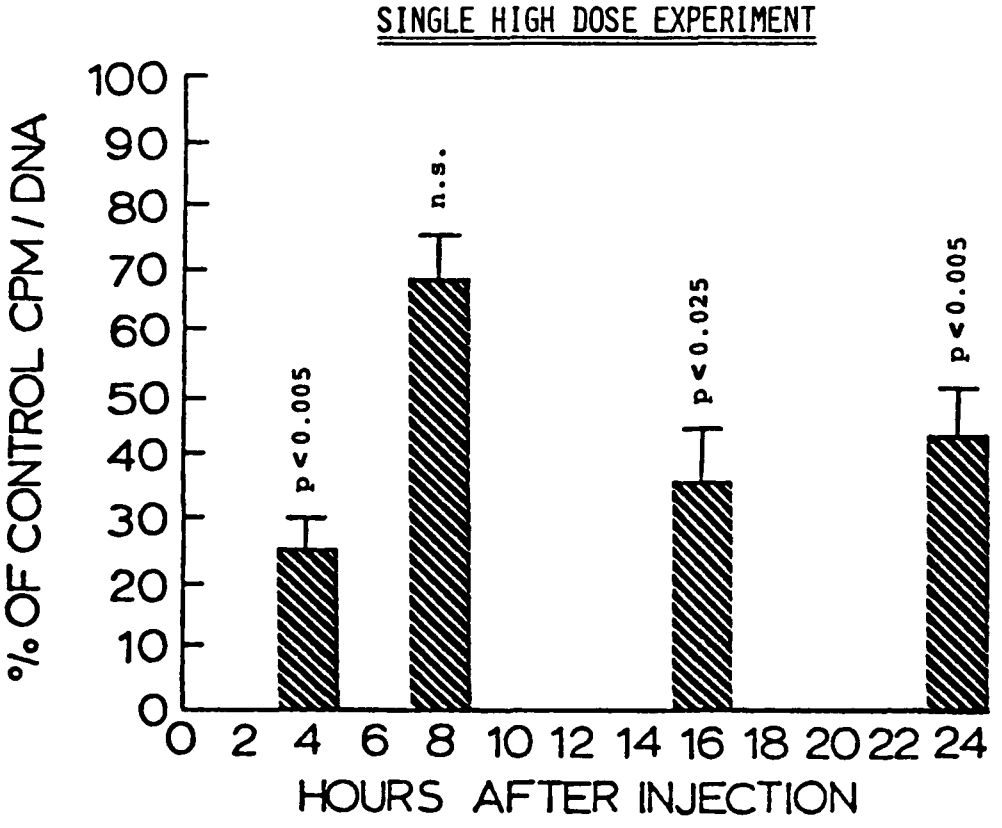


Fig. 3-6 Comparison of percent of ^{3}H-thymidine incorporation into mucosa of ovariectomized mice at times after a single 10ng per gram body weight injection of 17β-E_2 as compared to control (100%).

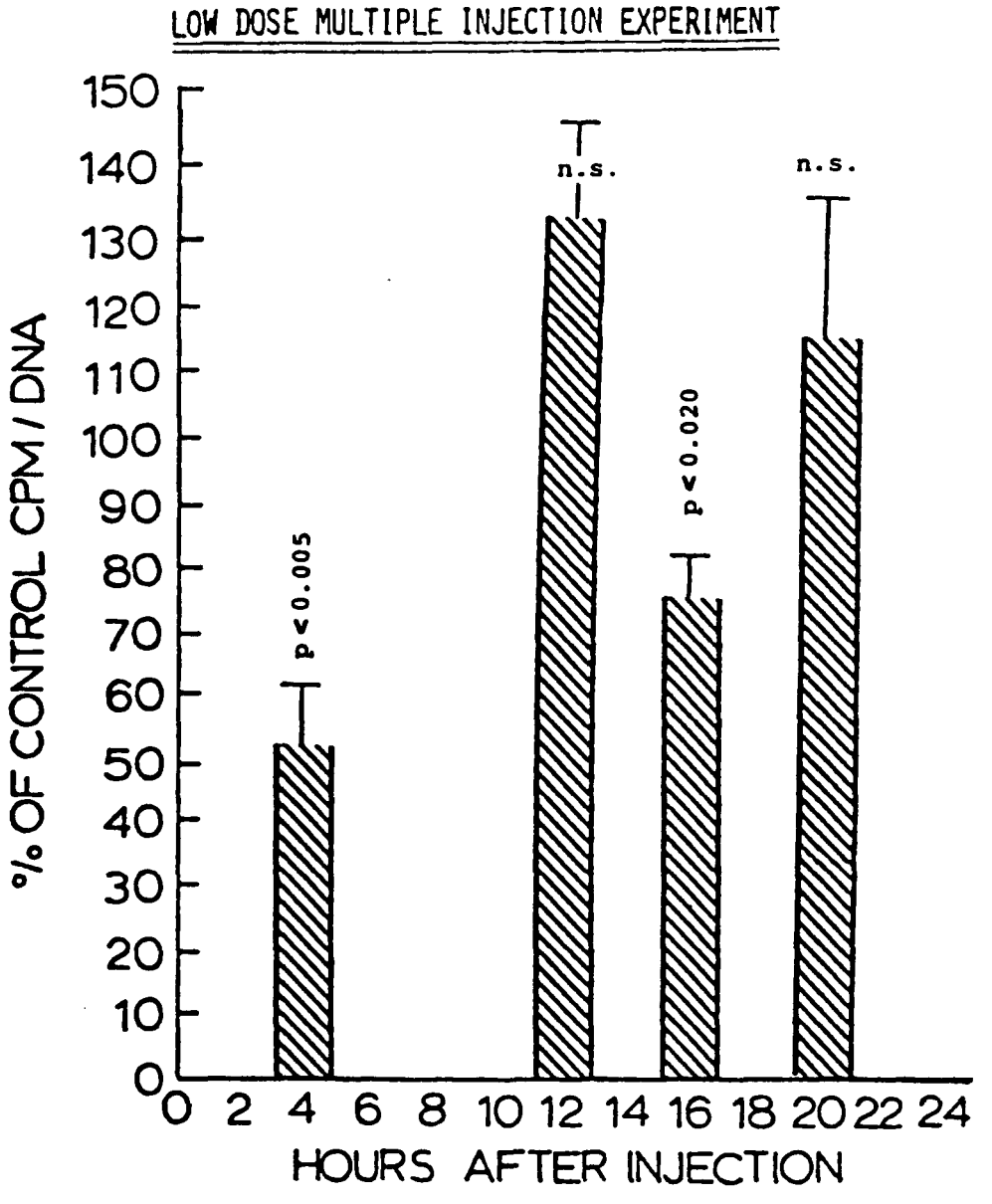


Fig. 3-7 Comparison of percent of ^{3}H-thymidine incorporation into mucosa of ovariectomized mice at times after the last injection (see Fig. 3-1) of 1 ng per gram body weight 17β-E_2 as compared to control (100%).

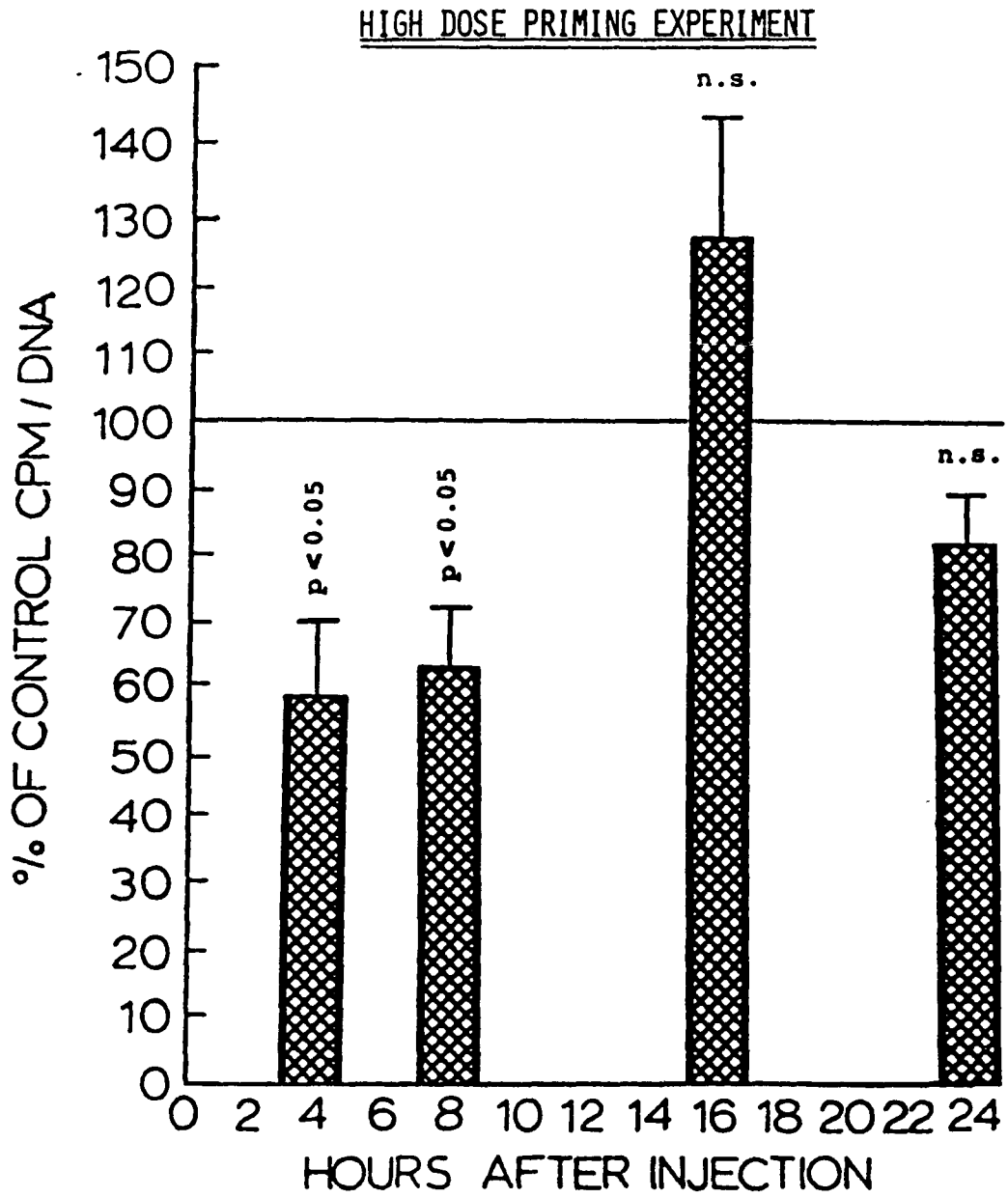


Fig. 3-8 Comparison of percent of ^{3}H-thymidine incorporation into mucosa of ovariectomized mice at times after the last injection (see Fig. 3-2) of 10 ng per gram body weight 17β-E_2 as compared to control (100%).

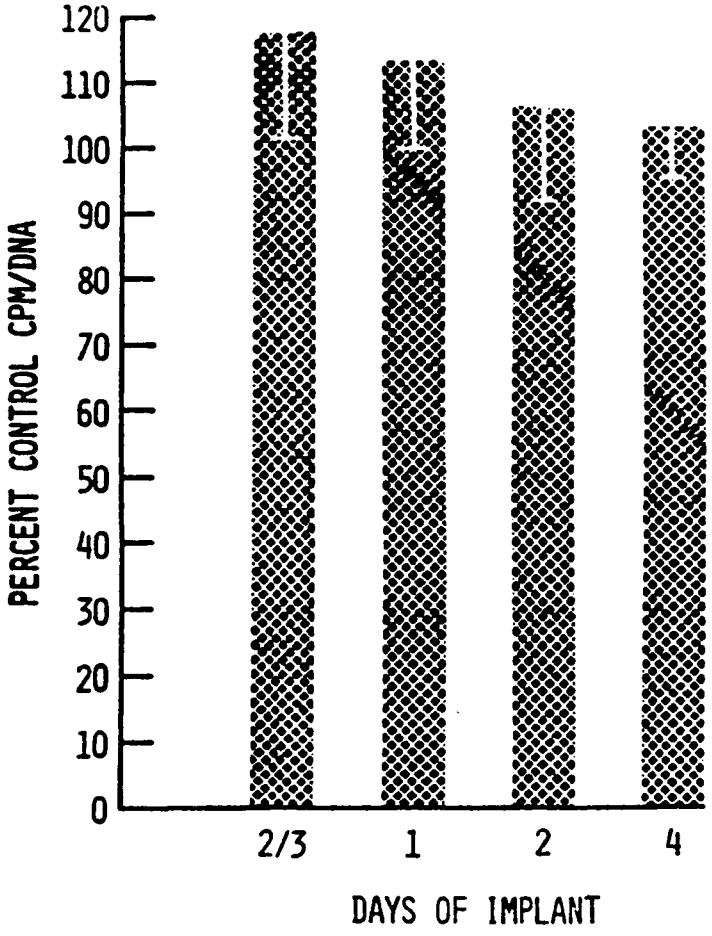


Fig. 3-9 Comparison of percent of ^{3}H-thymidine incorporation into mucosa of ovariectomized mice at times after subcutaneous estrogen capsule implantation as compared to control (100%).

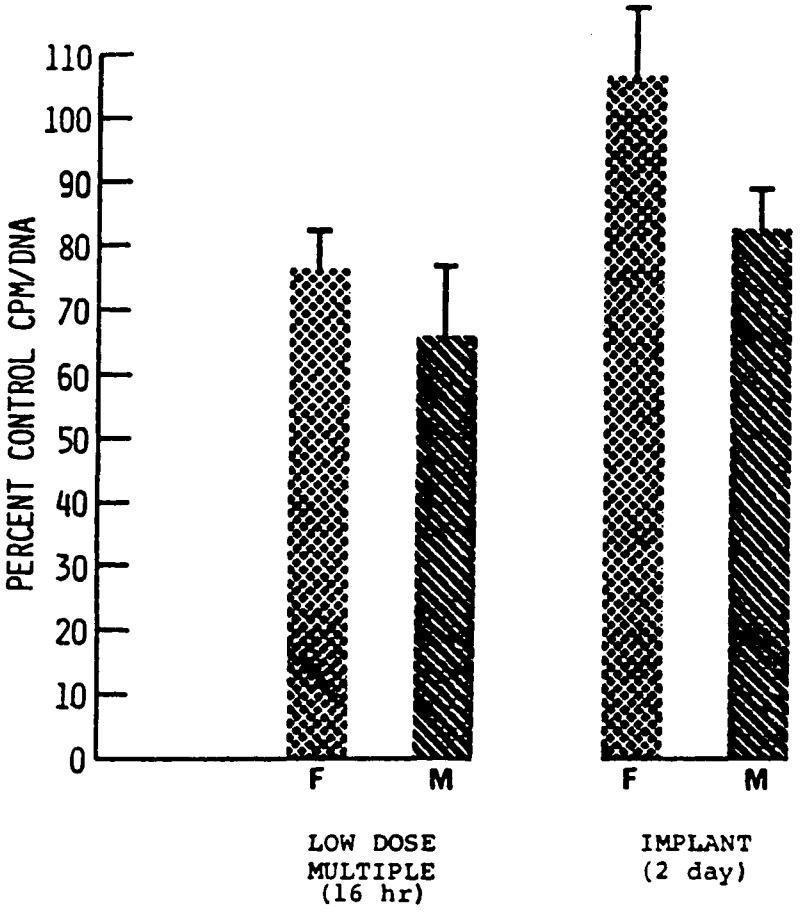
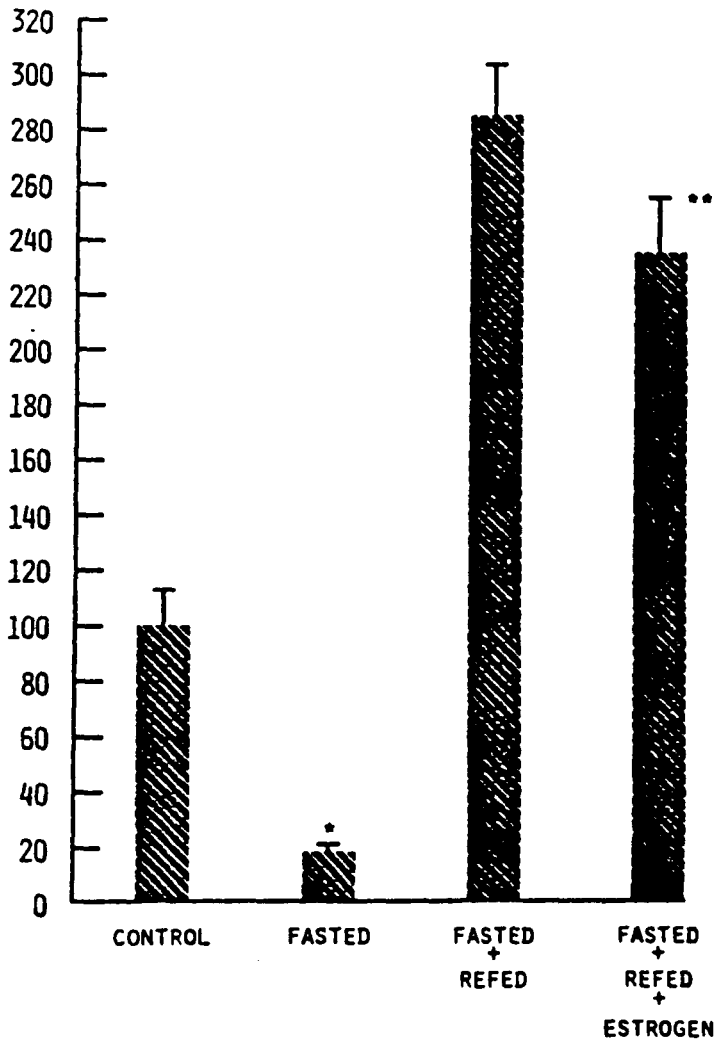


Fig. 3-10 Comparison of percent of ^{3}H-thymidine incorporation into mucosa between intact male and ovariectomized female mice as compared to control (100%) after two different estrogen treatment regimens.



* $p < 0.005$ AS COMPARED TO CONTROL

** $p < 0.05$ AS COMPARED TO THE FASTED-REFED GROUP

Fig. 3-11 ^{3}H-thymidine incorporation into mucosa of fasted, refed and refed estrogen-treated ovariectomized mice as compared to control.

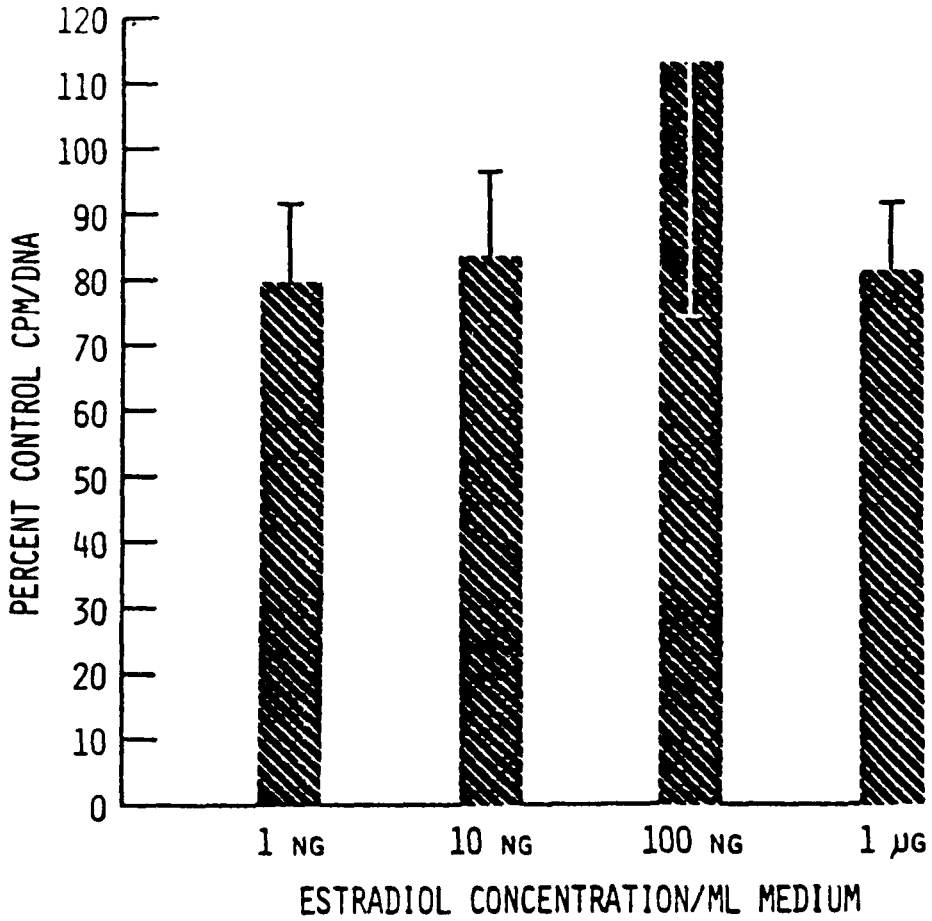


Fig. 3-12 Comparison of percent of ^{3}H-thymidine incorporation into mucosa of descending colonic explants from an immature female rabbit cultured for 18 hours in the presence of ^{3}H-thymidine and one of four different concentrations of 17β-E_2.

Section 4

EXPERIMENT:

AN AUTORADIOGRAPHIC CELL KINETIC STUDY OF COLONIC EPITHELIUM FROM OVARIECTOMIZED ESTROGENIZED MICE

MATERIALS AND METHODS

Twelve young adult virgin female CF-1 mice of 10-12 weeks of age (Charles River Breeding Laboratories, Wilmington, Mass.) were used. Approximately one week after arrival, the mice were ovariectomized bilaterally by a dorsal approach. Three or more weeks after the operation, the animals were divided into three groups of four animals each: (1) untreated (used as controls); (2) injected once with 10 ng per gram body weight (gbw) of 17β-estradiol (17β-E_2) and killed 4 hours later; and (3) injected once daily with 10 ng/gbw of 17β-E_2 for 4 days and killed 4 hours after the last injection. The details of the experiment are similar to what has been described previously.

All the mice were killed between 9:00 and 11:00 h. One hour before killing they were injected with 1 μCi/gbw of ^{3}H-thymidine (New England Nuclear, Boston, Mass.; specific activity 20 Ci per mmole). The distal portion of the descending colon, approximately 5 mm in length, was removed and placed in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) overnight in the cold.

Following fixation, the colonic tissue was dehydrated in graded concentrations of acetone and embedded in Epon 812 (Ladd Research Industries, Burlington, Vt.). Semi-thin (1 μm thick) sections were serially cut, and prestained with the periodic acid-Schiff technique and iron hematoxylin and processed for autoradiography as previously described in detail.

The criteria used for selection of crypts and the methods of cell population analysis have also been detailed previously. In this experiment, the greatest overall variance was also found to be among the observations within each animal. This finding supports the decision to use a large number of crypt columns for the analysis. As noted previously, a nested analysis of variance was employed which took into consideration the variation of observations within each animal, the variation within animals within each group and the variation between groups. This statistical analysis was carried out using the computer system of the City University of New York under the guidance of and with the technical assistance of Dr. Harry Smith and his colleagues in the Department of Biostatistics at this institution. In addition to the analysis of variance, a Student's t-test was also employed where noted.

RESULTS

Various parameters of cell population kinetics of the colonic crypt analyzed in the three groups of ovariectomized mice (one untreated and 2 treated with estrogen) are listed in table 4-1.

The crypt size, i.e. the number of epithelial cells lining an average crypt column, appeared to be somewhat reduced after single (6.3%) or multiple (12.2%) injections of estrogen as compared to the untreated ovariectomized mice, but the differences were not statistically significant when using the nested analysis of variance. A Student's t-test analysis, however, indicated that these were significant changes (see appendix C). In the mice given a single injection of estrogen, the decrease in the crypt size was notable due to a reduction in both the columnar (16.6% decrease) and mucous (11.5% decrease) cell populations, while the vacuolated cell population decreased by only 2.4%. In analyzing the reduction of crypt size in the mice given multiple injections of estrogen, it was found that columnar cells decreased by 32.3% vacuolated cells by 8.8% and mucous cells by 4.1%. Therefore, the colonic crypts of the estrogen-treated mice contained proportionally more vacuolated cells and fewer columnar cells than colonic crypts of the ovariectomized controls. The percentage decrease in columnar cells was greater with multiple injections than with a single injection. When the two groups of estrogen-treated mice were compared, the crypt size of the multiple injection group was 6.3% less than the single injection group with the difference reflecting a decrease in cells belonging to the vacuolated-columnar cell line.

Estrogen treatment of ovariectomized mice caused a significant reduction in both the relative and absolute number of labeled cells in colonic crypts, as compared to the controls. The relative ($P < 0.001$) and absolute ($P < 0.005$) reduction in labeling was more significant after a single injection than the relative ($P < 0.005$) and absolute ($P < 0.025$) reduction in labeling that was observed after the multiple injection regimen.

The proliferative activity of vacuolated cells and mucous cells appeared to be more or less equally affected by estrogen treatment, no matter which regimen of estrogen treatment (single or multiple) was used.

TABLE 4-1

Cell population changes in crypts of ovariectomized estrogen-treated mice as compared to ovariectomized mice*

CELL POPULATION PER CRYPT COLUMN	UNTREATED (MEAN±S.E.M.)	SINGLE INJECTION (MEAN±S.E.M.)	MULTIPLE INJECTION (MEAN±S.E.M.)
TOTAL	27.43 ±0.39	25.69 ±0.29	24.07 ±0.27
COLUMNAR	4.89 ±0.15	4.08 ±0.15	3.31 ±0.11
VACUOLATED	18.20 ±0.29	17.77 ±0.26	16.60 ±0.25
MUCOUS	4.34 ±0.18	3.84 ±0.15	4.16 ±0.16
% COLUMNAR	17.67 ±0.53	15.70 ±0.52	13.76 ±0.49
% VACUOLATED	66.57 ±0.64	69.21 ±0.72	69.00 ±0.71
% MUCOUS	17.98 ±0.24	14.95 ±0.56	18.19 ±0.24
TOTAL LABELED	3.41 ±0.17	0.98 ±0.08^{y}	1.38 ±0.09^{z}
LABELED VACUOLATED	3.18 ±0.17	0.91 ±0.08^{y}	1.28 ±0.08^{z}
LABELED MUCOUS	0.23 ±0.04	0.07 ±0.02	0.10 ±0.02
% TOTAL LABELED	12.05 ±0.55	3.85 ±0.34^{x}	5.69 ±0.36^{y}
% LABELED VACUOLATED	11.27 ±0.54	3.59 ±0.32^{x}	5.27 ±0.35^{y}
% LABELED MUCOUS	0.78 ±0.14	0.26 ±0.08	0.42 ±0.10
LABELED VACUOLATED/ VACUOLATED+COLUMNAR**	15.41 ±0.64	4.20 ±0.37^{x}	6.36 ±0.41^{y}
LABELED MUCOUS/MUCOUS**	4.86 ±0.93	1.63 ±0.54	2.60 ±0.70

Note: P-value as compared to ovariectomized group: x = P<0.001, y = P<0.005, z = P<0.025

* Contrasts by nested analysis of variance.

** Expressed in percent form.

Section 5

DISCUSSION

UTERINE RESPONSE TO ESTROGEN TREATMENT

The uterotropic action of estrogen was clearly demonstrated in the present investigation by the changes in the wet and dry weights of uterine tubes taken from ovariectomized mice treated with various doses and regimens of estrogen. The uterine response to estrogen can in general be classified into two categories: an early imbibition response (Astwood, 1938; Szego and Roberts, 1953; Martin et al., 1973) and a later growth response (Martin et al., 1973). Immediately following an injection of estrogen, there is a dramatic increase in the water content (Astwood, 1938; Szego and Roberts, 1953; Martin et al., 1973) and hyperemia (Hechter et al., 1940; Szego and Roberts, 1953) of the murine uterus, which reaches a peak at about 4 hours after estrogen administration. The imbibition response is closely associated with the facts that estrogen dilates the uterine vasculature (Holden, 1939; Szego and Roberts, 1953) and causes an increased uterine capillary permeability (Hechter et al., 1941), both of which seem to be related to uterine histamine release, (Szego, 1965) and an increase in the number of uterine eosinophils, which have been shown to specifically bind 17β-estradiol (Tchernitchin, 1972). The early imbibition response, therefore, does not seem to be mediated by the genome and, thus, differs from the genome-mediated growth response which occurs at a later time (climaxing 24-36 hours after the administration of estrogen).

The uterine growth response following estrogen administration entails a sequence of events involving the synthesis of RNA, protein and DNA and includes both hypertrophic and hyperplastic changes. There is evidence that the early imbibition response involves different estrogen receptor sites, or binding systems, than the later growth response (Tchernitchin, 1972). It is noteworthy that various estrogen compounds elicit different degrees of imbibition and growth response: 17β-estradiol elicits the most dramatic growth response in target organs of all the natural estrogens, while estriol elicits the weakest growth reaction but has the greatest effect on uterine water imbibition (Szego and Roberts, 1953; Hisaw, 1953; Tchernitchin, 1972).

The relative significance of the imbibition and growth responses following various estrogen treatments were assessed in a crude way by determining the changes in the wet and dry weights of the uteri. After a single injection of 17β-E_2, the uterine dry weight remained essentially the same up to 24 hours, whereas the wet weight increased steadily. This indicated that the imbibition response played the predominant role in uterine weight gain during this period. Following multiple injections of 17β-E_2, however, the greatest increase in the uterine water content occurred early (∿4 hours) and then tapered off, while the dry weight gain, which is a rough indicator of the growth response, was greatest between 12 and 20 hours after the last injection. The uterine response in the priming regimen was similar to that seen in the multiple injection regimen,

but was less obvious. When employing the estrogen implants, i.e. continuous infusion, the wet weights of the uteri increased most dramatically after one day and again after 4 days, but from 4 to 8 days, although a sizeable increase was seen, it was much less than seen during the first 4 days of implantation. Dry weight was seen to increase only slightly after 1 day but was almost doubled after 4 days. After 8 days of implantation uterine dry weight seemed to plateau or even slightly decline.

The uterine response to estrogen also seemed to be dose-related. In this investigation, the maximal response was obtained after a high dose (10 ng per gbw) with the response being somewhat less with a low (1 ng per gbw) or very high (50 ng per gbw) dose.

Although Parkes (1937, 1943) studied absorption rates and durations of action of various estrogen compounds and their esters, and Hisaw (1959) investigated the comparative effectiveness of various estrogens on uterine fluid imbibition and growth, a systematic investigation of uterine response to the different doses, regimens and vehicles of estrogen has yet to be carried out.

EFFECT OF ESTROGEN ON COLONIC EPITHELIAL CELL PROLIFERATION

In her review of hormonal effects on the cell cycle, Epifanova (1971) concluded that not only does estrogen stimulate cell proliferation in target organs, but in non-target ones as well. The present investigation revealed, however, that in ovariectomized mice, 17β-estradiol inhibited the incorporation of ^{3}H-thymidine into colonic epithelial cells, most notably at 4 hours after the last or only injection of every estrogen treatment regimen employed. This surprising finding in this non-target organ was confirmed by autoradiographs of the colon in which the number of ^{3}H-thymidine-labeled cells (per crypt column) was significantly reduced at 4 hours after the only or last injection of the single and multiple injection regimen, respectively.

In trying to analyze the mode of action of estrogen on the colonic epithelium, several facts are worthy of consideration. In the present investigation 17β-estradiol was dissolved in a water-alcohol vehicle so that relatively rapid absorption and excretion of the steroid would occur, unless it was specifically bound. As for its metabolism, estrogen is metabolized in the intestine a short time after its administration (Collins et al., 1970; Amland and Stϕa, 1975; Aldercreutz et al., 1976; Honjo et al., 1976; Collins et al., 1976), however, the function of these metabolites is unknown. On the other hand, a major group of urinary estrogen metabolites, the catechol estrogens, has been suggested to have an important role in

hypothalamic-pituitary regulatory functions (Paul and Axelrod, 1977). One of the well known effects of the neurohypophyseal hormones is arteriolar vasoconstriction, however, recently the vasoconstrictor response, due to either neurohypophyseal hormones or catecholamines, was shown to be enhanced in the mesenteric arterioles of estrogenized male and female rats (Altura, 1975). At first this effect may seem contradictory to the vasodilatory effect which estrogen has on the uterine vasculature, but they may actually be complementary if one considers the following hypothetical schema: with an intrauterine hypervolemia immediately following estrogen treatment, a transient general hypovolemia and splanchnic vasoconstriction may quickly follow. In this situation, some of the blood (which normally flows to the intestinal mucosa, but is presented with vasoconstriction) may be shunted away via mucosal arteriovenous anastomoses, which have been described in rat, rabbit, dog and man (Grayson, 1951; Grim, 1963). In other words, if this sequence of events was indeed true, less ^{3}H-thymidine would reach the mucosa and the present findings may thusly be explained.

To test the above hypothesis, determining the tritium content in the acid-soluble fraction of the mucosa would be of help, since it would represent the relative amount of thymidine in the intracellular (uptake) and intercellular components of the intestinal mucosa. The present data demonstrated that the radioactivity in the acid-soluble fraction of the mucosa varied with experimental conditions, and furthermore, it varied more or less inversely with the amount of

^{3}H-thymidine incorporated into the DNA of proliferating cells. Hence, the uptake of ^{3}H-thymidine by the colonic epithelium did not seem to be adversely affected by a circulatory adjustment, which may occur after estrogen treatment. Alternatively, the decrease in ^{3}H-thymidine incorporation might occur as a result of increased degradation of thymidine (Miyamoto and Tesayama, 1971). However, if this were the case after estrogen treatment, both the uptake and incorporation of ^{3}H-thymidine would have been decreased.

Since ^{3}H-thymidine was available in the colonic mucosa, we turn our attention to several other possible explanations for the decreased incorporation of ^{3}H-thymidine into colonic epithelial cells after estrogen treatment: (1) suppression of DNA synthesis in cells in the S phase; (2) promotion of the passage of cells through the S phase; (3) prevention of the passage of cells from the G_1 to the S phase; and (4) the combined effect of any of the above.

Although DNA synthesis can be specifically inhibited by some antimetabolites (Dethlefsen and Riley, 1973; Der et al., 1975; Margolis et al., 1971; among others), no hormone has been shown to possess such a mode of action. Analysis of autoradiographs prepared from the colon of ovariectomized mice revealed that the number of ^{3}H-thymidine-labeled cells decreased after estrogen administration but the number of silver grains per labeled nucleus did not seem to differ between the treated and untreated animals. Therefore, it is unlikely that estrogen suppressed the DNA synthesis of cells in the

S phase.

Like testosterone (Tuohimaa and Niemi, 1968; Wright et al., 1972), estrogen has been shown to shorten the duration of the S phase (and of the cell cycle in general) in target as well as in non-target organs (see Epifanova, 1971). Galand et al. (1972), using the double-labeling method, demonstrated a substantial decrease in the duration of the S phase (and consequently the duration of cell cycle) in the duodenum, jejunum, ileum and colon of ovariectomized animals following estrogen treatment. Bullough (1946) showed an increase in the number of mitotic cells in the crypts of the intestines from intact female mice, as compared to the controls, 12 hours after estrogen administration. Hence, it is highly likely that under the influence of estrogen, colonic epithelial cells were hurried through the S phase. This may explain, at least in part, why ^{3}H-thymidine incorporation was decreased 4 hours after the estrogen treatment. However, the decrease in ^{3}H-thymidine incorporation was too great to be explained solely by the shortening of the S phase. This leads to the possibility of an estrogen-induced block in the transition of cells from the G_1 to the S phase of the proliferative cycle.

It has been demonstrated that in the uterine epithelium repeated injections of estrogen resulted in a decrease in ^{3}H-thymidine incorporation (Epifanova, 1967; Lee, 1972; Stormshak et al., 1976). This effect was interpreted as an estrogen-induced G_1-S block. In addition to this, another steroid, hydrocortisone, has been shcwn to

cause a G_1-S block in the epithelium of the mouse forestomach (Frankfurt, 1968). Thus, it seems probable that estrogen effected a G_1-S block in the colonic epithelium. If this in fact, is the case, one could explain, in conjunction with the shortening of the S phase, the decrease in ^{3}H-thymidine incorporation into the colonic mucosal.

Although additional experimental evidence is necessary to definitively establish a G_1-S block by estrogen, the existence of such a G_1-S block, and subsequent release, can also explain other findings in the present investigation. Following a single injection of estrogen (10 ng/gbw) the inhibition of ^{3}H-thymidine incorporation was sustained for at least 24 hours. However, recovery from the inhibition occurred by 16 hours in the priming regimen, and by 12 hours in the multiple injection regimen (in the latter, there was a second inhibition by 16 hours after the last injection). Such an early recovery from the inhibition of ^{3}H-thymidine incorporation in the priming or multiple injection experiment can be explained by a blockage and subsequent release of cells at the G_1-S transition. It is probable that the G_1-S block by estrogen may be progressively weakened in the colon by repeated injections of estrogen, which is reminiscent of a tachyphylactic effect. The latter is also evident in the relationship between the regimen of estrogen and the degree of inhibition of ^{3}H-thymidine incorporation at 4 and 16 hours after the (last) estrogen injection as shown in figures 3-4 and 3-5.

The question may arise as to whether the effects of estrogen

on the colonic epithelium, as discussed above, were due to the direct action of the steroid, or not. In an attempt to resolve this question, an organ culture study using rabbit colonic explants and four different estrogen concentrations was carried out. As figure 3-12 shows, this study did not seem to correlate with the in vivo experiments. In view of the possibility that an earlier effect may have actually been missed due to the culture time employed, no definitive conclusion can be made at this time. With regard to the possibility of my findings being due to an indirect effect of estrogen, it should be noted that evidence exists which indicates that estrogen can lower serum gastrin levels (Albinus et al., 1976; Lichtenberger et al., 1976) and, if you will recall, gastrin stimulates cell proliferation in the intestinal mucosa (Williams et al., 1972; Pansu, 1974; Johnson and Guthrie, 1974; Johnson et al., 1975; Mak and Chang, 1976). Thus, there is a possibility that the present estrogenic effect on the colon is mediated via gastrin, although no one has yet demonstrated gastrin's activity in the mouse. Further studies are clearly indicated in this regard.

No matter what the mode of action of estrogen is, it has been shown to inhibit colonic epithelial cell proliferation, not only in the female, but in the male mouse as well, and not only did it inhibit proliferation in the normally active colonic epithelium, but when proliferative activity was enhanced or induced (by refeeding fasted animals).

COMPARTMENTAL ANALYSIS AND THE EFFECT OF ESTROGEN ON COLONIC EPITHELIAL CELL DIFFERENTIATION

In the descending colon of the mouse, a compartmental analysis of proliferation and differentiation of epithelial cells in the crypt has been made possible by the demonstration that the main cell line consists of proliferating (vacuolated) cells and differentiated (columnar) cells, which can be separated on a morphological and topographical basis (Chang and Leblond, 1971a). In such analysis, the following assumptions are inevitable: (1) epithelial cells migrating out of the crypt have differentiated to the same degree; (2) the rate of migration of epithelial cells out of the crypt is not significantly different from time to time; and (3) in spite of the steady-state balance between cell production and cell migration out of the crypt, the crypt is a dynamic structure and its cell population dynamics are to some extent modulated by the microenvironment, thus, transient changes in cell populations may occur. There is evidence to support assumptions (1) and (3) (Chang and Leblond, 1971a; Chang, 1971), but the rate of migration of cells out of crypt is known to vary from time to time (Chang, 1971). Nevertheless, the present compartmental analysis should not be noticeably affected by such a variable.

One of the problems inherent in this compartmental analysis, however, concerns a group of epithelial cells located in the transitional zone between the definitive vacuolated and the columnar cells along the cryptal wall (Chang and Leblond, 1971a). These epithelial cells still contain a few vacuoles which are small in size, and so, they have

been classified as vacuolated cells in the present study. However, these cells are most likely transforming vacuolated cells which have passed through their terminal mitoses, and with loss of the remaining vacuoles they will become the fully differentiated columnar cells. Since the number of these transitional cells is very small (Chang and Leblond, 1971a), their classification as vacuolated cells was not expected to affect the outcome of the present investigation.

The effect of ovarian hormones on the colonic crypt can be assessed in ovariectomized animals as compared to intact ones. Three weeks after bilateral ovariectomy, the colonic crypt appeared to reach a new steady-state, which was characterized by a smaller crypt size (total cells per crypt column), a decrease in both the relative and absolute number of differentiated cells and an increase in the relative number of proliferating cells. The new steady-state status of the crypt in the ovariectomized animal obviously had an intrinsic capacity to maintain proliferation and differentiation of epithelial cells, but the maintenance of the normal population size of differentiated cells, as well as the crypt size, depended upon the ovarian hormones. On the other hand, the relative increase in the proliferative activity in the crypt of the ovariectomized animal can be explained by (1) deprivation of estrogen (recall that estrogen has been shown to inhibit ^{3}H-thymidine incorporation into colonic epithelium) and (2) shrinkage of the differentiated compartment of the crypt which may control the production of cells in the crypt by a local negative feed-back mechanism (chalone?).

The sole effect of estrogen on proliferation and differentiation

of colonic epithelial cells can be investigated by giving estrogen to ovariectomized animals and employing compartmental analysis on the colonic crypts in autoradiographs. As noted previously, estrogen decreased the number of epithelial cells in DNA synthesis in both the single and multiple injection regimens. Moreover, after estrogen treatment, the number of differentiated columnar cells decreased; consequently, the relative number of proliferative vacuolated cells increased. A similar decrease was also noted in the mucous cell population. These data lead us to the conclusion that estrogen does not promote differentiation of epithelial cells in colonic crypts.

ROLE OF OVARIAN HORMONES ON COLONIC EPITHELIAL CELLS IN INTACT FEMALE MICE

The compartmental analysis of colonic crypts in mice during the estrous cycle supports, at least in part, the contention of Chang (1971) that the colonic crypt is a dynamic structure in which the crypt size, the rate of cell production, the rate of cell transformation from a proliferative to a differentiated state, the rate of migration of cells out of crypt and the rate of extrusion of cells from the surface epithelium may vary with the changes in the microenvironment of crypt. The regulation of cell dynamics in the colonic crypt by microenvironmental factors appears to be extremely complex due to the interactions of multiple factors or modulators.

It should be recalled that the duration of the various phases of the cell cycle may also vary according to microenvironmental factors, including hormones (Epifanova, 1971). Galand et al., (1967) have demonstrated that the duration of S phase was shortened in ovariectomized mice treated with estrogen as compared to untreated ones. Hence, it is highly probable that the cell cycle parameters, and so the cell cycle time, may vary from stage to stage during estrous cycle, depending upon the concentrations of the ovarian hormones in the blood.

Another complication to the present analysis is that so far there has been no investigation into the blood levels of 17β-estradiol during the estrous cycle of the mouse. In rat, however, estrogen blood levels have been determined in ovarian venous blood (Hori et al., 1968; Miyake,

1968; Yoshinaga et al., 1969; Shaikh, 1971) and in peripheral blood (Brown-Grant, 1970; Horikoshi and Suzuki, 1974; Dupon and Kim, 1973; Hashimoto et al., 1968; Butcher et al., 1974). The studies done by Horikoshi and Suzuki (1974), who measured the peripheral blood estrogen of adult cycling rats by radioimmunoassay (RIA), and by Miyake (1968), who determined the estrogen levels in ovarian venous blood using the modified vaginal tetrazolium assay of Martin (1960) were in good agreement: the peak estrogen level was observed to occur in the morning of proestrus.

Progesterone levels were also determined in rats by Miyake (1968), using a combination of thin-layer and gas-liquid chromatographic methods, and by Horikoshi and Suzuki (1974) using RIA. These studies were in accord, finding the highest progesterone level late on the day of proestrus and a second small peak late in the stage following estrus. Michael (1976), too, measured blood progesterone levels, but in the cycling mouse. Her findings paralleled those of the two former progesterone studies. Thus, it seems that the progesterone levels vary similarly in the mouse and rat during the sex cycle. It seems likely, then, that estrogen variation during the estrous cycle will also be similar in both animals. For the sake of uniformity, then, I will rely on the data from Horikoshi and Suzuki (1974) and Miyake (1968).

A composite graphic presentation of 17β-estradiol and progesterone levels during the estrous cycle, using the data obtained from Horikoshi and Suzuki (1974) and Miyake (1968), is shown in figure 5-1, together with the changes in the size of the vacuolated, columnar and mucous cell

populations. A precise temporal notation was intentionally omitted from this and all other figures relating to the estrous cycle since, in the course of the present investigation, I have confirmed the previous finding that the stages of the cycle are of unequal durations and that each stage may itself vary in its duration (Bertalanffy and Lau, 1963). Table 5-1b indicates the changes in the parameters shown in table 5-1a during the transition from one stage of the sex cycle to the next. On the basis of these data, I have attempted to correlate changes in the estrogen and progesterone levels to changes in the various cell populations of the colon during the estrous cycle: the intention is to define what appears to be the role that the ovarian hormones play in modulating the colonic epithelial cell kinetics during the cycle.

A glance at figure 5-1 seems to indicate that a rise in the progesterone blood level is associated with an increase in the columnar and mucous cell populations, the mature cell cell populations, although further studies are required to definitively establish a cause and effect relationship. One may also speculate that other factors, such as luteinizing hormone, may influence the process of differentiation in the colonic epithelium.

The following is an attempt to analyze each transition from one

state of the estrous cycle to the next. In the D_1-D_2 transition, there was a concurrent increase in the blood estradiol level and in the relative number of vacuolated cells, together with a concurrent decrease in the blood progesterone level and in the relative number of columnar and mucous cells. This may indicate that under the influence of estrogen, vacuolated cells are hurried through the proliferative cycle, but the majority of the daughter cells remained as vacuolated cells in the absence of progesterone, the promoter of differentiation.

In the D_2-P transition, the blood estrogen level rose continuously and peaked in early proestrus, while the number of vacuolated cells (per crypt column) decreased slightly and the columnar cells increased slightly. Also in this transition, there was a concurrent increase in both the blood level of progesterone and the number of mucous cells. The slight decrease in the vacuolated cell population may have been a result of (1) the G_1-S block by estrogen as discussed previously, and (2) the transformation of vacuolated cells to columnar cells under the influence of progesterone. The significant increase seen in the number of mucous cells may have been due to a stimulatory effect of progesterone. According to Chang and Nadler (1975), mucous cells originate by transformation from poorly differentiated vacuolated cells (stem cells) in the lower portion of colonic crypt and then undergo an average of two mitotic cycles as mucous cells before they become non-dividing, mature goblet cells. Which mechanism (the transformation from vacuolated cells or the division of mucous cells) played the more important role for the increase in the number of mucous cells during this transition? Because the change in the labeled mucous cells in this transition was negligible, the transformation

from vacuolated to mucous cells appeared to play the more important role for this expansion of the mucous cell population. More supporting evidence that this was probably the case was that in spite of a moderate increase in the labeling index of vacuolated cells, the number of vacuolated cells decreased slightly. Since the increase in the number of columnar cells could not account for the total decrease and production (as seen from the increase in labeling index) in the number of vacuolated cells, transformation of vacuolated cells into mucous cells probably occurred.

In the P-E transition, the blood level of progesterone peaked in late proestrus, whereas the blood estrogen level fell steadily. The number of vacuolated cells decreased while the number of columnar cells increased (obviously due to the transformation of vacuolated cells to columnar cells) while under the influence of progesterone. A slight decrease in the size of the mucous cell population was also observed which may be attributed to the migration rate being greater than the rate of production. The slight increase in the labeling index of mucous cells might be related to the fact that the newly-transformed mucous cells, which were formed at the D_2-P transition, were in the 'prime' of their proliferative lives. The sustained higher labeling index of vacuolated cells might be related to (1) the release of cells previously blocked at the G_1-S transition, and (2) the triggering of proliferative activity of vacuolated cells by a local negative feed-back mechanism due to a significant decrease in the size of the mature columnar cell population (chalone?).

The E-M_1 transition was a relatively quiescent period with regard to both cell proliferation and differentiation. The blood levels of both estrogen and progesterone were relatively low and stable. A major change was a decrease in the number of mucous cells which may be attributed to the fact that the rate of production of mucous cells was exceeded by the rate of migration of mucous cells out of crypt. In support of this is the fact that both the labeling index of mucous cells and transformation of vacuolated cells to mucous cells appeared to be decreased.

During the M_1-M_2 transition, there was a second rather small progesterone peak, associated with the greatest increase in the number of mucous cells seen during any of the other transitions. As was previously suggested for the D_2-P mucous cell increase, this increase in the number of mucous cells can be attributed to both the promoted transformation from vacuolated to mucous cells and the mitoses of immature mucous cells. The blood estrogen level remained low through this period. The crypt size became smaller due to the decrease in the absolute numbers of vacuolated and columnar cells, which may account for the slight increase in the labeling index of vacuolated cells.

The M_2-D_1 transition was another relatively quiescent period in terms of cell proliferation and differentiation: the blood level of 17β-E_2 was at its basal level and the progesterone level declined.

In summarizing the above analyses and findings, the proliferation and differentiation of epithelial cells in the colonic crypt are modu-

lated by ovarian hormones. My analyses indicate that estrogen and progesterone have different modes of action on the colonic epithelium. Estrogen appears to modulate cell proliferation by shortening the duration of the DNA synthesis phase and of the cell cycle as a whole, and also by blocking the G_1-S transition.

Further analysis of the action of the ovarian hormones on colonic epithelial cells during the estrous cycle suggests that progesterone promotes cell differentiation. If we consider the fact that the estrogen blood level rises in the morning of proestrus followed by a progesterone rise that evening and correlate the effects of these two hormones in the colon, as described above, a cooperative relationship may be inferred. That is, the estrogen serves to push the cycling cells through the cycle and in addition keeps them from re-entering the S phase; therefore, it is entirely possible that at the time of progesterone secretion, the cells which were pushed through their terminal division, and in their 'critical' or 'dicho-' phase, would have an extended opportunity to be promoted to differentiate. The analysis also suggests that a high level of progesterone promotes differentiation of cells in the vacuolated columnar cell line, while only a moderate rise in the blood level of progesterone triggers the transformation of vacuolated cells into mucous cells. In addition, progesterone may to some extent stimulate mucous cell mitoses, though this may be a result of transformation, since the young mucous cells are mitotically active. With regard to the mucous cell transformation being more receptive to lower levels of progesterone than columnar cell transformation, it may be of interest to note that production, i.e., transformation and mitosis, of mucous cells occur in

the lower portion of the crypt, whereas columnar cells differentiate toward the top of the crypt. I raise this point in light of the fact that the mucosal arteriole courses parallel to the crypt toward the lumen and it seems more than likely that in its path it leaves behind a concentration gradient of the blood contents, with the most concentrated zone at the level of the crypt base. Though speculative, teleologically, it fits the picture as well, since the most mitotically active cell population (the 'stem cells') which is located in the base of the crypt, should have a nutritional advantage.

SPECULATIVE CORRELATIONS OF THE PRESENT FINDINGS WITH RESPECT TO PHYSIOLOGY AND EPIDEMIOLOGY

The present finding that the number of differentiated absorptive columnar cells in murine colon increased in the postovulatory period (under the influence of progesterone), may be of physiological significance. Although there are differences between the various segments of the intestine, it seems unlikely that the maturational effect of progesterone on absorptive columnar cell differentiation would be limited to the colon. If the number of absorptive columnar cells in the whole of the intestine increases in the presence of progesterone, as in the colon, the amount of water and nutrients absorbed from the gut would likewise increase. Such a sequence of events might actually be vantageous to the body in preparation for pregnancy. This speculation may be attested to by the well known fact that women experience a weight gain due to the retention of water during the secretory phase of the menstrual cycle.

I would also like to point out that a number of epidemiological surveys indicate that the incidence of gastrointestinal cancer is, in general, higher in males than in females, with the trend indicating a decline in the incidence rates among females, and with increasing, or at best maintenance of, incidence among males (Cutler and Devesa, 1973; Waterhouse, 1974; Silverberg and Holleb, 1975; Cutler and Young, 1975; Seidman et al., 1976; McLennan et al., 1977; Snyder et al., 1977). These studies have clearly shown that the most marked sex difference lies in the incidence of rectal cancer with cancer incidence in other intestinal

segments showing a much less marked or no sex difference at all. A report from Connecticut (Snyder et al., 1977) which compared cancer incidence rates from 1940 to 1973 concluded that,

"...male rates in the rectum have been about 40% higher than female rates across the years. In the sigmoid colon male rates were nearly the same as female rates in the first time period but have increased to about 20% higher than female rates. Male rates for descending colon cancer have also risen from being less than female rates to exceeding them by a third. Transverse colon male/female rate ratios have risen from less than 1.0 to a slightly more than 1.0, as have the ratios for ascending colon...Older men have had larger increases than older women at all sites."

Also of interest is the report indicating that there is a sex difference in the incidence of colorectal polyps as well, where males show a greater overall incidence as well as a greater multiplicity (Helwig, 1947; Blatt, 1961; Arminski, 1964; Stemmermann, 1973; Correa, 1975; Correa et al., 1977).

These sex differences may be related to the fact that estrogen usage in medicine has greatly increased in most developed countries over the last 20-30 years. This speculation is greatly supported by the report of Snyder et al., (1977), which indicated that prior to the '40's and '50's the intestinal cancer incidence rates among women almost paralleled those of men, however, in recent years the two rates are seen to diverge, with incidence in males continuing to rise and incidence among women achieving a plateau, or even slightly declining. Thus, the modulatory effect of estrogen on colonic epithelial cell proliferation may, in fact, be a protective one.

SPECULATIONS ON THE EFFECTS OF ESTROGEN ON TARGET AND NON-TARGET ORGANS

As mentioned earlier, Epifanova (1971) considers estrogen to be a mitogenic hormone in both target and non-target organs; in both cases estrogen has been shown to increase either the mitotic index or the ^{3}H-thymidine-labeling index and to shorten the cell cycle, although these effects were quantitatately much less in the non-target organs (see Epifanova, 1971). On the other hand, there is evidence to suggest that estrogen can inhibit cell proliferation in both target and non-target organs. It has been known for many years, though it has been somewhat ignored, that erythropoiesis in bone marrow is inhibited by the administration of estrogen to animals, eventually leading to anemia, atrophy of the marrow and in some cases death (Crafts, 1941; Vollmer and Gordon, 1941; Mirand et al., 1959; Dukes and Goldwasser, 1961; Mirand and Gordon, 1966; Gordon et al., 1968). It seems likely that these manifestations can be attributed to a decrease in blood cell production. The present investigation revealed that estrogen suppressed the incorporation of ^{3}H-thymidine into colonic epithelium, a finding which also suggests an inhibition of proliferative activity. As discussed previously, estrogen seemed to have exerted its effect on colonic epithelial cells by, 1. shortening the S phase of cells in the proliferative cell cycle and 2. blocking the cells at some point in the G_1-S transition; also recall that the degree of this estrogen modulation of the cell cycle depended on the dose and regimen of the estrogen administered. The

block phenomenon was also suggested to occur in the uterus after chronic estrogen treatment, as will be discussed shortly.

On the basis of what has been observed, in both the target and non-target organs after estrogen administration, the series of complex events which follows estrogen treatment in the target organ (uterus) may be given a new interpretation. The effects which estrogen has upon the uterus seems to involve three independent, but inter-related, responses: 1. an early water imbibition response, 2. a later growth response and 3. a modulation of the cycling cells (observed most readily with chronic treatment). The imbibition response may be a preparative phase for the later growth response and is closely related to histamine release, hyperemia, and vasodilation (Szego, 1965) as well as the increase in uterine eosinophils (Tchernitchen, 1972). It is interesting that specific estrogen binding has been demonstrated on the surface membrane of eosinophils (Tchernitchen and Tchernitchen, 1976) and endometrial cells (Pietras and Szego, 1977) shortly after estrogen administration, but no such binding was observed on intestinal epithelial cell surfaces (Pietras and Szego, 1977).

The later growth response is preceded by the induction of many biochemical processes (Ui and Mueller, 1963; Hamilton et al., 1968; Villee, 1974; Chan and O'Malley, 1976) with RNA and protein synthesis being among them. It is also well known that these biochemical processes are triggered as a result of the estrogen-cytosol receptor complex interacting with the genome (Toft and Gorski, 1966;

Hamilton, 1968; O'Malley and Schrader, 1976; Chan and O'Malley, 1976). With this in mind, the present study demonstrated through a series of experiments that neither RNA nor total protein of the colonic mucosa was affected by estrogen, thus, it seems likely that the effect that estrogen had on the colonic epithelium was not associated with the genome, besides, there is no evidence that the cytoplasmic estrogen receptor is present in the intestinal mucosa. The hyperplastic part of the growth response is seen much later than the onset of the biochemical processes and is generally assumed to be due to the recruitment of cells from a dormant (G_0) cell population (Epifanova, 1971). It is this genome-mediated recruitment of most of the G_0 cells which accounts for the many-fold increase in ^{3}H-thymidine labeled cells and mitotic figures in the uterine epithelium after acute estrogen treatment. It has been shown, however, that with continuous (chronic) estrogen administration there is a marked decrease in this uterine response (Epifanova, 1967; Lee, 1972; Martin et al., 1973; Stormshak et al., 1976), which Stormshak et al. (1976) have called a 'refractory' phenomenon.

It has been suggested that this 'refractory' state of the endometrium is due to a G_1 block or a return of cells to G_0. Of interest is the fact that this refractoriness was shown to be independent of the hormone's cytosol receptor availability. To re-emphasize, after acute estrogen administration, endometrial cells, the majority of which are normally in a resting state (G_0), are stimulated to enter the proliferative cycle. Hence, when additional

estrogen was given in the continuous treatment studies, a reduction in ^{3}H-thymidine incorporation was observed despite the fact that a majority of the cells were already in the proliferative cycle. This situation seems somewhat analgous to the circumstances of the present study. In the intestine, the majority, if not all, of the epithelial cells are continually cycling. After estrogen treatment the cells seem to be prevented from entering the S phase, i.e. a G_0 or G_1 block, just as was suggested to explain the uterine refractoriness. Thus, it is suggested that estrogen has another role, besides those classically appreciated in the uterus; it appears to be a modulator of cells in the proliferative cycle. Why this response is so apparent in the colon can be explained by the fact that its cells are continually proliferating, and so the effect is rapid and obvious. This explanation can also be applied to the hemopoietic system.

It is suggested, then, that what was referred to as a 'refractory' state is not that at all. As a matter of fact, Lee (1972) observed a second and third wave of mitoses and ^{3}H-thymidine incorporation at about one and three weeks after the primary wave in the uterus in her continuous estrogen administration experiments. It is interesting to recall that, in contrast to the uterus, a recovery from the suppression of ^{3}H-thymidine incorporation into colonic epithelium by estrogen took place 12-16 hours after the last of the multiple injections in the present study. Thus, it may be construed that once incurred, the blockage of cells in the G_1-S

transition by estrogen is much stronger in the 'target' organ than the 'non-target' organ. Perhaps this fact is related to the inherent need of the organ, i.e. the local factors prevail over the distal modulator. Thus, whichever force is stronger prevails. So, with acute estrogen treatment, the endometrial cells actively proliferate; the growth response, because of its preparative function, should overwhelm the modulatory effect of estrogen.

Thus, in brief, my analysis indicates that the action of estrogen in the target organ not only involves the classical imbibition and growth responses, but a more general action which modulates the cell cycle, whereas in the non-target organ, estrogen only modulates the cell cycle.

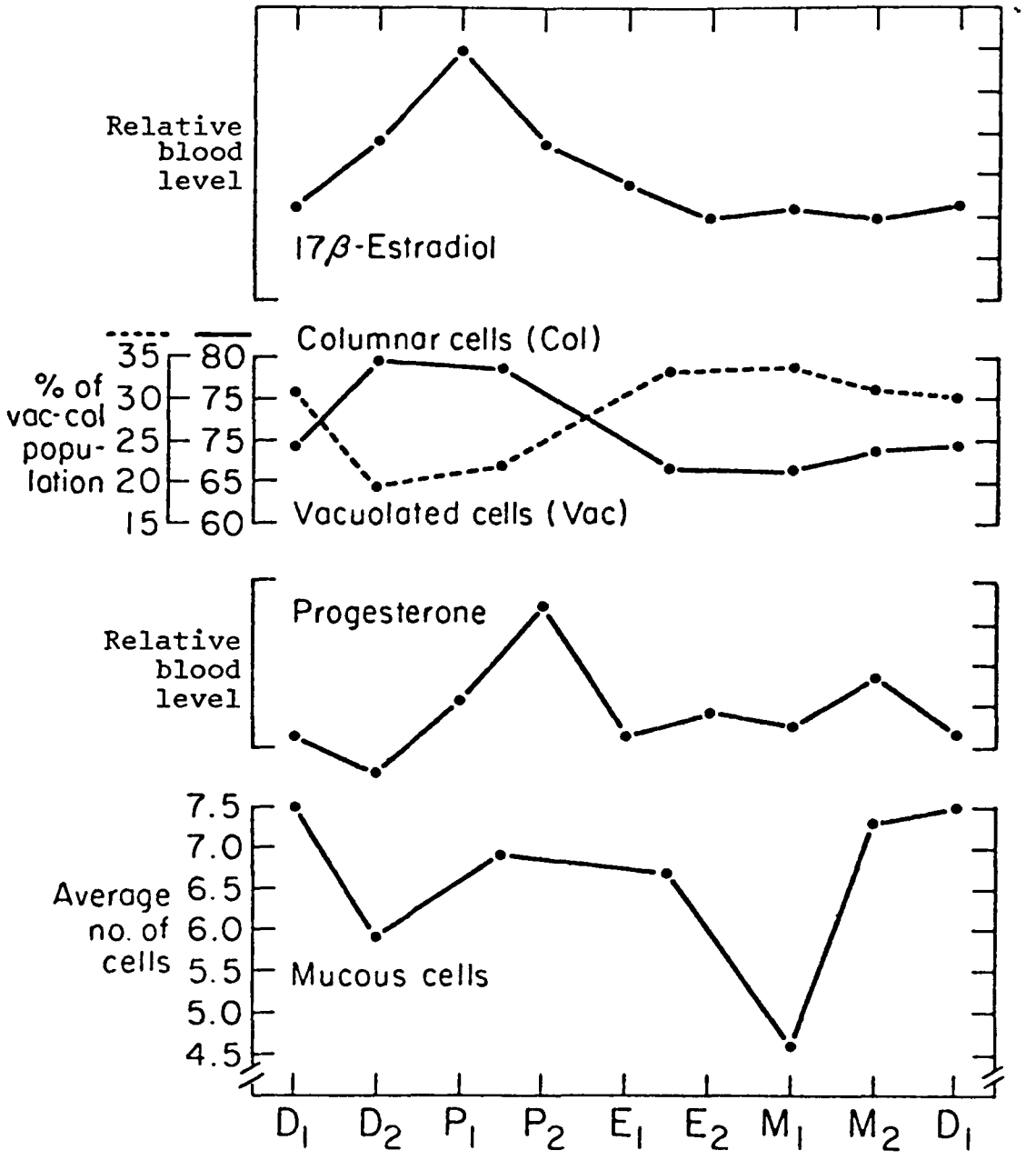
TABLE 5-1

Absolute and relative changes in 1. the vacuolated (vac), columnar (col) and mucous (muc) cell populations and 2. the labeling indices of both the vac-col and muc cell lines of the mouse colonic crypt during the estrous cycle and after ovariectomy.

a. STAGE	No. of VAC	No. of COL	No. of VAC+COL	% VAC VAC+COL	% COL VAC+COL	LABELED VAC VAC+COL	No. of MUC	LABELED MUC TOTAL MUC
D_1	19.5	8.7	28.2	69.1	30.9	9.1	7.5	4.7
D_2	22.9	5.4	28.3	80.9	19.1	8.8	5.9	5.0
P	21.9	6.1	28.0	78.2	21.8	10.2	6.9	5.5
E	19.6	9.8	29.4	66.7	33.3	10.2	6.7	7.2
M_1	20.3	10.4	30.7	66.1	33.9	8.0	4.6	4.3
M_2	18.7	8.6	27.3	68.5	31.5	9.6	7.3	7.2
MEAN	20.5	8.2	28.7	71.4	28.6	9.3	6.5	5.7
OVARIECTOMIZED	18.2	4.9	23.1	78.8	21.2	13.4	4.3	4.9

b. TRANSITION	% CHANGE				
	VAC	COL	LABELED VAC/VAC+COL	MUC	LABELED MUC/MUC
$D_1 \rightarrow D_2$	↑11.8	↓11.8	↓0.3	↓21.3	↑0.3
$D_2 \rightarrow P$	↓ 2.7	↑ 2.7	↑1.4	↑16.9	↑0.5
$P \rightarrow E$	↓11.5	↑11.5	---	↓ 2.9	↑1.7
$E \rightarrow M_1$	↓ 0.6	↑ 0.6	↓2.2	↓31.3	↓2.9
$M_1 \rightarrow M_2$	↑ 2.4	↓ 2.4	↑1.6	↑58.7	↑2.9
$M_2 \rightarrow D_1$	↑ 0.6	↓ 0.6	↓0.5	↓ 2.7	↓2.5
MEAN INTACT → OVARIECTOMIZED	↑ 7.3	↓ 7.3	↑4.1	↓33.8	↓1.5

Fig. 5-1 Comparison of the variations in 17β-estradiol blood concentration, percent of both columnar and vacuolated cells of the vacuolated-columnar cell population, progesterone blood concentration and the number of mucous cells during the estrous cycle. The hormonal levels indicated at each point was derived by averaging the all determinations within each stage of the rat sex cycle as reported by Miyake (1968) and Horikoshi and Suzuki (1974).


Relative blood level
17β-Estradiol
% of vac-col popu-lation
35
30
25
20
15
80
75
75
65
60
Columnar cells (Col)
Vacuolated cells (Vac)
Relative blood level
Progesterone
Average no. of cells
7.5
7.0
6.5
6.0
5.5
5.0
4.5
Mucous cells
D1 D2 P1 P2 E1 E2 M1 M2 D1

END OF TEXT

APPENDIX

APPENDIX A

Changes in cell populations of crypts in mice at the various stages of the estrous cycle.

CELL POPULATION PER CRYPT COLUMN	STAGE (MEAN±S.E.M.)					
	D_1	D_2	P	E	M_1	M_2
TOTAL	35.74±0.43	34.19±0.38	34.86±0.43	36.07±0.59	35.30±0.43	34.50±0.39
COLUMNAR	8.69±0.38	5.44±0.25	6.09±0.27	9.81±0.34	10.38±0.35	8.55±0.23
VACUOLATED	19.54±0.29	22.87±0.37	21.89±0.34	19.56±0.34	20.32±0.30	18.68±0.32
MUCOUS	7.51±0.22	5.88±0.20	6.88±0.27	6.70±0.26	4.60±0.18	7.27±0.21
% COLUMNAR	23.60±0.84	15.59±0.72	17.10±0.67	26.69±0.68	28.75±0.72	24.59±0.59
% VACUOLATED	55.49±0.87	66.90±0.77	63.65±0.96	55.09±0.86	58.16±0.80	54.17±0.73
% MUCOUS	20.90±0.54	17.51±0.59	19.25±0.64	18.23±0.63	13.08±0.49	21.24±0.63
TOTAL LABELED	2.98±0.15	2.77±0.13	3.16±0.18	3.61±0.18	2.81±0.21	3.15±0.17
LABELED VACUOLATED	2.61±0.15	2.45±0.13	2.82±0.16	3.07±0.17	2.58±0.20	2.66±0.16
LABELED MUCOUS	0.37±0.05	0.32±0.04	0.34±0.06	0.54±0.07	0.23±0.04	0.49±0.05
% TOTAL LABELED	8.30±0.42	8.19±0.38	9.21±0.50	9.62±0.46	7.63±0.54	9.10±0.48
% LABELED VACUOLATED	7.28±0.40	7.23±0.37	8.29±0.47	8.28±0.41	7.05±0.52	7.67±0.44
% LABELED MUCOUS	1.02±0.13	0.98±0.14	1.00±0.17	1.34±0.17	0.61±0.10	1.42±0.15
LABELED VACUOLATED/ VACUOLATED+COLUMNAR*	9.13±0.48	8.78±0.45	10.19±0.58	10.20±0.52	7.99±0.58	9.62±0.54
LABELED MUCOUS/ MUCOUS*	4.65±0.64	5.00±0.77	5.54±0.96	7.18±0.92	4.31±0.75	7.20±0.82

Note: D_1 = Early diestrus P = Proestrus M_1 = Early metestrus * = Expressed
D_2 = Late Diestrus E = Estrus M_2 = Late metestrus in percent form.

APPENDIX B

ESTROUS CYCLE STAGE CONTRASTS OF CELL POPULATIONS IN CRYPTS OF MICE[a]

CELL POPULATION PER CRYPT COLUMN	D_1/D_2	D_1/P	D_1/E	D_1/M_1	D_1/M_2	D_2/P	D_2/E	D_2/M_1	D_2/M_2	P/E	P/M_1	P/M_2	E/M_1	E/M_2	M_1/M_2
TOTAL	y	-	-	-	z	-	y	-	-	-	-	-	-	z	-
COLUMNAR	u	u	z	v	-	-	u	u	u	u	u	u	-	v	u
VACUOLATED	u	u	-	-	-	-	u	u	u	u	u	u	w	-	v
MUCOUS	u	-	x	u	-	v	x	u	u	-	u	-	u	-	u
% COLUMNAR	z	z	-	-	-	-	v	u	w	v	u	y	-	-	-
% VACUOLATED	u	u	-	z	-	z	u	u	u	u	u	u	w	-	u
% MUCOUS	u	-	v	u	-	z	-	u	u	-	u	z	u	v	u
TOTAL LABELED	-	-	x	-	-	-	u	-	-	-	-	-	w	-	-
LABELED VACUOLATED	-	-	z	-	-	-	v	-	-	-	-	-	-	-	-
LABELED MUCOUS	-	-	-	y	-	-	x	-	x	z	-	-	u	-	u
% TOTAL LABELED	-	-	z	-	-	-	y	-	-	-	z	-	w	-	z
% LABELED VACUOLATED	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
% LABELED MUCOUS	-	-	-	x	-	-	-	z	z	-	z	-	u	-	u
LABELED VACUOLATED/ VACUOLATED+COLUMNAR	-	-	-	-	-	-	z	-	-	w	-	-	w	-	z
LABELED MUCOUS/MUCOUS	-	-	z	-	x	-	-	-	-	-	-	-	x	-	w

Note: [a] Contrasts by Student's t-test (d.f.=318)

u = P<0.001 x = P<0.020
v = P<0.005 y = P<0.025
w = P<0.050 z = P<0.050

APPENDIX C

Cell population changes in crypts of ovariectomized estrogen-treated mice as compared to ovariectomized mice*

CELL POPULATION PER CRYPT COLUMN	EXPERIMENTAL GROUP (MEAN±S.E.M.) A (UNTREATED)	B (SINGLE INJECTION)	C (MULTIPLE INJECTION)	CONTRASTS A/B	A/C	B/C
TOTAL	27.43±0.39	25.69±0.29	24.07±0.27	v	v	v
COLUMNAR	4.89±0.15	4.08±0.15	3.31±0.11	v	v	v
VACUOLATED	18.20±0.29	17.77±0.26	16.60±0.25	n.s.	v	w
MUCOUS	4.34±0.18	3.84±0.15	4.16±0.16	z	n.s.	n.s.
% COLUMNAR	17.67±0.53	15.70±0.52	13.76±0.49	n.s.	n.s.	n.s.
% VACUOLATED	66.57±0.64	69.21±0.72	69.00±0.71	x	y	n.s.
% MUCOUS	17.98±0.24	14.95±0.56	18.19±0.24	n.s.	n.s.	y
TOTAL LABELED	3.41±0.17	0.98±0.08	1.38±0.09	v	v	w
LABELED VACUOLATED	3.18±0.17	0.91±0.08	1.28±0.08	v	v	w
LABELED MUCOUS	0.23±0.04	0.07±0.02	0.10±0.02	v	x	n.s.
% TOTAL LABELED	12.05±0.55	3.85±0.34	5.69±0.36	v	v	v
% LABELED VACUOLATED	11.27±0.54	3.59±0.32	5.27±0.35	v	v	v
% LABELED MUCOUS	0.78±0.14	0.26±0.08	0.42±0.10	w	z	n.s.
LABELED VACUOLATED/ VACUOLATED+COLUMNAR**	13.41±0.64	4.20±0.37	6.36±0.41	v	v	v
LABELED MUCOUS/MUCOUS**	4.86±0.93	1.63±0.54	2.60±0.70	w	n.s.	n.s.

Note:

v = $P < 0.001$
w = $P < 0.005$
x = $P < 0.010$
y = $P < 0.020$
z = $P < 0.050$
* Contrast by Student's t-test
** Expressed in percent form

APPENDIX D

Cell population changes in crypts of ovariectomized estrogen-treated mice as compared to intact mice*

CELL POPULATION PER CRYPT COLUMN	INTACT (MEAN±S.E.M.)	SINGLE INJECTION (MEAN±S.E.M.)	MULTIPLE INJECTION (MEAN±S.E.M.)
TOTAL	35.11±0.18	25.69 ±0.29[w]	24.07 ±0.27[w]
COLUMNAR	8.16±0.14	4.08 ±0.15[x]	3.31 ±0.11[w]
VACUOLATED	20.48±0.14	17.77 ±0.26[z]	16.60 ±0.25
MUCOUS	6.47±0.10	3.84 ±0.15[y]	4.16 ±0.16[z]
% COLUMNAR	22.30±0.30	15.70 ±0.52[z]	13.76 ±0.49[x]
% VACUOLATED	58.90±0.40	69.21 ±0.72[y]	69.00 ±0.71[y]
% MUCOUS	18.40±0.30	14.95 ±0.56	18.19 ±0.24
TOTAL LABELED	3.08±0.07	0.98 ±0.08[x]	1.38 ±0.09[y]
LABELED VACUOLATED	2.70±0.07	0.91 ±0.08[x]	1.28 ±0.08[z]
LABELED MUCOUS	0.38±0.02	0.07 ±0.02[x]	0.10 ±0.02[y]
% TOTAL LABELED	8.70±0.20	3.85 ±0.34[x]	5.69 ±0.36
% LABELED VACUOLATED	7.60±0.20	3.59 ±0.32[y]	5.27 ±0.35
% LABELED MUCOUS	1.10±0.10	0.26 ±0.08[x]	0.42 ±0.10[z]
LABELED VACUOLATED/ VACUOLATED+COLUMNAR**	9.30±0.20	4.20 ±0.37[x]	6.36 ±0.41
LABELED MUCOUS/MUCOUS**	5.70±0.30	1.63 ±0.54	2.60 ±0.70

Note: P-value as compared to intact group: w = P<0.001
x = P<0.005
y = P<0.010
z = P<0.025
* Contrasts by nested analysis of variance.
**Expressed in percent form.

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