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Age and Generation Cycle of Oral Epithelium

Nabil John Barakat
Loyola University Chicago

AGE AND GENERATION CYCLE OF ORAL EPITHELIUM

by

Nabil J. Barakat

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LIFE

Nabil John Barakat was born on March 25, 1940, in Cairo, Egypt. He graduated from Holy Family's Jesuit College in 1958. From 1958 to 1959 he attended the Faculty of Sciences, Cairo University.

He received his Bachelor of Dental Surgery degree from the School of Dentistry, Cairo University, in 1963. Upon graduation, he started a rotating internship at Cairo's University hospital.

In January 1965, he began a two-year Graduate program at Loyola University leading to a Master of Science degree in Oral Biology. Clinical training was done in the Oral Surgery Department under Dr. Nicholas Choukas. During his course of studies, he instructed Physiology and Pharmacology at the school.

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CHAPTER I

INTRODUCTION

In the adult mammal, large number of cells are produced and lost everyday. All cell populations increase in number during the period of growth of the organism. In some cell populations, the rate of proliferation is greater than that needed to sustain growth; this means that some cells are lost and replaced by newly formed cells. Such cell populations are called "Cell renewal systems" (Leblond 1956). However, upon completion of the growing period for a given renewal system a steady state is established; Cell (renewal) balances cell loss.

The first author to raise the question whether physiologic regeneration of epithelia actually slows with age was Ortiz Picon (1933), who studied the epidermis of abdomen and back of mice from birth to 33 months of age. At 6 months the rate of division was twice as high as at one month and remained higher than at one month for the entire period studied. In 1949, Bullough found the mitotic frequency in mouse epidermis to be considerably higher in middle-aged than in young animals even before they have reached full size. Marwah (1956) found the mitotic frequency in human gingiva to be 50% higher in the older age groups.

These findings contradict the common concept of aging, according to which bodily processes slow down and the regenerative capacity of the tissues diminishes with age. These observations would point to the possibility that the mechanisms by which normal cellular proliferation is controlled are reduced in the older individual. The incidence of malignancy is known to increase with age. Marwah states that one might suspect the full emancipation from control of the full fledged malignant cell is prepared for by the partial emancipation from control which occurs with age.

The present study will attempt to estimate the generation cycle of the oral epithelium in 600 day old mice. In particular this study employs the "direct graphic method" to study the renewal of cell population.

CHAPTER II

REVIEW OF THE LITERATURE

A. HISTOLOGY OF THE ORAL MUCOSA

Kutuzov and Sicher (1953) described in detail the dorsum of the mouse tongue.

Macroscopically, the dorsum of the tongue can be divided into 3 regions, each characterized by a specific type of papillae:

1. Anterior portion of simple conical papillae.
2. Intermolar eminence characterized by the giant papillae.
3. Posterior portion beset with the filiform papillae.

The stratified squamous epithelium of these three areas is keratinized.

Sonntag (1924) pointed out that though animals comprising the Order of Rodentia are very diversified the lingual characters do not differ very much specifically.

In 1959, Medak gave a detailed account of the oral epithelium of the mouse. He described it as having a lamina propria consisting of connective tissue cells and fibers, a basement membrane with collagenous and reticular fibers and a stratified squamous epithelium.

The basal layer of the epithelium has a single row of columnar cells. They are perpendicular to the basement membrane. The cytoplasmic nucleic acid contents are higher in these cells than the cells forming the rest of the epithelium. These nucleic acid contents decrease peripheralward.

The stratum spinosum has large polygonal cells with star-shaped outlines and intercellular connections. Keratohyaline granules are found in the cells of the stratum spinosum in later stages of cellular differentiation. In 1967, Cutright stated that the keratohyaline granules, which are eventually shed with maturing cells, do not contain any DNA component.

Medak (1956) described the stratum granulosum as having no residual cytoplasm and the unstained granules are of uniform size only adjacent to the compact keratin areas. There is evidence of phosphamidase activity in the keratohyaline granules. Cutright (1967) noted clear spaces in the nuclei of cells approaching the keratin layer and attributed it to the normal loss of their nuclear DNA in the course of keratinization.

Leblond (1956) described the morphological change in the basal cell layer. As these cells move outwards they change from columnar to polyhedral and finally to flattened, granule containing cells, which then transform abruptly into a homogenous glassy layer.

This, in turn, changes into soft keratin.

In 1967, Cutright stated that the normal oral epithelium has the property to achieve cytoplasmic differentiation while undergoing nuclear degeneration coupled with a mechanism by which the DNA released from keratinizing cells is reutilized by newly proliferating cells.

Normal histology of aging oral soft tissue:

Wentz el Al (1952) found an absence of age changes in the thickness of epithelium and length and configuration of the epithelial ridges of the gingiva. Klingsberg (1960) found no major alterations in the keratin layer and the overall epithelium with increasing age.

Marwah (1956) found some age changes in human gingiva as follow:

(1) a decrease in incidence of a granular layer (2) a decrease in incidence of parakeratosis and (3) a decrease in the association of a granular layer with parakeratosis, (4) an increase in cell density by a third. He attributed that increase in cell density to be due in part to lower water content of the tissue and in part to the smaller size of cells.

Burzinski et Al (1965) reported that there was no significant difference in the palatal tissues with aging between sexes of the same age groups, and in palatal tissues of 1-year-old and 4-year-old animals in non-collagenous to collagenous ratios.

B. CELL RENEWAL SYSTEMS

I. Renewal of Cell Population

In 1956, Leblond reviewed in detail the process of the renewal of cell population. He attributed the lack of interest of classical histologists in the subject to their belief that new cells were produced by "amitosis" as well as by "mitosis" and therefore the true rate of proliferation could not be estimated. However, in 1955, Ris pointed out that amitosis consists of a mere nuclear fragmentation, a process which generally precludes further proliferation. Thus mitosis is the only means of cell production, and the number of new cells formed in this tissue.

Howard (1953) divided the "generation cycle" into the following phases; S phase (DNA synthesis time) during which sufficient DNA is synthesized to form a new complement of chromosomes, G_2 phase which is an interval between S phase and beginning of mitosis, M phase in which mitosis occurs and a G_1 phase (resting phase) between termination of mitosis and commencement of the S phase. The period between the two mitosis is called an "interphase." In 1963, Patt pointed out that the duration of G_1 (resting phase) varies widely, in typical mammalian cell systems, and is mainly responsible for the very large differences in cell cycle time among various tissues.

The time required for one generation cycle to occur is referred to as the "generation time" which should be clearly distinguished from the "turnover time" which is defined as the time taken for the replacement of a number of cells equal to that in the whole population (Leblond 1956).

In 1960, Messier considered the stratified squamous epithelium of the tongue as an example of renewing cell population with a "restricted proliferation site." New cells are always formed in the stratum germinativum of the epithelium, since only cells in this layer are labeled (that is, synthesizing DNA prior to mitosis) one hour after isotope injections. He further stated that cell "migration" is common in the tongue epithelium as shown by the migration of a labeled basal cell to the upper strata. Cutright (1967) concluded that the greater the speed of epithelial cell turnover, the more cells migrate.

Cowdry (1942) reported that the daughter cells of a cell dividing in the reproducing layer may either become members of the surface layers in which no mitosis occur (Post mitotic cell) or remain in the germinative layer and there undergo further mitosis (vegetative intermitotics and differentiating intermitotics).

In 1965, Leblond pointed out that two daughter cells of a mitosis come out independently and at random, thus the division of a basal cell may give two basal, or one basal

and one spinous, or two spinous cells. He considered the coming out of the basal layer as the critical step, after which the evolution of the cell is irreversible up to keratinization and shedding. He concluded, therefore, that on the average half of the cells arising from mitosis divide again and half transform into spinous cells, thus, there is no change in the number of cells of the epithelium and the steady state of the cell population is maintained.

In 1967, Cutright demonstrated that the maintenance of epithelial homeostasis is helped by the reutilization of the DNA, released from keratinizing cells, by the newly proliferating cells. He pointed out that this recycling could be important when rapid regeneration is needed, since it makes the epithelium less dependent on blood-born DNA precursors and provides a local pool of essential DNA metabolites.

Henry (1952) used the "colchicine method" to estimate the rate of regeneration of the rabbit's oral epithelium and found it to be "8.5" days. Since then, many workers have gathered "data" on the "turnover time" of the oral epithelium. They estimated it to be between 3 days (Gottesberge 1963) and 5.8 days (Cutright 1967) for the various portions of the oral mucosa. These values are summarized in Table I.

Toto (1962) conducted an investigation of the generation cycle of the epithelium of the tongue in mice in which he concluded that the duplication stage lasts for ten hours. In 1964, Dhawan found that the synthesis time is eight hours for the palate and dorsal surface of the tongue, and eight and one-half hours for the ventral surface of the tongue, in the 60 day old mice.

Leblond (1956) pointed out that variations in the estimation of the duration of mitosis were probably due to observational difficulties, since prophase and telophase are hard to delimit in living cells. Henry (1952) concluded that the mitosis required 64 minutes in the oral epithelium of rabbit. In case of mice's oral mucosa it was found to require 40 minutes by Medak (1959). This finding is in agreement with those of Toto (1962) and Dhawan (1964).

In 1908, Minot studied the epidermis of rabbit embryos at varying age ranges, he found different mitotic indices in various tissues, thus establishing evidence of differential rates of growth. He further, described the mitotic index (MI) as the number of cells to be found at any given moment in the active process of division out of a total of 1,000 cells. Mitotic index is used to measure the renewal time. Messier (1960) pointed out that mitotic index is not altogether satisfactory to assess the rate of

cell proliferation in a tissue because the counts of mitosis in sections are proportional not only to the number of cells entering mitosis, but also to the time taken by these cells to complete visible mitosis, also, mitotic figures are difficult to recognize in small and elongated cells.

II. Causes of Cell Renewal

In 1963, Bertalanffy assumed that cell renewal is a protective mechanism. He stated that many epithelia are almost continuously exposed to stress of various kinds; e.g., mechanical (oral epithelium), enzymatic (duodenal epithelium), bacterial agents (respiratory epithelium), to site a few; therefore, were there no cell renewal, cells of various epithelia might soon become abraded by mechanical contact or digested by proteolytic enzymes. Vulpe (1954) found that irritation due to urine causes mitosis in the transitional epithelium of the bladder. Lesher (1964) estimated that the turnover time of duodenal epithelium of the germ-free mice is longer than that of conventional mice. He postulated that the germ-free environment reduces attrition of cells on the villi and the resulting decreased need for renewal is recognized and responded to by decreased cell production. In 1956, Leblond demonstrated that most renewal systems, though responsive to extrinsic forces, are capable of renewing in their absence, e.g., cells were renewed even in

isolated intestinal loops not exposed to the mechanical stress that may be exerted by ingested food. Accordingly, the ability to proliferate is an inherent property of the cells of the renewal system.

Leblond (1965) pointed out that the addition of new cells by mitosis tends to increase population pressure in the basal layer as shown, often, by the racket shape of the cells leaving this layer, as if squeezed out by pressure.

Bierman (1955) suggested that cells may be eliminated by hormonal factors as seen in case of lymphocytes by the adrenal cortical hormone (hydrocortisone).

Harvey (1963) stated that the production rate in hematopoietic tissues is death-controlled. That is, adjusted by variations in the mortality of newly formed cells. Bertalanffy (1963) concluded that the component cells of tissues with cell renewal are replaced at constant rates governed by the steady state, whether they are stressed at any particular time or not.

III. Methods of Determination of the Generation Cycle of Cells

There are various methods for determination of the rate of regeneration of cells preferred by different workers for different reasons. Some of the methods used will be described.

Mitotic Index - is the simplest approach. Estimation of M. I. involves little interference with the tissue and therefore, a minimum of artifacts. An important disadvantage of this method is that no measurement of time is involved and therefore the rate at which the cells of a tissue are replaced cannot be calculated. It finds its main application when relative measurements are needed, for instance, in comparing the effect of various agents on the renewal of epidermal cells. (Leblond 1956).

Colchicine Method - Colchicine, a plant alkaloid, has the property of blocking mitotic divisions at metaphase and was used by Henry (1952) in oral mucosa of rabbits. It has the main disadvantage of altering the rate at which cells enter mitosis.

X-ray - Lesher (1961) suggested the use of x-ray irradiations for this study of the regeneration of cells. It stops early prophases in the material treated in this manner.

Cell Labeling - Cells can be labeled with radioactive isotopes, vital dye, etc., and then traced through their life cycle. The turnover time of cell population can then be calculated by measuring either the rate at which the labeled cells form, or the time they spend in a particular compartment of their migration path, or the rate at which they are lost from the tissue. (Leblond 1956)

Volkman (1950) assessed the renewal time of the epidermis by measuring the rate at which India ink injected into the deep layers was carried to the surface.

Leblond (1965) states that the advent of radio-autography gave histology a new dimension, time. When cells double their DNA prior to mitosis, they take up various DNA precursors. If labeled precursors are injected into animals, they are incorporated in dividing cells, which are then tagged and may be traced in radioautographs.

Phosphate-P^{32}, Adenine-C^{14}, and C^{14} formate have been used in this manner (Lajtha 1954). However, the first one of these precursors is taken up into a variety of substances besides DNA, the second and third into both RNA and DNA and only "Thymidine" is taken into DNA alone (Reichard 1951). Amano (1959) observed that, after thymidine-H^3, radioautographic reactions were present only over nuclei, and that pretreatment of the sections with the enzyme Deoxyribonuclease prevented the occurrence of reactions. It may, therefore, be stated that the only radioactive substance present in sections after labeled thymidine injection is DNA, and that any cell found to be labeled is about to divide by mitosis. Thymidine has the advantage of being available with a tritium (H^3) label, which produces Beta-ray with such a low energy that they are

recorded in the photographic emulsion very close to their site of emission, the result is a fine radiographic resolution (Taylor 1957).

Messier (1960) used thymidine H^3 for his studies of the cell proliferation and migration in male rats and mice. The frequency of labeled cells is proportional to the duration of the duplication stage. A close relationship exists between the labeled thymidine uptake and mitosis in the tissues. He further states that the radioactive index (percentage labeled cells) is a rough index of the rate of cell division, since it is influenced by the rate of incorporation of the label into the intermediate substance leading to DNA synthesis and by the duration of the period of DNA synthesis. Nevertheless, the radioactive index has two advantages: (1) labeled cells are more readily identified than mitotic figures in many tissues and are many times as numerous; (2) the cells tagged by thymidine-H^3 could be traced long after mitosis had been accomplished.

There is a difference of opinions, among scientists, as to the extent of injury to chromosomes caused by thymidine-H^3. Schoenheider (1960), reviewed the method of autoradiography, assumed that the chemical behavior of the labeled substance is identical with that of its stable counterpart. It is like a "tracer."

Leblond (1965) stated that the autoradiography shows movement under physiological conditions, on the other hand Quastler (1959) pointed out that this method of investigation is not physiological, because of the growth factor in young animals, the stress of injection and the injury to the chromosomes caused by triated thymidine. In 1962, Toto concluded that there is no minimal threshold for triated thymidine at which injury to the chromosomes does not occur.

Lesher (1961) used both the graphic method and the stage duration index one to determine the generation time. He concluded that the graphic method is superior to the stage duration index one because the latter requires precise knowledge of the size of the proliferative pool and of the duration of a particular stage of the generation cycle which are difficult to obtain. Its only advantage is that it can be applied without observing the complete generation cycle. Lesher (1961), Toto (1962) and Dhawan (1964) calculated the generation cycle of cells by adding the DNA synthesis period (S), a rest period (G_2), the duration of mitosis (M), and the interphase (G_1).

Lesher (1961) reported that the generation time computed by the stage duration index formula:

$$\frac{\text{Number of Cells in Mitosis}}{\text{Number of Cells in Interphase}} = \frac{\text{Duration of Mitosis}}{\text{Duration of Interphase}}$$

is unreliable proportioned to the degree to which these

estimates of the mitotic duration are unreliable.

In 1967, Cutright used a pure histologic and a mathematical technique to determine the turnover time of the Stratified squamous epithelium. The histologic method expressed the renewal time as the time required for the last initially labeled cell to pass from the stratum germinativum to the keratin layer which was used as an end point because it normally contains no living cells and thus no labeled nuclei. On the other hand, the mathematical method used the radioactive index (percentage labeled cells) of the basal layer and calculation is also based on the time needed for DNA synthesis, 7.5 hours. The following formula was used:

$$\frac{100}{\text{Radioactive Index}} \times \frac{7.5}{24 \text{ hours}}$$

He pointed out that this approach is based on the assumption that all newly formed cells derive from the basal layer and that, therefore, the percentage of proliferating cells within the basal layer reflect the turnover rate. He considered the mathematical method to be superior to the histologic one.

TABLE I

TURNOVER TIME OF ORAL EPITHELIUM

Author and Year	Method Used	Source of Material	Region	Findings In Days
Henry (1952)	Colchicine	Rabbit	Oral Mucosa	8.5
Toto (1962)	Autoradio-	Mice	Tongue Epithelium	4.1
Koburg (1962)	Autoradiography	Mice	Hard Palate	4.4
Bertalanffy (1963)	Colchicine	Rat	Buccal Mucosa Tongue Epithelium	4.3 4.9
Gottesberge (1964)	Autoradiography	Mice	Gingival Epithelium	3.5
Dhawan (1964)	Autoradiography	Mice	Palatal and Dorsal Tongue Epithelium Ventral Tongue Epithelium	4 4.1
Cutright (1967)	Autoradiography	Rat	Post Dorsal Tongue Epithelium Anterior Tongue Epithelium Ventral Tongue Epithelium Hard Palate-Gingiva, attached	3.2 3.5 5.1 4.3

C. FACTORS AFFECTING MITOTIC ACTIVITY

Wherever mitotic activity is present in adult mammalian tissues its rate is commonly variable from hour to hour and from day to day. It is always evident, however, that there exist strict limits to this variation and that these limits are characteristic for each particular tissue. Among the factors known to influence the daily variation in mitotic activity are:

Hormonal: Many workers in the past have determined that endocrinological influences are important to the rate of mitotic activity. Earthly (1951) showed that "thyroxine" in small doses depresses mitotic activity and that "testosterone" in male enhances the rate of cell division. In 1950, Bullough suggested a possible mechanism for thyroxine's action. Energy required for mitosis is supplied by glycogen. Now, thyroxine has the well known ability to deplete tissues of glycogen, and consequently decrease mitotic activity. Leblond (1955) stated that "Growth hormone" accelerates mitosis. Bullough (1961) showed that mitotic activity is significantly increased in "adrenalectomized" or "hypophysectomized" animals. Bullough (1946) presented evidence that the "estrone" level in the female mouse affected the renewal of epidermal cells. However, in 1961, he stated that while the mitotic rate of a tissue may be influenced by hormones, it is not controlled

by them. The findings for some of the investigators are summarized in Table II.

Physical Stimulation: Carleton (1934) found that continuous exposure of animals to light caused an alteration in the rhythmical periodicity of mitosis. In 1955, Peters suggested that irritation causes cell proliferation, since it is noticed that at the edges of a wound the mitotic activity may rise to more than ten times the normal rate. Bullough (1960) found that even gentle massage causes a marked increase in the epidermal mitotic rate both invitro and invivo.

Temperature: Bullough (1949) concluded that mitotic activity is high during lowered temperatures and low during higher temperature. Blumenfeld (1939), in a study of mitosis in the epidermis of the albino rat, speculated that the early morning increase was related to a high body temperature at that time. In 1950, Storey found a rise of mitotic activity at a temperature of 25° - 30°C. in rats epidermis. However, Leblond (1956) is of the opinion that in an integrated animal there is probably no effect because of the internal temperature regulation mechanism.

Blood Sugar Levels: Diller (1946) pointed out that starvation lowers mitotic activity in the rat intestine. In 1949, Bullough observed the existence of a direct relationship between mitotic activity and blood sugar levels.

He found that epidermal glycogen content increases during sleep when the mitotic rate increases and considers the glucose or glycogen to be the critical substance effecting the mitotic activity in the epidermis of the adult mouse and of man. In 1954, he reported evidence that a moderate increase in insulin concentration may lead to a raised epidermal mitotic rate. O'Connor (1954) reported that the mitotic activity of a tissue is dependent on the rate of carbohydrate metabolism.

Glucose Oxidation: Orban (1942) using a repeated application of 30% hydrogen peroxide which in turn produced monomeric oxygen, found the basal cell layer of human gingival epithelium to form from four to six layers of cells with a remarkable increase in mitosis. In (1961) Gargiulo after single exposures of 30% hydrogen peroxide for a thirty-day period, found an apparent prolongation of the mitotic period in the attached interdental gingival epithelium of humans. This seemed to be more specific in prophase. This apparently gives evidence that the mitotic process is retarded by the colchicine-like effect of hydrogen peroxide. Medawar (1947) found that anoerobic conditions prevent both epidermal mitotic activity and epidermal migration. By re-introducing oxygen it is possible to obtain a normal rate of division in the same cells. In 1951, Bullough concluded that mitotic activity could be increased by stimulating the

glucose oxidation. The number of mitoses can be increased by stimulating the rate of energy production from glucose oxidation. This has its effect immediately prior to the prophase which appears to be a critical time in the histophysiology of mitosis. However, Glickman (1950) discouraged the assumption that the artificial introduction of oxygen would appreciably hasten the normal cellular processes in the course of gingival healing.

Stress: Bullough (1952) demonstrated that stress is an influencing factor in mitotic activity and that the glucocorticoid hormones play a role in the antimitotic mechanisms. In 1954, Macapanpan found an increase in activity of fibroblasts in the periodontal ligament space when abnormal force was exerted on the teeth.

Radiation: Chase (1961) found that the mitosis-inhibition effects of ionizing radiation on the normal oral epithelium of the buccal mucosa in human caused a reduction in the number of cells. In 1963, Patt stated that different transitions in the proliferative cycle are blocked for different lengths of time by a given dose of ionizing radiation.

Chemicals: Henry (1952) reported that "colchicine" arrests all mitoses especially in the metaphase stage. In 1958, Orr demonstrated an increase in the mitotic activity of the epidermis and hair follicles by painting the skin

with "croton oil" or "Methylocholanthrene."

Mitotic Rhythmicity: Many investigators have shown the existence of a diurnal mitotic cycle in both animals and man. As yet, however, there is no agreement as to the timing of peak mitotic activity.

Bullough (1947) reported that there are higher mitotic activity with sleep and lower mitotic activity with those of wakefulness. Experimentally, the proliferation could be either increased by inducing sleep with an anesthetic or decreased by having an animal run in a revolving box to keep him awake. In 1961, he attributed this diurnal mitotic cycle to the mitotic inhibition caused by adrenalin. Any mouse when awake is evidently under sufficient stress to cause an increased adrenalin output and consequently a reduced epidermal mitotic rate; conversely a sleeping mouse with less circulating adrenalin shows a raised epidermal mitotic rate. On the other hand, Muhlemann (1954) found a night low and day high in retromolar epithelium and periodontal membrane of five month old black male rats. Henry (1952) found a maximal activity of 7.2 and a minimal activity of 3.8 mitosis per 1,000 cells in the oral mucosa of a 3½ month old rabbit at 1:00 P.M. and 10:00 P.M., respectively. A summary of these values is found in Table III.

Other Factors: In 1960, Marwah found that "chronic inflammation" increases mitotic activity in 50% of the cases observed in both age groups.

Halberg (1954) showed an inhibition of mitotic activity in the connective tissue of the interdental papillae of rats which were treated with large doses of "cortisone."

Thuringer (1924, 1928) found regional "differences." He counted one mitosis for 2,414 cells on the scalp, for 378,325 cells on the leg, and 268,275 on the ear.

Storey (1949) described "seasonal variations" in the mitotic activity of epidermis. He recorded the turnover time of epithelium during summer (9.8 days), in the autumn (16.3 days) in the winter (18.8 days).

Marwah (1956) reported that the presence of a "granular layer" and the presence of "parakeratosis" influence mitotic activity. The former markedly reduced the mitotic rate, the latter increased it slightly.

TABLE II

HORMONES AND ITS EFFECT ON MITOTIC ACTIVITY

Author	Year	Hormones	
		Stimulate	Inhibit
Bullough	1946	Estrone	
Earthly	1951	Testosterone	Thyroxin
Bullough	1952	Testosterone	Glucocorticoid
Bullough	1954	Insulin	
Halberg	1954		Cortisone
Leblond	1955	Growth Hormone	
Bullough	1961		Adrenalin Nor-Adrenalin

TABLE III

MITOTIC RHYTHMICITY

Author and Year	Source of Material	Organ	Time of Mitotic Cycles	
			Maximal Activity	Minimal Activity
Carleton (1934)	Mice	Hair Follicle	8:00 P.M.	12:00 A.M
Blumenfeld (1939)	Rat	Skin	8:00 A.M.	Early Evening
Bullough (1947)	Mice	Skin	6:00 A.M. 2:00 P.M.	10:00 A.M. 8:00 P.M.
Henry (1952)	Rabbits	Oral Buccal Mucosa	1:00 P.M.	10:00 P.M.
Halberg (1954)	Rats	Retromolar Epithelium and Perio-dontal Membrane	Night	Morning
Mullemann	Rats	Retromolar Pad	Day	Night

D. AGING AND MITOTIC ACTIVITY

In 1933, Ortiz Picon investigated the rate of division in the epidermis of abdomen and back of mice from birth to 33 months of age. He found that at six months the rate of division was twice as high as at one month and remained higher than at one month for the entire period. Bullough (1949) reported that mitotic frequency in mouse epidermis is considerably higher in middle-aged than in young animals even before they have reached full size. On the other hand, Kiljunen (1956) found that the mitotic index in the epidermis of young rats is higher than that of older ones.

In 1961, Lesher investigated the effect of age on the generation time of the mouse duodenal epithelial cell. He found that the fraction of the crypt population in the S-stage is practically independent of age.

The first mitotic index of human oral mucous membrane was published by Marwah (1956). He reported that the frequency of mitosis in epithelium of human gingiva is 50% higher in older age groups (50 to 78 years) than in younger age groups (25 to 55 years). A study on human attached gingival epithelium by Gargiulo (1961) showed age changes in the same direction. In 1956, Marwah stated that this finding would seem to contradict the common concept of

aging, according to which bodily processes slow down and the regenerative capacity of the tissue diminishes with age. He concluded that the concept of aging as a general slowing down of physiologic regenerative process cannot be maintained for epithelia.

In 1956, Falzone indicated that age changes which are not demonstrable in resting tissue can be discerned under condition of stress. Carter (1956) tested the reactions of gingiva to a brushing stimulus at different ages. He found that brushing led to a significant increase in the thickness of cornified cells and the height of the epithelial rete pegs. He, also, noted that within the range studied (early adult to middle age) aging per se was without significant influence on the thickness of the cornified layer or of the epithelium. On the other hand, Klingsberg (1960) investigated the adaptive potentiality of the oral epithelium, to an acute stress, with aging of the individual. He noted a significantly greater increase in the adaptive capacity on the part of the younger animal as compared to the older one.

In 1961, Lesher reported that the generation time and heterogenicity of the cell population is increased.

He concluded that whatever mechanism is responsible in old age, the reproduction of epithelial cells in the intestinal crypt of mice is changed.

A review of the literature reveals no studies reported on the effect of age on the generation cycle of oral epithelium.

CHAPTER III

MATERIALS AND METHODS

Materials

Twenty-four 600 day old mice, a strain from C57, Black and Albino, weighing an average of 37 grams, and being fed a diet of Wayne Lab Blox for mouse or rat were injected intraperitoneally with thymidine-H^3 (specific gravity 1.9 curies per milli-mole) at a dose rate of one microcurie per gram of animal weight.

The time of injection was recorded for each mouse to assure precise timing at sacrifice. The mice were sacrificed at intervals of a quarter of an hour, 1, 8, 10, 12, 14, 24, 30, 44, 54, and 100 hours.

The tongues were dissected out, cut mid-sagittally, fixed in formalin, dehydrated with ascending series of alcohol, and embedded in paraffin. Sections of 3 micron thickness were made.

Methods

Autoradiograms were prepared by a modified Fitzgerald's (1959) technique using Kodax NTB3 emulsion; the exposure time of 30 days at $4^{\circ}C$ was used. The slides were developed in Kodak D-19 developer for 5 minutes at $18^{\circ}C$. The slides were rinsed in distilled water and fixed in acid fixer with hardener (Kodak). The slides are then

washed in running water, cleaned and the tissue sections stained, through the overlying emulsion layer, with (1) Hematoxylin and eosin or (2) Nuclear-fast red and indigo carmine.

The times at which the first labeled prophases and telophases were seen were recorded. Owing to the difficulty of identifying prophases and telophases in heavily labeled cells, only metaphases and anaphases were scored. One hundred mitotic figures for each interval of time were observed for each of the specimens under present investigations and the percentage of labeled mitotic figures was determined.

The stages of mitosis are identified according to DeRobertis (1954) standards, as follows:

1. Prophase is identified by an increased visibility of chromatin threads, and an increased basophilia of nuclei.
2. Metaphase is recognized by the absence of a nuclear membrane and the chromosomes arranging themselves along the equatorial plate.
3. Anaphase is characterized by the separation of the daughter chromosomes in the undivided cell body.
4. Telophase is identified by the division of the cell body into two daughter cells.

A curve plotting the percentage of labeled mitotic figures as a function of time was prepared. The two coordinates representing 50% labeling on the ascending and descending limbs of the curve were located and the time interval recorded as the DNA synthesis time.

Radioactive index: Under high dry magnification "400 basal cells" contiguously were counted in the one hour interval sections. The number of labeled nuclei observed in each of the 400 cells was then recorded. The count of labeled cells was divided by 4 to give the percentage of labeled cells.

The following formula was used to estimate the turnover time:

$$\frac{100}{\text{Radioactive Index}} \times \frac{\text{DNA synthesis time}}{\text{24 hours (1 day)}}$$

Where the radioactive index is the percentage of labeled cells at 1 hour period. The DNA synthesis time is taken from the curve.

CHAPTER IV

FINDINGS

The stratified squamous epithelium on the dorsal surface of the tongue in 600 day old mice, at any given sacrifice period showed always some cells in preparation for mitosis.

At the fifteen minute interval, following injection of the T-H^3, some labeled cells are observed in the basal cell layer of the epithelium. These labeled cells are randomly distributed.

Within the first hour of injection, the time that triated thymidine is available, it is bound in the DNA of the cells in the S-phase from which it cannot escape during the period under consideration.

At one hour interval, the basal cell layer of the dorsal surface of the tongue showed a radioactive index (percentage of labeled cells) of 10 which represents the percentage of the cell population in the S stage at injection.

Only cells in the stratum germinativum are labeled one hour after isotope injection. Also nuclei of fibroblasts and salivary glands contained the isotopes. (Figure 3)

In the course of 8 hours the number of labeled mitotic figures rose rapidly from zero to 100 percent (ascending limb), where it remained for about 4 hours (plateau), then fell rapidly to 50% at 14 hours (descending limb). Graph 1

No labeled mitotic figures were found either at 24, 30, 44, 54, or at 100 hours following the injection of the triated thymidine. (Table IV)

The first labeled prophases are observed at 15 minutes and the first telophases at one hour following the injection with triated thymidine. (Figure 1, 2)

The time interval between the coordinates extrapolated on the curve as representing fifty percent labeling is 12 hours (Graph I).

Between the 8 and 14 hour interval labeled cells are observed in the stratum germinativum, often paired and showing a reduction of grains over the nuclei, appearing approximately half as many grains, as the originally labeled cells. The paired cells were, usually, arranged as follows: 2 basal, one basal and one spinous or two spinous. (Figure 4)

Many cells had migrated from the stratum germinativum to the upper layer of the stratum spinosum at the 24 hour period. (Figure 5)

Labeled cells are observed near the stratum corneum at the 44-54 hour period. Clear spaces are noted in the nuclei of cells approaching the keratin layer. Individual tritium exposed silver grains are observed outside the nuclei of the migrating cells undergoing keratinization. (Figure 6, 7)

One hundred hours following the injection of TH^3, a marked decrease in number of heavily labeled cells is observed as compared to the 44-54 hour periods. Many cells in the stratum germinativum contains nuclei displaying 2-4 tritium exposed silver grains. (Figure 8)

CHAPTER V

DISCUSSION

The stratified squamous epithelium lining the dorsal surface of the tongue is constantly renewed by multiplication of cells in the stratum germinativum. This finding is supported by Leblond (1965) who stated that cell proliferation occurs within the basal layer of the epithelium, since only cells in this layer of the stratum germinativum are labeled one hour after isotope injection. Medak (1959) also pointed out that in the oral epithelium of mice, the basal cells contributed 63%-98% in the renewal of the cell population.

At any given time, there are always some cells in the S phase. It is during this stage that the injected triated thymidine enters the DNA molecule in place of the naturally occuring thymine. The percentage of labeled cells (radioactive index) found one hour following injection represents all stages in DNA synthesis as determined by the availability of the triated thymidine during this time. Some labeled cells represented by the ascending limb are in late stage of DNA synthesis period, those represented by the descending limb are in an early phase of synthesis and those shown on the plateau are in some interval in between.

The number of cells in the S stage at the time of injection is 10% of the cells in the germinative layer. It indicates the rate of homeostatic epithelial cell replacement. This observation is very close to the radioactive index, at the one hour period, estimated by Toto (1962, 1966) and Cutright (1967) for the stratum germinativum of the dorsal epithelium of the tongue in the 300 and 400 days old mice and 100 day old rats. Therefore, the fraction of the cell population in the S stage at the time of injection is practically independent of age. It is concluded that there is no increase in mitotic activity occuring with aging. Furthermore, Lesher (1967) investigated the generation cycle of the mouse duodenal epithelium. He found out that the fraction of the crypt population in the S stage at the time of injection is smaller in the older animals than in the younger ones. These findings disagree with previous reports of an increased mitotic activity with age (Ortiz Picon) (1933), Bullough (1949), Marwah (1956) and Gargiulo (1961).

The radioactive index increases as the time of sacrifice increases. This is due to the division of the labeled cell population as indicated by the paired labeled cell showing approximately half as many grains per nucleus as the originally labeled cell.

Mitotic division, migration and maturation of the epithelial cells occur completely at random to assure the maintenance of a uniform epithelium and a steady state of the cell population is, consequently, achieved. Bertalanffy (1962) stated that in spite of mitotic formation of cells, in the adult, organs with cell renewal increase neither in size nor weight. This is because identical number of cells are continuously being extruded from the organs.

The first labeled prophases are observed at fifteen minutes following the injection of triated thymidine. The interval of time between the end of DNA synthesis and prophase, which is known as G_2, must be less than 15 minutes but more than zero i.e., approximately 12 minutes. The first labeled telophases are seen at one hour, the period of approximately 48 minutes is estimated to be required for mitosis. These findings are close to those calculated by Toto (1962, 1966) and Dhawan (1964) for the dorsal surface of the tongue epithelium of adult and young mice. They estimated a G_2 period of twenty minutes and forty minutes required by the cell division.

The DNA synthesis time for the dorsal surface of the tongue is 12 hours as determined by extrapolation on the curve.

Toto (1962) found it to be 10 hours in the adult mice and Dhawan (1964) found it to be 8 hours in the 60 days old mice. It is concluded therefore that the DNA synthesis time increases with age. This observation is supported by Lesher (1961, 1967) who found out that the S phase is longer in the older animals than in the younger ones.

Cutright (1967) used the following formula

$$\frac{100}{\text{Radioactive Index (at 1 hour period)}} \times \frac{\text{DNA Synthesis Time}}{\text{24 hours (1 day)}}$$

to calculate turnover time. In the present studies, the calculations based on such a method show that the generation cycle for the dorsal surface of the tongue epithelium, in the 600 days old mice, to be 5 days (120 hours). No autoradiographic studies have been published which could be used for comparison. Toto (1962, 1965), Dhawan (1964) and Cutright (1967) used the autoradiographic technic to estimate the turnover time of the dorsal surface of the tongue epithelium in 300 days, 400 days, and 600 days old mice and 100 days old rats. They found it to be 4.1 days, 3.7 days, 4 days and 3.2 days, respectively. It is concluded that the physiologic regeneration of epithelia slowed with age. This finding agrees with the common concept of aging, according to which bodily processes slow down and the regenerative capacity of the tissues diminishes

with age.

Lesher (1961), Toto (1962, 1965) and Dhawan (1964) calculated the generation cycle of cells by adding the DNA synthesis (S), a rest period (G_2), the duration of mitosis (M), and the interphase (G_1). Using this method, the G_1 phase is estimated to be 107 hours. The preparation of the cells for DNA synthesis occurs in G_1, it is suggested that this preparation period is prolonged.

Epithelial cells in the process of maturation undergo karyolysis, karyorhexis and the formation of keratohyaline granules. The loss of their nuclear DNA in the course of keratinization is indicated by the scattering of labeled particles of DNA around degenerating nuclei as soon as karyolysis begins.

The labeled DNA particles, scattered in the intercellular spaces, may be taken up by the "basal cells" in the S phase i.e., the DNA extruded from maturing epithelial cells may be reutilized. Cutright (1967) stated that the reutilization of intra-epithelially released DNA is independent of the circulation, since, it occurs in tissue explants and does not include the cells of the submucosa. Furthermore, the labeled DNA is incorporated first by those epithelial cells which lie near DNA-releasing cells.

He concluded that squamous epithelium, with its unique property of nuclear degeneration during physiologic maturation, thus conserves the fragmented nuclear material for the local use of newly formed cells. This recycling may be important when rapid regeneration is needed, since it makes the epithelium less dependent on blood-borne DNA precursors and provides a local pool of essential DNA metabolites.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Twenty-four 600 day old mice, a strain of C_{57}, were injected with triated thymidine intra peritoneally.

They were sacrificed at intervals of from 15 minutes through 100 hours.

The tongues were dissected out and cut mid-sagitally.

Autoradiograms were prepared and stained with (1) Hematoxylin and Eosin or (2) Nuclear - fast red and indigo carmine.

The number of labeled cells in the stratum germinativum 1 hour after injection was determined.

One hundred mitotic figures for each interval of time were counted for each of the specimens under investigations and the percentage of labeled mitotic figures was determined.

A curve plotting the percentage of labeled mitotic figures as a function of time was prepared.

The DNA synthesis time is 12 hours; G_2, the interval between end DNA synthesis and prophase, 12 minutes; mitotic time 48 minutes; interphase, 107 hours. The estimated generation cycle of the dorsal surface of the tongue epithelium is 5 days (120 hours).

The stratified squamous epithelium lining the dorsal surface of the tongue is constantly renewed by multiplication of cells in the stratum germinativum.

At any given time, 10% of the epithelial cells, of the dorsal surface of the tongue in 600 day old mice, are in preparation for mitosis.

Mitotic division, migration and maturation of the epithelial cells occur completely at random to assure a uniform epithelium and maintain a steady state of the cell population.

There is no substantial difference in the duration of G_2 and Mitosis (M) in the old animals as compared to the younger ones.

The synthesis time, interphase (G_1) and the generation time of the oral epithelium are increased with age.

Epithelial cells in the process of maturation loose its nuclear DNA into the surrounding intercellular spaces.

The DNA extruded from maturing epithelial cells may be reutilized by the basal cells in the S phase.

The physiologic regeneration of oral epithelia slows with age.

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Figure 1 At 15 minutes interval. (A) the first prophase is observed.
(H & E X 750)

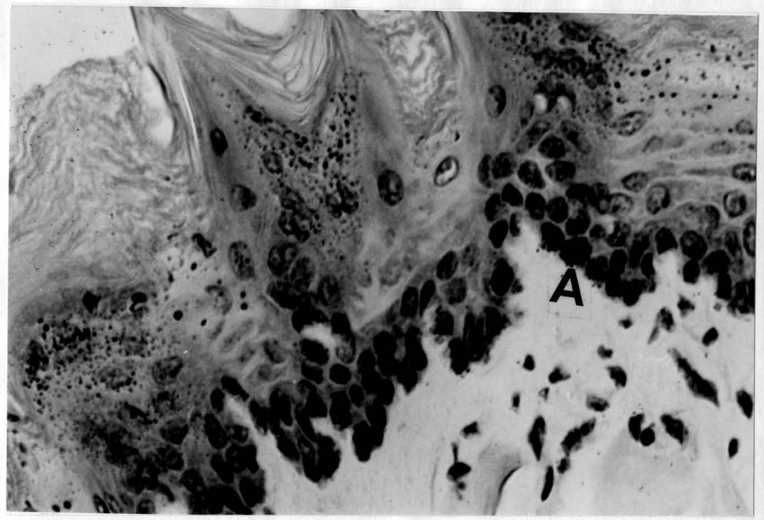
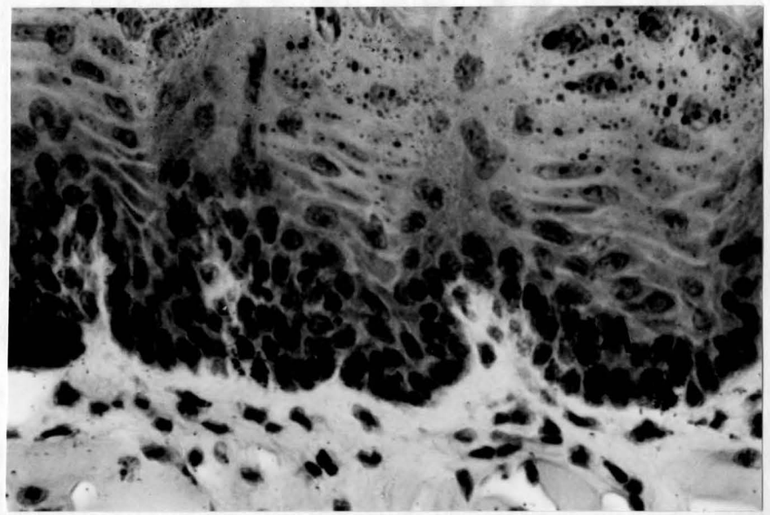


Figure 2 At 1 hour interval. (A) the first telophase is observed.
(H & E X 750)



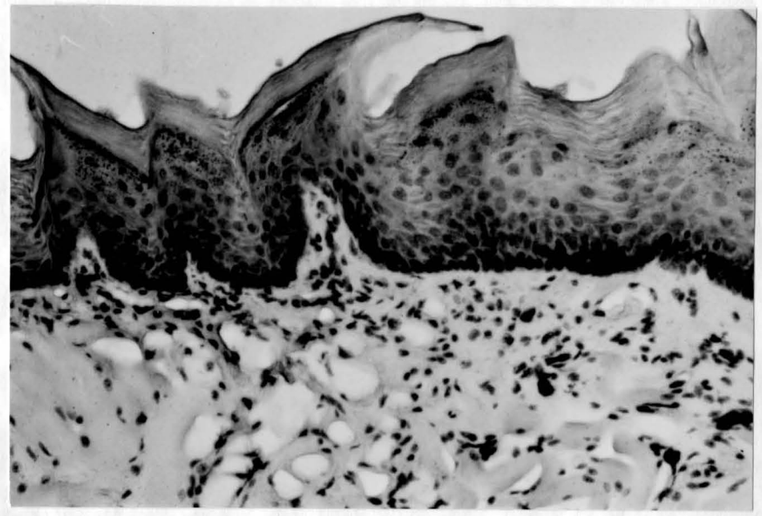


Figure 3 At 1 hour interval. All labeled cells are in the stratum germinativum.
(H & E X 300)

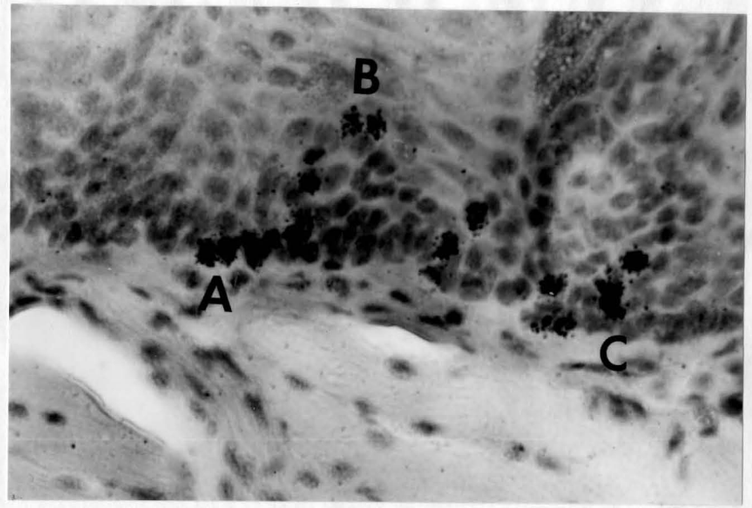


Figure 4 At 12 hours interval. All mitotic figures are labeled.
Daughter cells can be seen: (A) 2 basal cells
(B) 2 spinous cells
(C) 1 basal and 1 spinous cell

(H & E X 750)

Figure 5 At 24 hours interval. (A), (B) & (C) represent labeled cells migrating from the basal layer to the stratum spinosum.
(H & E X 750)

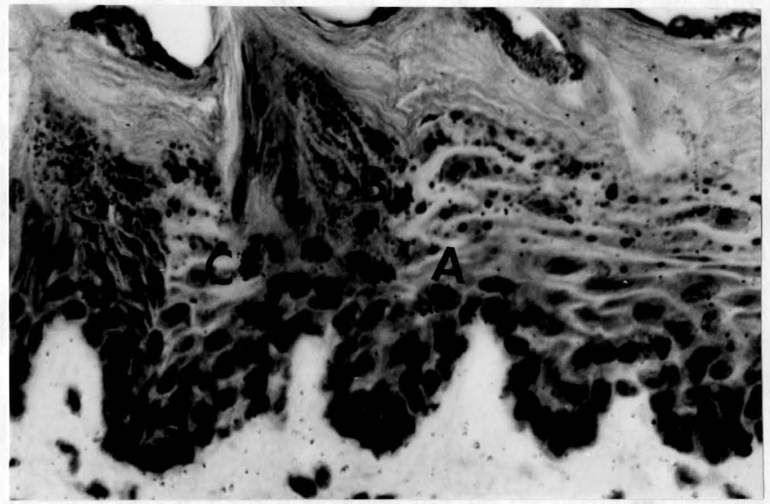
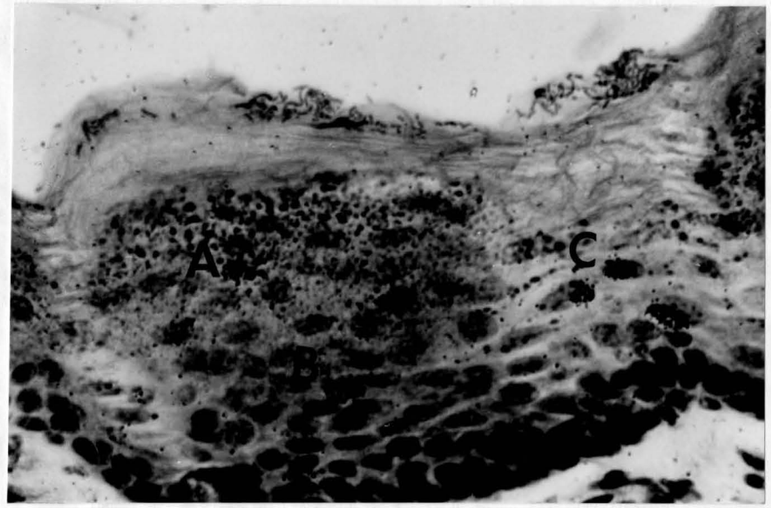


Figure 6 At 44 hours interval. (A), (B) & (C) represent migrating labeled cells in the upper layers of the stratum spinosum. (H & E X 750)



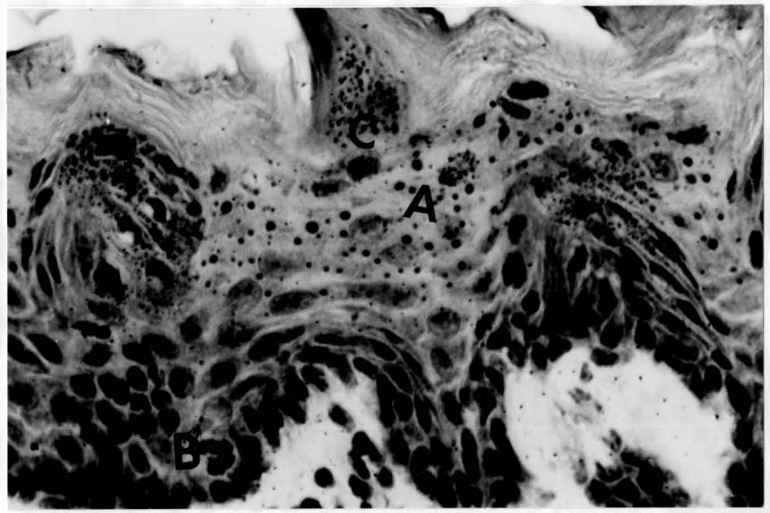


Figure 7 At 54 hours interval. (A) & (C) represent labeled cells in the process of degeneration and showing scattering of labeled DNA particles outside their nuclei. (B) represents an unlabeled mitotic figure (early Telophase). (H & E X 750)

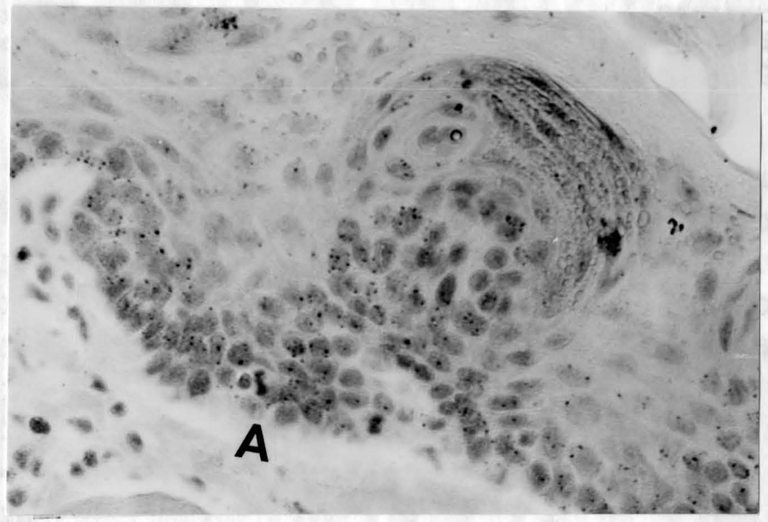


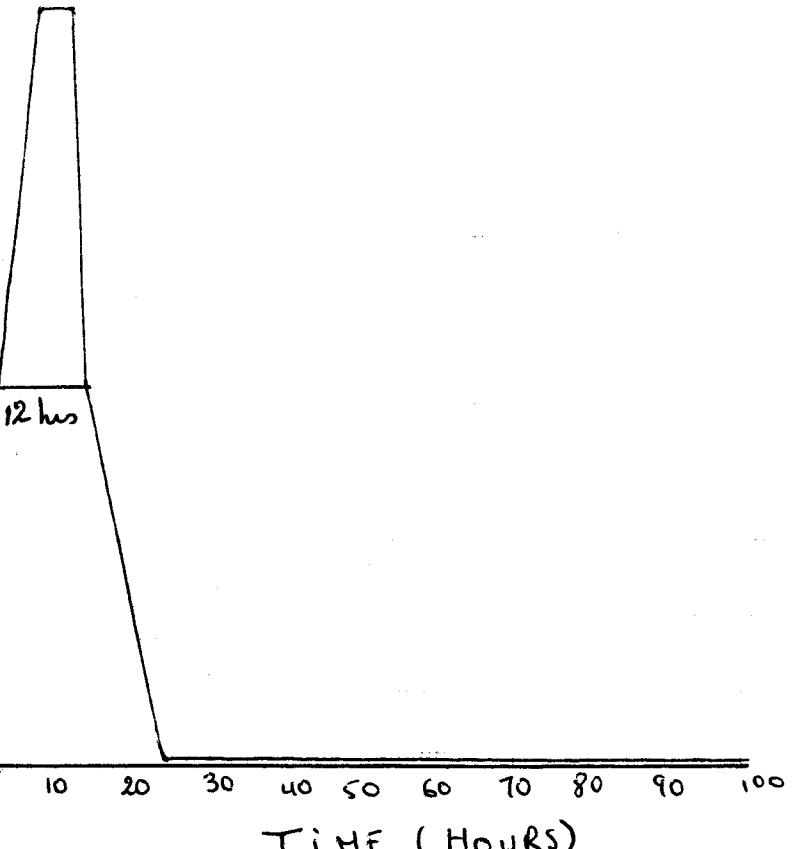
Figure 8 At 100 hours interval. Scattered labeled DNA particles can be seen in the stratum germinativum. (A) represents a labeled (2 grains) mitotic figure (Metaphase). (H & E X 300)

TABLE IV

EPITHELIUM - DORSAL SURFACE OF THE TONGUE IN 600 DAY OLD MICE

THE NUMBER OF MITOTIC FIGURES COUNTED IN EACH INTERVAL OF TIME IS ONE HUNDRED.

Hours (Following the Injection of Triated Thymidine)	Mean Labeled Mitotic Figures	Percentage Labeled Mitotic Figures
¼	0	0
1	40	40
8	100	100
10	100	100
12	100	100
14	50	50
24	0	0
30	0	0
44	0	0
50	0	0
100	0	0


12 hrs
10
20
30
40
50
60
70
80
90
100
TIME (HOURS)

APPROVAL SHEET

The thesis submitted by Nabil J. Barakat has been read and approved by the director of the thesis. Furthermore, the final copies have been examined by the director and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 25, 1967
Date

Patrick D. Tate
Signature of Advisor