CELL PROLIFERATION IN HUMAN ORAL MUCOSA

VOLUME 1 OF TWO VOLUMES

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CONTENTS

VOLUME 1
(Contd.)

PAGE

VOLUME 2

CHAPTER 1. (Contd.)

CHAPTER 2. (Contd.) Page

CHAPTER 3 (Contd.) Page

CHAPTER 4 (Contd.) Page

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PREFACE

The work in this thesis was undertaken in the Department of Oral Medicine and Pathology in the University of Glasgow Dental School during the period February 1973 to January 1976, when the author was on study leave from the University of Ceylon. The work was supported by a Scholarship Award under the Colombo Plan Scheme of the British Council.

Some of the techniques used in this thesis are modifications of previously published work and some are original techniques developed by the author in conjunction with the other members of staff in the Department of Oral Medicine and Pathology.

The applicationsof these techniques as described in the present study were undertaken by the author personally, except for atypia scoring described in Chapter 6, which was undertaken by Dr. D. G. MacDonald. Tissue microtomy was carried out by the technical staff under the direct supervision of the author.

The studies described in this thesis are either entirely original or greatly extend previously reported investigations.

Parts of the work of this study have been presented at scientific meetings:-

1. "Cell proliferation in hamster oral epithelium. Comparison of in vitro and in vivo labelling techniques"
   International Association of Dental Research, British Division, Liverpool, April 1974. (with D. G. MacDonald)
2. "Cell kinetics of normal human buccal epithelium"
   International Association of Dental Research, General Session, London, April 1975. (with D. G. MacDonald and J. S. Rennie)

3. "Compartment analysis in human buccal epithelium"
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4. "Practical methods in quantitation - cell kinetics and morphometry"
   The British Society for Oral Pathology, September 1975.

SUMMARY

Studies of cell proliferation and migration - cell kinetics - have been described extensively on animal tissues and to a more limited extent in human tissues. These reported studies are reviewed and the deficiencies in available knowledge are highlighted. One of the main difficulties in studying the kinetics of human tissues is that the most widely used technique in animal work, namely parenteral injection of radioactive isotopes to label specific cells, is not generally applicable in man. The initial work in the present study was concerned with investigation of techniques for quantitating cell kinetics and the development of an in vitro radioactive labelling technique suitable for use with human tissues. The reliability of this technique was evaluated in the animal study where the in vitro labelling was shown to give comparable results to the classical in vivo labelling techniques.

The in vitro radioactive labelling technique was then used on biopsies from normal cheek mucosa obtained at six times of the day. This allowed diurnal variations in cell kinetic parameters to be quantitated. In conjunction with this investigation a stereologic study of the epithelium was undertaken.

The cell kinetic and stereologic techniques were next used to study eight cases of leukoplakia of cheek, a clinical disorder characterised by keratosis. The findings in the leukoplakia lesions were contrasted with those in biopsies from clinically normal mucosa in the same individuals. Epithelial cell production was found to be increased although the viable cellular layers of the epithelium were atrophic in the leukoplakias.

The control biopsies, although obtained from clinically normal mucosa, were shown to exhibit several abnormalities. The degree of increased cell producation in leukoplakia in these control specimens was shown to have a positive correlation with the severity of epithelial atypia.

The further applications of kinetic studies and epithelial morphometry are outlined and the difficulties encountered in the use of in vitro radioactive labelling techniques and other morphometric methods in the study of cell proliferation are discussed.

GENERAL INTRODUCTION

The mouth is lined by a stratified squamous epithelium which, like other lining epithelia in the body, is continually undergoing cell renewal. Diseases involving lining epithelia frequently manifest as disturbances in cell production and cell loss to produce hyperplastic or atrophic lesions. In premalignant and malignant lesions an increased rate of cell production is often said to be present. This assertion is mainly based on subjective observations and presently there is increasing interest in quantitating the parameters of cell production. The study of the proliferation and life cycle of cells is popularly known as cell kinetics and this includes estimations of such parameters as cell production and loss, and cell movement within and between tissues. A pre-requisite to the study of cell kinetics in disease states is an accurate evaluation of normal tissues.

MacDonald (1971a) in a review of cell renewal of oral epithelium indicated that the results of many studies on cell proliferation of the oral epithelium are incomplete in that they fail to consider all the variables which may affect cell kinetics. Karring (1973), reviewing the mitotic activity of oral epithelium, regarded the available results as unreliable because many of the assumptions on which the estimates were based were not valid. Both these authors have indicated the need for re-evaluation of previously published work.

The available knowledge on cell renewal patterns of oral epithelium stems mainly from studies conducted on animals. These studies have usually used in vivo incorporation of radioactive isotopes as the markers of cell proliferation or migration. These in vivo methods cannot be readily applied for studies in man. Although in vitro methods of radioactive labelling to estimate cell kinetic data have been described in the

literature, application of these methods to the study of oral epithelium has been limited. Therefore it was felt that the development of an in vitro system of radioactive labelling to allow quantitation of the cell kinetic parameters of human oral epithelium was desirable. If such a system could be shown to be reliable it was the aim of the present study to assess the cell production kinetics in normal human buccal mucosa with appropriate consideration of as many as possible of the variable factors known to influence kinetic parameters.

At present the histopathological diagnosis of oral epithelial premalignancy and cancer is based on subjective evaluation of the microscopical features of tissues and the constituent cells. Both premalignant and malignant lesions of oral epithelium involve alterations in cell kinetics. A quantitative assessment on cell kinetics in the normal and disease states could lead to a better understanding of how altered cell kinetics modify normal cytology and architecture in diseased epithelium.

The aims and design of the present study can be summarized as follows:-

In Chapter 1 the general concepts of cell proliferation kinetics are presented. Although the literature directly concerned with the description and measurement of the cell proliferation of oral epithelium is limited, the number of publications which are relevant to the general field of study of cell kinetics is very large. Chapter 1 therefore includes a general review of this literature and an evaluation of the current concepts of epithelial cell proliferation. The available data on cell production in oral mucosa is also discussed.

Several investigative methods have been described for quantitating the cell kinetics of lining epithelia, especially in skin and the gastrointestinal tract. It was considered appropriate to review these techniques briefly and Chapter 2 contains a discussion of the methods of studying cell renewal.

The primary aim of the present study was to develop a suitable in vitro system for the estimation of cell production in human oral epithelium by the use of radioactive labelling methods. It was necessary in the first instance to evaluate the method using experimental animals. In Chapter 3 a preliminary study in the hamster is presented in which the in vitro method is compared with the classical method of in vivo radioactive labelling.

Chapters 4 and 5 consist of experimental studies on human buccal mucosa using the in vitro labelling technique and other quantitative morphometric methods, in order to define the normal cell kinetic parameters with particular reference to the study of diurnal variations. The data so derived allows of some speculation as to the intrinsic control mechanisms of oral epithelial cell production.

Chapter 6 describes a limited assessment on the use of in vitro labelling techniques to examine cell proliferation in a disease state, namely leukoplakia. The ultimate aim of this part of the study will be to develop a system of quantitation applicable to the diagnosis of mucosal lesions.

In Chapter 7 a general discussion on experimental findings is presented with some remarks on the problems involved in the study of cell proliferation in epithelial tissues.

CHAPTER ONE

CELL PROLIFERATION AND CELL POPULATION KINETICS

1.1 INTRODUCTION

Cell proliferation has been studied in many biological systems. Tissues with abundant mitoses were recognised as early as 1894 by Bizzozero who studied the crypt cells of small intestine. Since that time, cell biologists investigating tissues demonstrating mitoses, have remarked on the importance of understanding their dynamic nature. With recent advances in research methods, there has been considerable progress in the analysis of cell systems. Quastler (1959, 1960), introduced the idea of enumerating different parameters for the estimation of cell kinetics and detailed quantitation in more recent investigations has improved the general understanding of the subject.

Before discussion of cell kinetics in oral epithelium can be undertaken, it is necessary to describe the general concepts of dynamic cell systems.

1.2 TISSUE GROWTH, CELL RENEWAL AND CELL POPULATIONS

1.2.1 General Concepts

Bizzozero (1894) proposed a classification of tissues into three types; these being permanent, stable and labile. This was based on the degree to which various specialised somatic cells preserved their ability to divide by mitosis during postnatal life. Permanent cells were considered to have lost the capacity to multiply about the time of birth. Stable cells ceased proliferation, about the time somatic growth stopped, but retained the ability to divide throughout adult life, so that regeneration could take place in the event of an injury. The labile cells were described as those which multiplied throughout life under normal physiological conditions.

Bizzozero's classification illustrates the growth potential of cells comprising different tissues in the body and enables generalisations to be made about their behaviour in pathological states such as wound healing or in excessive functional activity.

Leblond and his co-workers have classified cell proliferation in a different manner with reference to cell populations. A cell population was defined by Leblond (1964) as a group of cytologically similar cells presumably with a similar function. The term was also used to refer to all the cells coming from common stem cells.

Leblond and Walker (1956), Leblond, Messier and Kopriwa (1959) and Leblond (1964) classified the cell populations of postembryonic growing rats as static, expanding and renewing by studying mitotic activity and deoxyribonucleic acid (DNA) replication and investigating the extent to which cells completing DNA synthesis were retained in or were lost from the tissue. Cells in DNA synthesis were identified by radioactive labelling methods. In order to use DNA synthetic activity as a marker of cell proliferation, the assumption was made that all cells replicating DNA would proceed to divide at a later time. All cell populations in the embryo were regarded as expanding. The characteristics of the three types of cell populations these authors described in immature growing animals require discussion.

1.2.2 Static Cell Populations

Static cell populations are defined as those in which mitotic activity or DNA replications cannot be detected. DNA replication studies of static cell populations using radioactive labelling techniques in six months old rats indicated that there were no cells in DNA synthesis, but in animals labelled at three days of age radioactivity was still present after six months (Leblond, Messier and Kopriwa, 1959).

All cells belonging to static populations are highly differentiated and appear to have the potential to live for a long time, perhaps as long as the organism itself survives (Goss, 1967). Postnatal growth of tissues with static populations must be due to an increase in cell size without an increase in cell number. Neurons of the central and peripheral nervous systems, and striated and cardiac muscle cells belong to static cell populations.

1.2.3 Expanding Cell Populations

Expanding cell populations described in the growing rats are those demonstrating evidence of cells replicating DNA and undergoing mitotic division in numbers accounting for the increase in organ or tissue size co-ordinated with somatic growth. Leblond (1964) presented evidence to demonstrate that expanding cell populations incorporate DNA labels during the growth period and retain such labelled cells throughout adult life in the rat without any significant cell loss. Parenchymal cells of liver, acinar and islet cells of pancreas, cells of proximal convoluted tubule of kidney, cortical and medullary cells of adrenals are among those included in expanding cell populations by Leblond, Messier and Kopriwa (1959). These cell types belong to static cells according to Bizzozero's (1894) classification. However, as Leblond and co-workers (1959, 1964) used growing rats for these experiments, their conclusions are not directly applicable to adult non-growing animals and man (Cameron, 1970). In the adult animals most of the tissues cited above maintain a steady size because of a negligible rate of cell division, and of cell loss. The potential for expansion of individual populations probably decreases progressively with age but cell proliferation may be resumed with adequate stimuli such as in the liver following partial extirpation.

1.2.4 Renewing Cell Populations

Renewing cell populations were defined by Leblond and Walker (1956) and Leblond (1964) as groups of cells in which abundant DNA synthesising cells and mitoses can be detected in numbers exceeding that required for somatic growth. This high cell production is balanced by a high cell loss. Cells arising from stem cells undergo maturation and loss and therefore are replaced throughout life. Haemopoietic cells of bone marrow, lymphopoietic cells of thymus, spleen and lymph nodes, epidermis, cornea, oral epithelium, gastro-intestinal tract epithelium, spermatogonia and some of the reproductive system lining epithelium belong to renewing cell populations.

1.2.5 Cell Populations in Non-growing Adult State

The proliferative behaviour of cells in adult non-growing mammals

is obviously different from that of growing rats and therefore Leblond's (1964) classification is not directly applicable to mature animals or man.

Cameron (1970) reviewed the features of cell renewal in the organs and tissues of non-growing adult mice. He proposed a classification of cell types consisting of three cell populations in the normal adult state. These are, static cell populations demonstrating no cell proliferation; cell populations which demonstrate some cell proliferation but do not renew all cells during the life span of the animal; and a third type, renewal cell populations. Renewal cell populations may be sub-divided into two major groups. These are rapid renewal populations which renew in 30 days or less and slow renewal populations which include the parenchymal cells of most organs. These slow renewal populations although they undergo cell renewal, incorporate radioactive label into DNA only slowly and do not replace themselves within 30 days but do so within the life span of the animal.

Most of the cell populations which were considered as expanding by Leblond (1964) in the growing rat, were found to be of slow renewing type in the adult mouse (Cameron, 1970). Oral epithelium as it renews in less than 30 days was classified among the rapid renewal cell populations.

Cameron (1970, 1971) has pointed out that as these characteristics used to classify cell populations are based on proliferative behaviour of cells in mice, this classification also may not be appropriate for describing cell renewal patterns in mammals that live longer,or in man.

1.3 CHARACTERISTICS OF CELL RENEWAL SYSTEMS

1.3.1 The Concept of Compartments

A renewing cell population may be divided into compartments of similar cells (Quastler, 1960). These compartments may be characterised by the morphology, function and sometimes location of the constituent

cells. The group of cells engaged in proliferation or new cell production is known as the progenitor cell population and forms the progenitor cell compartment. A proportion of these cells undergo maturation to form the compartment comprising what have been called the functional cells. The identification of these compartments may be by cell morphology alone where compartments are intermingled, or by both cell morphology and location when the compartments are clearly defined spatially.

Quastler and Sherman (1959) and Quastler (1960) studying intestinal epithelium in the mouse described the cell renewal system at that site as consisting of two compartments designated as progenitor and functional. This simple two compartmental cell renewal system is illustrated diagramatically in Figure 1. 1. The analysis of cell kinetics of a two compartment cell renewal system becomes simpler if the following features can be assumed (Quastler and Sherman, 1959):-

(i) cells are born only in the progenitor compartment
(ii) cells die only or almost only in the functional compartment
(iii) cells never return from the functional to the progenitor cell compartment.

Some investigations have assumed a two compartmental system for stratified squamous epithelia consisting of a progenitor cell compartment and a mature cell compartment.

1.3.2 Tissue Compartments in Oral Epithelia

In a non keratinized oral epithelium, using histological sections stained with haematoxylin and eosin it is possible to identify two main cell populations which correspond to the progenitor and mature cell compartments described earlier. In regions showing keratinization the cornified cells can be considered as a separate third compartment where no viable cells are present.

The relationship of progenitor and maturation compartments to the conventional histological strata of many stratified squamous epithelia is not clear cut. Histologically the stratum basale or the basal cell layer, is described as the deepest cell layer in an epithelium apparently in contact with the basement membrane. However, the parabasal cells which are morphologically similar to the basal cells should be included in the progenitor cell compartment. The rest of the keratinocytes in the stratum spinosum belong to the maturation compartment.

1.3.3 The Progenitor Cell Compartment

Cell division occurs in the basal cell layer or the deeper cell layers in a stratified squamous epithelium and these consti tute the progenitor cell compartment. Identification of the progenitor cell compartment is based mainly on location and morphology e.g. small cell size of the constituent cells. In certain epithelia the features distinguishing these cells from the cells of the maturation compartment may not be clear cut. The presence of mitotic figures in histological sections and labelled nuclei in the autoradiographs prepared from radioactive labelled tissues, to a certain extent delineate the distribution of the progenitor compartments.

Early publications on the site of cell division gave contradictory results. Pinkus (1927) believed that cell division in epidermis occurred solely in the basal cell layer, while Thuringer (1924, 1928) studying the human scalp and prepuce reported that mitoses were more abundant in the middle or deeper third of the stratum spinosum and that only 10% - 12% of the mitoses were present in the basal cell layer at these sites. Most textbooks of histology mention that mitoses occur in the stratum germinativum, taken both as the basal cell layer and the lower spinous cell layer. Our present knowledge on cell formation and the closely linked process of cell migration stems from the work on rat oesophageal epithelium by Leblond, Greulich and Pereira (1964) and Pereira and Leblond (1965); and a study

on mouse epidermis by Iverson, Bjerknes and Devik (1968). Both these groups of workers suggested that the progenitor cell compartment is limited to the basal cell layer and that any cell leaving the basal cell layer is destined towards maturation. Suprabasal dividing cells are not present in either rat oesophagus or mouse epidermis which are relatively thin epithelia with a limited number of cell layers. This finding cannot be uniformly applied to other stratified squamous epithelia which have a higher mitotic rate or are thicker. A suprabasal dividing cell population has beendemonstrated in hamster palatal epithelium by Thilagaratnam (1969), and in rat palate and ventral tongue epithelium by Sharav and Massler (1967). Pinkus (1954) and MacDonald (1971 a) have remarked on the importance of serial sections for analysing the location of mitoses. These authors confirmed that a suprabasal dividing population can exist but the size of this can be overestimated if serial sections are not used to exclude those mitoses which, although appearing suprabasal in one section, are seen to be basal in an adjacent section. Löe, Karring and Hara (1972) reported a study based on serial sections in rat oral mucosa and human gingiva and came to the conclusion that it is highly probable that cell division is restricted to the three cell layers closest to the basement membrane.

From the evidence presented, it is clear that in certain epithelia, the progenitor cell compartment is not limited to the basal cell layer. Calculations based on the assumption that the progenitor cell compartment consists only of cells in the stratum basale may be considerably in error (MacDonald, 1971 a). To date, no data is available on the proportion of the epithelium formed by the progenitor cell compartment, in human oral mucosa.

The progenitor cell compartment is often assumed to consist of a homogeneouscell population with all cells engaged in or preparing for cell division. However, sub-populations such as non-proliferative cells

may be present in this compartment (Lajtha, 1963; Quastler, 1963). The life cycle of these cells is different from the reproductive cells (Section 1.3.4) and possible methods of identification of these non-proliferative cells are discussed in Section 2.4.4. Unrelated cell types such as melanocytes or Langerhans cells may also be present in this location, but as these form only a very minor proportion compared to the bulk of the keratinocytes, their presence probably does not add any significant inaccuracy to the size estimation of the progenitor cell compartment.

Recently Potten (1974) studying mouse epidermis, has grouped the progenitor cells into epidermal proliferative units based on the morphological finding of a central stem cell surrounded by approximately 10 basal cells. The central stem cell was found to be cycling at a different rate from the other basal cells and this has led to the hypothesis of the presence of a hitherto unknown stem cell in the epidermal progenitor cell compartment. In oral epithelia the presence of such stem cells has not been described.

1.3.4 The Cell Cycle of Dividing Cells

In order to understand the renewal of cells in a tissue, knowledge of the life cycle of individual cells is required.

Until 1953 the only microscopically distinguishable stage in the cell cycle of dividing cells was mitosis. Therefore the cell cycle was broadly divided into mitosis and interphase. Howard and Pelc (1953) by studying the synthesis of DNA in bean root meristem of Vicia faba suggested the presence of a discrete synthesis phase in the interphase of a cell preparing for division. Using autoradiographic techniques these authors demonstrated the incorporation of ^{32}P during DNA synthesis.

This observation led to the concept of a cell cycle consisting of four phases. These are illustrated diagramatically in Fig. 1.2. The demonstrable stages are mitosis (M) which is readily distinguished by histology and the DNA synthesis (S) phase during which replication of DNA in the nucleus occurs before division and therefore is demonstrable by DNA labelling techniques. There is a time lapse between completion of DNA synthesis and the beginning of mitosis and this gap is known as G_2. Similarly daughter cells produced by mitosis do not start replicating DNA immediately and this post-mitotic, pre-synthetic gap is called the G_1 phase. The G_1, S and G_2 phases consitute what was previously known as the interphase in a proliferative cell.

Non-cycling cells may be present in a proliferative cell compartment and these resting cells are described as being in a G_o phase (Lajtha, 1963; Quastler, 1963). Whether these cells constitute a population with very long G_1 phase is not known yet. Using cell kinetic methods it is sometimes possible to prove the absence of cells in G_o phase in a cell compartment, but it is much more difficult to prove their presence (Van Putten, 1974). This is because they cannot be shown by biochemical methods, to be different from G_1 cells. If a G_o phase is included in the cell cycle of a proliferative compartment, these cells could be considered as reserve cells having the potential to enter the proliferative cycle when suitably stimulated.

The life cycle of cells in the progenitor and maturation compartments are obviously different. It is only the progenitor compartment that contains cells in all phases of cell cycle. The maturation compartment contains cells that have a DNA content equal to G_1 cells.

In interphase, the stage at which a cell leaves the G_1 phase to re-enter the proliferative cycle by beginning DNA replication or, alternatively, leaving the proliferative cycle to begin maturation, is not clearly distinguishable. Bullough (1963) suggested the term dicophase derived from διχο (expressing

doubt between ways ahead) to this "phase of decision". The exact location of this phase between the G_1 and S phases is not known. S and M phases have been the areas of major interest, as these are the phase markers used in kinetic investigations. However, the control mechanisms of the cell cycle appear to operate in the G_1 and G_2 phases.

1.3.5 Analysis of Cell Proliferation Kinetics

Having discussed some of the morphological and functional characteristics of a cell renewal system, it is now possible to enumerate certain parameters which can be used to analyse cell kinetics in such systems. Our present knowledge and terminology of the concepts used for such analysis is largely derived from the criteria laid down by Quastler (1959, 1960, 1963) and Quastler and Sherman (1959) who indicated that the analysis of cell kinetics can be made in terms of size, time and flow parameters, with reference to either cells in tissue compartments or cells in cell cycle phases.

Size:- In a detailed analysis, the size parameter is used to refer to the actual or the relative number of cells in a cell compartment or in a phase of a cell cycle. Alternatively the size parameter may be estimated in terms of the volume occupied by the cells, instead of counting cell numbers in a tissue compartment.

Time:- In cell compartment analysis, the time parameter generally refers to the transit time, which is the average time spent by a cell in a compartment between its entry and leaving the compartment. In cell cycle estimations the time parameter defines the average time spent by the dividing cells in a particular phase of the cell cycle. These can be designated as T_s to refer to the duration of DNA synthesis and T_m to the

time spent by cells in the mitosis phase.

Flux:- The flux or the flow of cells describes the rate of entry and leaving of cells into and out of a cell compartment or a cell cycle phase.

When cells in a compartment are homogeneous the application of these measurements of kinetics is fairly simple and meaningful. However, when a heterogeneous cell compartment is analysed, usually only average or mean values are obtainable and these mean values may be considerably influenced by small numbers of cells with characteristics differing markedly from the main population (Quastler, 1960).

The estimations of size, time and flow, are more frequently used with reference to the cell cycle phases than to cell compartments. In such estimations the total cells in each phase of the cycle are considered as forming homogeneous sub-populations of cells. Using the size, time and flow parameters it is possible to estimate the overall cell production of renewal systems. These methods of estimation will be described under measurement of cell renewal in Section 2.6.

1.3.6 Steady State of a Cell Renewal System

Normal tissues undergoing cell renewal maintain a steady state. Using the parameters discussed in the earlier section 1.3.5, it is possible to describe the features of a steady state.

The size distribution of cell compartments is maintained within constant limits in a steady state. In other words the cell production and cell loss are balanced and the flow of cells must be precisely regulated. The rate of entry of cells (influx) is equal to the rate of exit (eflux) of cells (Quastler, 1959).

The size of a cell compartment is proportional to the time cells spend in that compartment, provided the flux of cells is constant. This allows estimation of any one parameter, when the other two parameters are known. The assumption that the flux of cells is constant is true only in completely asynchronous cell populations, where the cells are distributed uniformly throughout the different phases of the cell cycle. However, some authors suggest that deviation from complete asynchrony to partial synchronization of cells in the S phase does occur in some cell renewal systems (Pilgrim, Erb and Maurer, 1963). These manifest mostly as diurnal variations in cell cycle parameters. Where such variations are present, it may be necessary to obtain 24 hour mean values of size, time and flux, to estimate the cell production rate. Even then, an assumption that a 24 hour balance in cell production and cell loss occurs during cell renewal has to be made.

1.4 HOMEOSTASIS IN EPITHELIAL RENEWAL SYSTEMS

Mitotic activity of renewal tissues in a steady state proceeds at different rates in various tissues, in such a manner as to maintain a constant size of the total cell population. In an epithelium the basic mean rate of cell production is related to the rate of cell ageing in the maturation compartment and cell loss from the surface and probably also to its thickness and functional activity. The control of cell production in renewal tissue appears to reside within the tissue itself. An understanding of the fundamental problem of mitotic homeostasis requires the elucidation of controlling factors and their nature and action upon the tissues.

1.4. 1 Evidence for the Presence of Controlling Factors

Abercrombie (1957) suggested the possibility of the presence of a mitotic stimulating substance which is responsible for continued cell proliferation. The abnormally high mitotic activity adjacent to a wound was attributed to the stimulating effect of this substance called 'wound

hormone' or trephone and it was thought that this substance was produced by the damaged tissues (Abercrombie, 1957).

Bullough and Laurence (1960) examined the local factors controlling mitotic activity by studying epidermal wounds in the mouse. By wounding hypodermis and dermis from below, without causing any damage to the epidermis, they found that epidermal mitotic activity over such wounds was normal. Therefore, they suggested that damage to the epidermis itself is necessary before the development of increased epidermal mitotic activity. Following epidermal wounding these authors found an increased mitotic activity and this was greatest at the wound edge usually within a distance of 1 mm.

Bullough and Laurence (1960), also critically examined whether high epidermal mitotic activity adjacent to a wound was due to a wound hormone stimulating mitoses, or due to local elimination of a mitotic inhibitor which is normally present in the epidermis. They used the mouse ear as the experimental model to test the above two possibilities as this site provides two closely adjacent epithelia on either side of the ear, one of which could be wounded, allowing observation of the effects on the opposite epidermis. Epidermal wounds of 3 mm square were created on one side of the ear and the mitotic activity of the skin epithelium opposite this wound was analysed. This revealed that the mitotic activity in the epithelium of the undamaged side was greater opposite the central area of the wound than in the area of the wound edges. From this they concluded that increased mitotic activity in adjacent areas of an epithelial wound was due to the removal of a local mitotic inhibitor, which normally controlled the epidermal cell production. From this evidence Bullough and Laurence (1960) and Bullough (1964) concluded that the control of mitotic activity in epidermis is by mitotic inhibition, due to the presence of a local factor in the tissue.

1.4.2 Some Properties of Mitotic Inhibitors

In 1964, Bullough, Hewett and Laurence, succeeded in extracting a mitotic inhibitor from epidermal tissue. Bullough and Laurence (1964), further investigated the nature of this proposed epidermal mitotic inhibitor by preparing an aqueous extract of macerated mouse epidermis and using this to demonstrate that the epidermal mitotic activity of mouse ear fragments in vitro, is suppressed by the addition of this macerate to the culture medium. It was found that the degree of suppression was proportional to the amount of macerate used.

Bullough and Laurence (1964) suggested that the epidermal mitotic inhibitor is tissue specific. This information was derived by using various macerated tissues such as kidney, liver, lung and brain to prepare aqueous extracts and using these to demonstrate that epidermal mitotic activity in vitro was unaffected by these extracts.

The epidermal macerate was found to be effective, even on some of the epidermally derived tissues such as corneal epithelium and sebaceous glands. It was found to be ineffective in reducing the mitotic activity of hair follicles. This may be related to the high mitotic activity in hair follicles, which possibly have a different mitotic control mechanism or agent from that of tissues with moderate mitotic activity.

Iverson, Aandahl and Elgjo (1965), and Elgjo (1969), using intraperitoneal injections of aqueous skin extract, demonstrated in vivo, the inhibition of epidermal mitoses in mice, thus confirming the results of Bullough and Laurence (1964) which were obtained by in vitro analysis.

The mitotic inhibitory power of the epidermal macerate was shown to be not specific to the strain or the sex of animals (Bullough and

Laurence, 1964). This was demonstrated using male and female mice from different strains for preparing the epidermal macerate and for obtaining the tissue fragments used for analysis.

The epidermal mitotic inhibitor is neither species nor class specific and therefore appears to be similar in all vertebrates. The non species specificity was demonstrated by Bullough and Laurence (1964) using epidermal macerate from mice, rat and guinea pig and the similarity of the vertebrate epidermal mitotic inhibitor from fishes to mammals was demonstrated by Bullough et al (1967) using skin macerates from guinea pig, pig, rat, hamster and cod fish.

Boldingh and Laurence (1968) using pig skin powder prepared in different ways, characterised the mitotic inhibitory agent as a protein or a glycoprotein with a molecular weight of 30, 000 to 40, 000.

1.4.3 Terminology and Definitions for Epidermal Mitotic Inhibitor

To represent this mitotic inhibitor present in epidermal tissues, the term chalone was suggested by Bullough (1962). He thought that the term hormone would be unsuitable because by definition a hormone is a substance which is produced by one tissue with a primary function of exerting a specific effect on a different target tissue (Starling, 1906). Also the effect of a hormone is stimulatory in nature rather than inhibitory. The term chalone derived from χαλάω (to make slack) as originally proposed by Schäfer (1916), appears to be more appropriate for a substance with an inhibitory effect.

Chalone was therefore defined as an internal secretion produced by a tissue for the purpose of controlling mitotic activity of the same tissue by an inhibitory mechanism (Bullough, 1967). Eleven chalone systems have been described so far (Laurence et al, 1972) namely in granulocytes, erythrocytes, lymphocytes, sebaceous glands, kidney, liver, fibroblasts, melanocytes,

endometrium and the epidermis. From the evidence collected in these different systems, the biological aspects of chalone have been characterised by these workers as follows:-

(1) Chalones compel a cell to proceed to maturation and thereby inhibit mitosis.
(2) This action is reversible.
(3) They act both in vivo and in vitro.
(4) Their action is tissue specific and is not species specific.
(5) Each chalone is present in the tissue on which it acts.

1.4.4 Mechanism of Action of Chalone

Bullough's concept of the presence of a mitotic inhibitor produced by differentiated cells and having an anti-mitotic effect on the undifferentiated cells, is widely accepted now as the basic mechanism for mitotic and functional homeostasis. This hypothesis is in accordance with the general growth control mechanism proposed by Weiss and Kavanau (1957), in which they stated that each differentiated cell in a growing tissue produced specific freely diffusible compounds (anti-templates) that catalysed the "generative mass". However, the mechanisms by which chalones operate are not fully evident. The negative feed-back mechanisms of growth control in epidermis proposed by Mercer (1962) and Iverson (1961) suggest that the information required for secretion of mitotic inhibitory agents is produced by the differentiating or keratinizing cell population. These factors regulating cell proliferation must act on the progenitor cell compartments controlling the entry of cells into the dividing cell cycle.

Bullough and Laurence (1961) originally postulated that the inhibitory factor operates in the G_2 phase by inhibiting the entry of cells into prophase. They showed that the number of epidermal mitoses developing after

experimental stress in mice was proportional to the duration of the stress episode. Once the inhibitory effect was removed, cells which had accumulated in the G_2 phase during the stress period, entered mitosi s in increasing numbers (Bullough, 1964). Defendi and Manson (1963) suggested that the control mechanism should be acting at an earlier phase in the cell cycle before the cells begin DNA synthesis, in preparation for division. Swann (1958) and Bullough (1962) had also suggested the possibility, that this control could be acting before a cell starts to manufacture proteins necessary for division or differentiation.

Gelfant (1962) endeavoured to determine whether epidermal cell proliferation is initiated in the G_1 or the G_2 phase of the cell cycle, and analysed the new cells entering mitosis by DNA labelling and mitotic arresting techniques after wounding mouse epidermis. He suggested the presence of a unique cell population present in the G_2 phase which was ready to divide at short notice. Such cells appeared as unlabelled mitoses following DNA labelling. Another cell population was present which entered DNA synthesis from the G_1 phase, one hour after the application of the stimulus and these cells appeared as labelled mitoses. A cell population with a long G_2 phase, with an ability to divide after a relatively short period on application of an appropriate stimulus as proposed by Gelfant, has been criticised by Bullough (1963), and has not received general acceptance.

Following the reports confirming a regular circadian rhythm both in mitotic activity and in DNA synthetic activity, the idea of growth controlling factors acting only at the transition from G_2 phase to mitosis, has been disputed. Working on a new hypothesis that there could be more than one controlling factor acting at different phases, Elgjo, Laerum and Edgehill (1971, 1972) described possible control mechanisms in cell proliferation in mouse epidermis by two individual local factors. One of these, known as the G_1 inhibitor, was thought to be produced by the differentiated cell layers and to have an inhibitory effect on the entry of cells into DNA synthesis.

The other, produced by progenitor cells which in mouse epidermis are the basal cells, was called the G_2 inhibitor and was postulated to have an inhibitory effect on epidermal mitosis. The effects of these two inhibitory agents were demonstrated by separating the basal cell layer from the rest of the keratinizing epithelium (Laerum and Boyum, 1970) and preparing extracts from the two fractions of epithelium. Elgjo and Hennings (1971) and Elgjo (1973) have also shown other properties in which these two agents differ.

These two mitotic inhibitors, G_1 and G_2, while having an overall effect on epithelial homeostasis, may also play a role in the circadian rhythms seen in cell kinetic parameters (Section 1.5).

1.4.5 Chalone Studies in Oral Epithelium

Hansen (1967) demonstrated that an epidermal macerate, when injected into mice, inhibited the mitotic activity of tongue and gingival epithelium. It is believed that a chalone mechanism is operative in oral epithelium and that this is similar to the epidermal chalone system.

In order to determine whether chalonesin skin and oral epithelium are similar in their biological characteristics, Laurence and Hansen (1971 and 1972) used extracts from pig skin, palate and gingiva to study the degree of mitotic inhibition produced by these extracts in mouse ear and tongue epithelium. They demonstrated a greater depression in mitosis in mouse tongue epithelium when 'oral epithelial chalone' was injected compared to that found when 'epidermal chalone' was administered. Mitoses in ear epidermis were less frequent when chalone extracts from epidermis were used as compared to chalone extracts from the palate. This suggested a possible regional specificity in chalone action, and indicated that the oral epithelia may have a slightly different chalone system from that of epidermis.

1.4.6 Newer Concepts of the Control of Epithelial Thickness

Bullough (1972) reviewed the mode of action of the epidermal chalone system in the control of epidermal thickness. In normal epidermis

chalone seems to have a dual function. It inhibits mitotic activity in the progenitor cells and maintains mature stratum spinosum cells in the post-mitotic state preventing them from reverting to mitosis. This implies that reduced levels of chalone could result in hyperplasia of the progenitor cell compartment and mitoses manifesting at abnormally superficial sites in epithelia. Such a reduction in the chalone concentration below a critical level, in epidermal cells, could arise as a result of a lack of synthesis of chalone or by a rapid chalone loss across an abnormal cell membrane. Excessive epithelial cell proliferation may also arise as a result of failure of cells to respond to chalone which is present in the tissue.

Bullough (1972) classified epidermal cell renewal systems into three phases, depending on the relationship of mitotic rate, rate of cell ageing and epidermal thickness. The mitotic rate was defined as the number of mitoses occurring per hundred basal epidermal cells per day, and post-mitotic ageing was measured as the number of days taken by an epidermal cell to pass from its last mitosis to its death in the base of the stratum corneum. Phase 1 was characterised by thin epithelia with a flat epithelial connective tissue interface and having progenitor cells limited to one (basal) cell layer. Covering epithelia with these features have a low mitotic rate. Normal mouse epidermis and hypoplastic or atrophic human epidermis were grouped in Phase 1. Epithelia with a higher degree of mitotic activity were grouped in Phase 2. In the lower range of this phase were those epithelia demonstrating progenitor cells still limited to one (basal) cell layer but with almost all of these cells engaged in cell production. The hyperplastic mouse epidermis and the normal human epidermis were classified in this lower range of Phase 2.

When the level of chalone in an epithelium is temporarily reduced below a critical level, an increased number of cells enter the cell division cycle. There is also a progressive shortening of the time spent in the various cell cycle phases. As a consequence, more daughter cells are

produced and this results in a multilayered progenitor cell compartment and folding of the epithelial connective tissue interface. These features are seen in the upper range of Phase 2 epithelia. Hyperplastic human epidermis was grouped in this category by Bullough (1972). The increased cell production in this situation, however, is balanced by a rapid cell maturation and cell loss.

Phase 3 comprises other pathological states such as carcinomata, where increased cell production is not balanced by the cell loss.

It is not yet clear to what extent this three phase categorisation of epithelial thickness can be applied to oral epithelium. From the available data on cell kinetics of animal oral epithelia, it appears that hamster cheek pouch epithelium with a single progenitor cell layer limited to basal cells, would fit into Phase 1. Cell renewal systems with a multilayered progenitor cell compartment such as hamster palate (Thilagaratnam, 1969) and the oral epithelia of rat and rabbit would belong to Phase 2. Human oral epithelium probably also belongs to the upper range of Phase 2.

So far the discussion on epithelial homeostasis has centred on a tissue level. Control mechanisms of cell proliferation need to be looked at from cellular and subcellular levelsas well. In this regard inhibitory and stimulatory effects on mitogenesis by cyclic nucleotides have been reported. Birk (1968) and Ryan and Heidric (1968) first presented evidence for the possible role of adenosine 3' 5' monophosphate (cyclic AMP) in the regulation of cell division by mitotic inhibition in cell cultures. Voorhees, Duell and Kesley (1972) and Marks and Rebien (1972) demonstrated similar effects in rat and mouse epidermis. Guanosine 3' 5' cyclic monophosphate (cyclic GMP), was described as having a stimulatory effect on mitoses by Hadden et al (1972).

Further studies on the levels of cyclic nucleotides in relation to cellular proliferation in human skin have been conducted by various groups of workers. Voorhees et al (1972 a, b), Hsia et al (1972) and Wright et al (1973) demonstrated decreased cyclic AMP levels in hyperplastic skin disorders. In 1973, Voorhees, Stawiski and Duell showed increased cyclic GMP levels in a similar condition. Voorhees et al (1974) concluded that an imbalance of the ratio of cyclic AMP and cyclic GMP levels may have a relevance to the pathophysiology of proliferative skin disorders.

By comparing the actions of chalones and cyclic AMP, Iverson (1969) hypothesised that chalones may act via the cyclic AMP system. Voorhees et al (1974) discussed the possible functions of cyclic AMP in epidermis and concluded that in addition to the effect of glycogenolysis, intracellular cyclic AMP produces mitotic inhibition by its effect on the transition of cells from the G_2 phase to mitosis. Elgjo (1975) demonstrated that epidermal G_2 inhibitor (Section 1.4.4) required a certain cyclic AMP level for its action in mouse epidermis, although this was not critical for the epidermal G_1 inhibitor.

Sutherland and Robison (1966) showed that adrenaline caused beta receptor stimulation of the plasma membrane bound enzyme adenyl cyclase and thereby the release of cyclic AMP. The metabolic and physiological actions of adrenaline therefore appear to be mediated by cyclic AMP.

Although chalone mechanism was originally thought to exist as a chalone-adrenaline complex (Bullough and Laurence, 1964), such a complex has not been demonstrated. It now appears that both chalone and adrenaline which control the functional and mitotic homeostasis in epidermal tissues are exerting this effect via the cyclic AMP system.

1.5 FACTORS AFFECTING CELL KINETICS OF NORMAL TISSUES

Apart from locally produced mitotic inhibitory factors discussed in Section 1.4.3, many other factors are known to affect the cell kinetic parameters of renewal systems. It is necessary to look at these factors in detail and consider how they affect the cell kinetic parameters, in order to control or compensate for these variables during a cell kinetic study. Furthermore, in tissues which show a variation in histological type, these variable features may be correlated with differences in cell production parameters.

For convenience the factors influencing cell kinetics can be broadly divided into two groups as (1) general factors and (2) variable features in oral epithelium that may be related to variations in cell kinetics.

1.5.1 General Factors

Lehmiller (1971), discussed the general factors affecting cell kinetics in different cell renewal systems. He listed these as hormonal factors, stress, diurnal variations, nutrition, temperature and age.

Hormones secreted by the adrenal gland, adrenaline from the medulla, and glucocorticoid hormones from the cortex, are known to inhibit mitotic activity. The effects of these hormones on epidermal mitosis are discussed in detail in Section 1.6.3. Sex hormones and therefore the oestrous rhythm was described as affecting cell kinetic parameters in mouse skin by Bullough (1943) and these effects were considered in detail by Epifanova (1971). Epifanova (1966) indicated that oestrogen and progesterone have a pronounced effect on cell proliferation of target tissues and also a similar but less pronounced effect on non-target epithelial tissues. These hormones may therefore be considered as mitogenic, in that they stimulate or trigger cells from resting phases into the dividing part of the cell cycle. However, a limited investigation in human females by Fisher (1967) did not reveal any simple correlation of mitotic rhythm of epidermis with the menstrual cycle.

Stress appears to be a major factor in altering the cell kinetics of a tissue. The effects of stress could act via the alteration of levels of stress hormones (Bullough, 1962) or by bombardment of the autonomic nervous system. During animal experimentation, stressful situations may arise from handling of animals, methods of cageing, physical and chemical trauma and by the use of injections and anaesthetic agents.

Cell cycle parameters are known to vary during a 24 hour period. The numbers of cells in particular phases and the time spent by cells in different phases can show consistent variations with time. These changes are referred to as diurnal variations. The diurnal variations are mostly related to the activity patterns of animals or man and are discussed in detail in Section 1.6.3.

Nutrition can affect cell kinetics in different ways. Starvation may elicit a stressful situation in laboratory animals and this may alter normal parameters. Usually food and water are made available to animals ad libitum in most laboratories, but filling the food jars may disturb the sleeping habits of nocturnal animals and therefore reflect as stress, sufficient to vary mitotic rhythms. When gastrointestinal tract epithelium is being studied, the time interval between the last feed and sacrifice of the animal may have a bearing on observed variations (Alov, 1963; Sigdestad and Lesher, 1972).

Variations in body temperature are known to affect cell kinetic parameters. In mouse ear, where the temperature is lower than the average body temperature (Sherman, Quastler and Wimber, 1961), cell renewal is known to progress more slowly than in other tissues. Increasing the room temperature was found to induce cell proliferation in mouse ear in vivo by Gelfant (1975). Schmid et al (1974) have

demonstrated the effects of variations of temperature on DNA replication during in vitro organ culture of rat epidermis.

Opinion on the effect of age on mitotic activity of epithelial tissues is divided as a controversy exists as to whether this increases or decreases with ageing. The general opinion among clinicians is that wounds heal faster in children than in the aged and this leads to the idea that mitotic activity is reduced with age. However, closer survey on available experimental evidence does indicate that mitotic activity may increase with age.

Meyer, Marwah and Weinmann (1956) examining human attached gingiva in two age groups showed a 50 per cent increase in the number of cells in mitosis with age. In human epidermis, Thuringer and Katzberg (1959) have claimed that mitotic activity increases from birth up to the fifth decade and thereafter remains stationary. Bertalanffy, Pusey and Abbott (1965) showed a 50 per cent increase in the rate of entry of cells into mitosis in senile rats as opposed to younger rats.

The reported work by Lavelle (1968) on rat oral epithelium and that by Ryan, Toto and Gargiulo (1974) on human gingival epithelium did not reveal any statistically significant differences in mitotic activity in different age groups. Karring and Löe (1973) found an increase in mitotic duration in rats belonging to an older age group, while the frequency of mitoses was found to be similar or decreased with age. They favoured the opinion that mitotic activity decreases with age.

Karring (1973) discussing the effect of age on mitosis considered that variations in epithelial cell density, which may occur as a result of ageing, may also alter the estimated frequency of mitosis by influencing the reference unit used for the calculation (Section 2.5.2). Löe and

Karring (1971) in human gingival epithelium, and Karring and Löe (1973) in rat oral epithelium, did not find any reduction in cell density with ageing. On the other hand, Meyer, Marwah and Weinmann (1956) and Ryan, Toto and Gargiulo (1974) found an increase in epithelial cell density with age. An increase in the epithelial cell density in the older age group would cause an underestimation in the calculated mitotic index, in the studies mentioned earlier. These reasons further support the hypothesis favoured by Meyer, Marwah and Weinmann (1956) that mitotic activity increases with ageing.

1.5.2 Control of Variable Factors in Studies of Cell Kinetics

The knowledge of factors which potentially alter the dynamics of cell proliferation mentioned in Section 1.5.1 can be utilised in the design of experiments so that the experimental conditions approximate as closely as possible to normal.

Control of stress and the range of normal diurnal variation appear to be of considerable importance as both these influence the observed data, probably through a common pathway by alterations in stress hormones. Handling of experimental animals should, therefore, be limited to a bare minimum. Cageing of animals needs to be arranged with due consideration to the cage size, number of animals in each cage and also by groups receiving similar treatment to be caged together so that disturbances are limited. When animals are subjected to physical and chemical trauma, such as by application of friction, or by chemicals such as carcinogens or injection of drugs, sham treated control animals should be used whenever possible to estimate the degree of alteration of cell proliferation by the additional experimental procedures.

As diurnal variations are mostly related to the waking and sleeping habits of animals it is necessary to control this variability to a

reproducible pattern. Halberg et al (1959) indicated that lighting regimes in the animal house, when properly regulated, could control this variation to a rhythmic pattern. Sigdestad and Lesher (1971) have shown that reversal in lighting regimes alters the established rhythmic variations in mitotic activity. Effects of diurnal variations on cell cycle parameters should be taken into account during analysis of results, and the need for this is discussed in Section 1. 6.

Maintenance of room temperature in animal houses close to that of average body temperature appears to be important and this principle is also applicable to the in vitro situation where the incubation temperature needs to be controlled.

As the effects of sex hormones and the influence of age changes on cell production are known, there is also a need for age and sex matched controls in cell kinetic studies.

1. 5. 3 Variable Features in Oral Epithelium Affecting the Rate of Cell Proliferation

The mitotic activity of oral epithelium and therefore cell proliferation is related to the thickness and the degree of keratinization. These two features are known to vary widely in different areas of the oral cavity. Alvares and Meyer (1971) reviewed these variable features and discussed the relationships of these to the mitotic rate and to functional and regional differences seen in the oral epithelium. The information for their discussion was derived from studies in mouse, rabbit and human oral mucosa conducted by Weinmann and Meyer's group during their pioneering work in the early 1960's.

As has been mentioned already, these variables appear to modify epithelial cell proliferation in some ways and certainly affect the interpretation and comparison of cell kinetic data. Therefore it is

pertinent to discuss these features briefly.

Mitotic activity and epithelial thickness:

The stratified squamous epithelium covering the different regions of the oral cavity in mice was shown by Meyer, Medak and Weinmann (1960), to vary in thickness. Epithelial thickness in four regions was examined; these being the floor of mouth, the central and lateral palate and the cheek mucosa. The thickness of the cellular layers was found to vary from 21.2 μm to 116.5 μm, the thinnest being the floor of mouth and thickest the buccal epithelium. Of the four regions examined the buccal epithelium had the fastest daily growth rate. They reported an approximately rectilinear relationship between the growth rates of the four regions and the thickness of the cellular portions, growth increasing slightly faster than thickness.

Gigoux (1962), in rabbits reported that the mitotic activity is higher in the thicker cheek epithelium than in the thin ventral surface of tongue. The relationship between epithelial thickness and mitotic activity was found to be non-linear. While the thickness varied by a factor of 5.3 in these two sites, the mitotic activity was 4.4 times higher in the thicker epithelium.

The correlation of epithelial thickness and proliferation rate was confirmed by Christophers (1972a)in guinea pig epidermis and later by Christophers and Laurence (1973) in the mouse, by a comparison of these features in epidermis using two different body sites, the ear and the sole of the foot. They found that the soles in mice were 3.4 times thicker than ear epidermis and had 5.3 times more cells in DNA replication preparing for division.

Subjectively, human oral epithelium appears to have a marked

variation in epithelial thickness in different regions and even in a given anatomical site. In a quantitative study, Meyer and Gerson (1964) reported that human buccal epithelium is 1.7 times thicker than the palatal epithelium. A detailed knowledge of the variation in thickness and its relation to cell proliferation in human oral epithelium is still not available.

Degree of keratinization and mitotic activity:

The importance of estimating the degree of keratinization in oral mucosa was first stressed by Weinmann (1940) and during subsequent studies by Weinmann and Meyer (1959) and Weinmann, Meyer and Medak (1960), based on the staining behaviour of tissues with Mallory connective tissue stain. They demonstrated five types of keratinization of oral epithelium, which they designated as complete orthokeratinization, complete parakeratinization, incomplete orthokeratinization, incomplete parakeratinization and non-keratinization.

Alvares and Meyer (1971) reviewed these features of keratinization, seen in different regions of human oral mucosa. It appears that areas showing non-keratinization roughly correspond to what is generally known as the lining mucosae in oral cavity. The masticatory epithelium appears to demonstrate varying degrees of orthokeratinization and parakeratinization.

The relationship of the degree of keratinization to mitotic behaviour of the normal epithelium in different sites is still not known. However, a two-fold increase in mitotic activity in areas showing metaplastic keratinization was reported by Gerson and Meyer (1964) and a four-fold increase in parakeratinized areas as compared to orthokeratinized areas was demonstrated by Renstrup (1963) in pathological lesions.

1.6 DIURNAL OR CIRCADIAN VARIATIONS IN CELL KINETIC PARAMETERS

1.6.1 Introduction

It is known that fundamental biologic phenomena in plants and animals exhibit regular changes or variations during the day and night. These variations are rhythmic or periodic in nature and have been found to correspond approximately to a day and night pattern. The term 'diurnal variation' was used to describe these rhythms which show a 24 hour periodicity and the term 'circadian rhythm' (from circus = about: dies – a day) was introduced by Halberg (1959) to refer to these rhythms, as the length of a cycle may slightly differ from an exact 24 hour period. The terms diurnal and circadian are generally used synonymously.

Human circadian rhythms in various physiologic processes such as wakefulness, psychomotor performance, breathing, body temperature, adrenocortical activity, urinary excretion and in blood cell counts have been described (Mills, 1966) and these features were discussed in detail by Halberg (1959) and Harker (1958). It is not yet certain whether these particular rhythms are due to exogenous signalling factors, such as environmental variations in light and darkness, temperature and social habits, or whether this control is endogenous in nature, in that a biological internal clock might be operative in determining these rhythms (Harker, 1964).

It is now well established that consistent circadian rhythms are present in the number of cells in mitosis and in DNA synthesis in many cell renewal systems. Various tissues have been studied to investigate the degree of diurnal variation in these parameters and the cellular and hormonal mechanisms involved in maintenance of these biorhythms.

1.6.2 Historical Review

Studies on mitotic rhythms in plants were described at the turn of this century (Kellicott, 1904; Karsten, 1918) and application of this

knowledge to animals and mammals was first made by Fortuyn-van Leyden (1916), who reported periodicity of nuclear division in the cat. Carleton (1934) reported that mitotic activity in mouse epidermis was greatest at night and least at 12 noon and was the first to relate this periodicity to light and darkness. Mitotic rhythms in human epidermis were first reported by Cooper (1939). Diurnal variations in mitotic activity of human epidermis were later studied by Scheving (1959) and by Fisher (1968b).

Diurnal variations in DNA synthesising cells in various mouse tissues were first reported by Pilgrim, Erb and Maurer (1963) and many subsequent studies by others on different animal epithelia have yielded similar results.

1.6.3 Hormonal Influences on Diurnal Variation in Mitotic Activity

The secretion of hormones which inhibit mitosis in various tissues has been described as showing diurnal variations. The mitotic inhibitory properties and diurnal variations in the output of these hormones have been correlated with the mitotic rhythms in epithelial tissues.

Adrenaline:

The inhibitoryeffect of adrenaline on mitotic activity was first described in rat corneal epithelium by Freidenwald and Buschke (1944). Later studies by Bullough (1952, 1955), Chaudhry, Halberg and Bittner (1956) and Bullough and Laurence (1961) have confirmed that adrenaline is a potent mitotic inhibitor, both in vivo and in vitro.

Bullough (1948 a) was able to relate the diurnal variations in mitotic activity of the epidermis in adult male mouse, to its waking and sleeping habits. Mitotic activity was maximal when the animals were resting and sleeping and was minimal during periods when the animals were awake and active. Furthermore, indices of mitotic activity were

found to be parallel in different mice and in different tissues of the same mouse. Experimentally induced rest (sleep) by injection of barbiturate resulted in a sharp rise of mitotic activity and enforced exercise by placing the mice in a slowly rotating box resulted in extremely low mitotic activity (Bullough, 1948 b).

The rate of adrenaline secretion is lowest during sleep and high during muscular exercise and emotional stress (Euler and Hellner, 1952; Euler and Laudberg 1954). It is also known that diurnal rhythms in adrenaline output are present in the rat (Euler, 1956; Dunn and Lin, 1974) and in man (Karki, 1956). Bullough and Laurence (1961) and Bullough (1962) first suggested that these two rhythms, the adrenaline output and epidermal mitotic activity, could be inter-related and drew the inference that the variations in the stress hormone concentration during rest and exercise were responsible for the diurnal variations in mitotic activity.

In order to confirm this effect of adrenaline on mitotic activity, Bullough and Laurence (1961) carried out adrenalectomy in adult male mice. These animals showed a marked rise in epidermal mitotic activity two days after adrenalectomy, with loss of normal diurnal rhythm. Subsequent injection of 10 mg of adrenaline produced a fall in epidermal mitotic activity.

Later, Bullough and Laurence (1964), from in vitro experimental data suggested that adrenaline alone has no power to reduce the mitotic activity but was able to do so by forming an unstable chalone-adrenaline complex (Section 1.4.3). They concluded that the diurnal mitotic rhythm reflected the fluctuations in the adrenaline content of blood. The mechanism of action suggested was that the epidermal mitotic rate rises as a result of the breakdown of chalone-adrenaline complex when the level of adrenaline in blood falls during sleep (Bullough, 1965).

However, reports from other workers have not been in complete agreement with Bullough's suggestions on the role played by adrenaline on mitotic inhibition. Evensen (1964), on the grounds that adrenaline is responsible for speeding up physiological activity in the body, doubted the concept that it should have the opposite effect on mitosis. His experiments on mice demonstrated that adrenaline had a promoting effect on the epidermal mitotic rate and he raised the possibility that the reason for lower mitotic counts being found in adrenaline treated tissue was because of loss of the power of the mitotic inhibitor colchicine to arrest mitoses which were speeded up by adrenaline (Evensen, 1964; Evensen and Heldaas, 1964)

Susequently Bullough and Laurence (1966) re-investigated the action of adrenaline on epidermal mitoses and concluded that adrenaline has a dual action on the mitotic process; in that it accelerates the rate of completion of already existing mitoses and slows down both the entry of new cells into mitosis and their rate of completion of division.

Alov (1959) and Scheving and Pauly (1967a) have shown that circadian rhythms in mitotic activity continue in the absence of adrenal glands and that the only observable change is a reduction in the mitotic rate.

Although the antimitotic action of adrenaline has been confirmed by many studies, Bullough's hypothesis that it plays a central role in maintaining the circadian rhythm of mitotic activity cannot be considered as proven as direct evidence to this effect is still not available. Critical experiments correlating circadian rhythms in stress hormones and epidermal mitotic rhythms have not been reported on individual animals. By comparing the periodic mitotic activity in different organs in the albino rat, Blumenfeld (1942) found that the 24 hour curves of

mitotic activity differed for each organ. Tvermyr (1969) reported a lack of parallelism of circadian mitotic rhythms in ear and dorsal mouse epidermis in the same animal. This does indicate that apart from body hormones there are other factors involved in the regulation of mitotic rhythms.

Glucocorticoid Hormones:

Glucocorticoid hormones have been shown to exert an anti-mitotic effect by Bullough (1952), and Bullough and Laurence (1968). Glucocorticoid hormone secretion exhibits a diurnal variation (Halberg, Peterson and Silber 1959). As in the case of adrenaline, although glucocorticoid hormones are believed to exert an antimitotic effect, their role in maintaining a circadian rhythm is still unproven. Fisher (1967) stated that glucocorticoid hormones are more important than adrenaline and noradrenaline in maintaining the diurnal mitotic rhythms in intact animals.

1.6.4 Diurnal Rhythms in the Number of Cells in Mitosis and S Phase

Evidence for the presence of a diurnal rhythm in the mitotic activity of human epidermis is available. Fisher (1967, 1968a) showed a nine-fold variation in the mitotic index in normal adult males throughout the day and night. The mitotic index was found to be lowest around 12 noon and was greatest between 1 a.m. and 7 a.m. Studies on the mouse, the rat and the hamster by others have demonstrated mitotic rhythms opposite to that described by Fisher (1967) in man. In general, in these nocturnal animals peak mitotic activity is during the daytime and the minimum mitotic activity is around midnight.

Since the observation of diurnal variations in the numbers of DNA synthesising cells in various mouse tissues by Pilgrim, Erb and

Maurer (1963), evidence has accumulated that this is a consistent finding in many other animal epithelial tissues (Pilgrim, 1967; Scheving and Pauly, 1967b; Grube, Auerbach and Brues, 1970; England and Burke, 1971). A detailed study by Brown and Berry (1968) showed that the diurnal variation in the number of cells in S phase in the hamster cheek pouch had a four-fold variation over a 24 hour period, with a peak at midnight and a minimum number during early afternoon. Diurnal variation of S phase cells in human epidermis was investigated by Kahn et al (1968) and only a slight increase in the number was observed at midnight, as opposed to a several-fold variation in epithelial tissues from animals.

The magnitude of any diurnal variations in mitotic activity or in the number of cells in S phase, in human oral epithelium is not known.

1.6.5 Pitfalls in Estimation of Cell Kinetic Data in the Presence Of Circadian Rhythms

Mills (1966), in a review on human circadian rhythms, stated that any observation without a statement of the time of the day at which it was made may be suspect. Pitfalls arising from non-recognition of circadian rhythms of S and M phases of the cell cycle were discussed by Scheving and Pauly (1973).

In order to eliminate the errors arising from circadian variations in cell cycle parameters most investigators sample their tissue at one time of the day. Although this would be reasonable for completely asynchronous cell populations, this method of sampling does not reveal absolute data for synchronous and partially synchronous cell populations (Section 1.6.6) demonstrating circadian rhythms. The extent of circadian variation in the parameters under study should be

known before assuming that data collected at one time of the day is representative of the total proliferative behaviour of the tissue. Where diurnal variation is large, a 24 hour mean value is required for each parameter used in cell kinetic estimations.

1.6.6 Relationship Between the Number of Cells in M and S Phases: Cell Flux and Phase Durations

The number of cells seen in one phase of the cell cycle at a particular time depends on the rate of entry of cells into that phase and on the time spent by cells in that particular phase. Diurnal variations in both of these could result in increased or decreased numbers of cells in a particular phase of the cell cycle at any one time during a 24 hour period.

Until 1963, it was generally believed that diurnal variation in the number of cells in mitosis, was a direct result of different rates of entry of cells into mitosis from the G_2 phase during a 24 hour period. Bullough (1962) related this rhythm to the stress hormone secretion in blood (Section 1.6.3). Alternatively, the effect of these hormones in increasing or decreasing the mitotic duration (Tm) was thought to be important in the causation of apparent mitotic rhythms (Evensen, 1963).

The observation by Pilgrim, Erb and Maurer (1963) of diurnal rhythms in the number of DNA synthesising nuclei, and their hypothesis that this was due to the effect of partial synchrony of cells entering the S phase, indicated a requirement for re-investigation of many previously held views in order to assess whether the diurnal variations reported in the number of cells in mitosis was a later

reflection of the partial synchronisation present in the S phase. If this were the case, the mechanism of regulation, of diurnal rhythm of the number of S and M phase cells, could be acting at the stage of the entry of cells from G_1 into the S phase or even prior to this in the cell cycle rather than at G_2 stage preceding mitosis.

Brown and Berry (1968) and Izquierdo and Gibbs (1972) investigated diurnal variations in the number of cells in mitosis (mitotic index) and the number of cells in S phase (labelling index) and described circadian rhythms of these two parameters in the hamster cheek pouch. By comparison, they found that the variations in mitotic rhythm were largely a reflection of diurnal variations in the number of cells in the S phase manifesting a few hours later as cells proceeded to later parts of the cell cycle. Grube, Auerbach and Brues (1970) in a similar study on mouse dorsal epidermis, reported that the flow of cells from G_1 to S phase is a changing parameter and that this alone is sufficent to cause diurnal rhythm in the number of cells seen in the S phase and in mitosis at any one time.

Only a few investigators have measured the duration of the M phase (T_m) or the S phase (T_s) accurately to estimate whether variations in the time spent by cells in these phases could contribute to the increased or decreased number of cells in these phases found at a particular time. Brown and Berry (1968) found a slightly longer T_m at a time when the mitotic index was greatest. Izquierdo and Gibbs (1972), Grube, Auerbach and Brues (1970) could not detect any variation in T_m or T_s. Tvermyr (1969, 1972) reported circadian rhythms in the number of cells in S and M phases and the duration of mitosis and the S phase in mouse epidermis. The number of cells in

the S phase varied roughly about four and a half times between minimum and maximum and the Ts at different times varied from 5 - 12 hours. The rate of cell entry into, and exit from, the S phase was also reported to show rhythmic variations.

At present, the influence of the changing flux of cells or possible variations in the duration of phases, leading to definite circadian rhythms in the number of cells seen in a particular cell cycle phase, is not clearly understood.

1.6.7 A Suggested role of G_1 and G_2 Inhibitors in the Maintenance of Circadian Rhythms

Elgjo, Laerum and Edgehill (1971, 1972) described two inhibitory factors produced by epithelium which could control the entry of cells into S and M phases (Section 1.4.4). A G_1 inhibitor produced by differentiated, mature cells acts on cells entering DNA synthesis in the proliferative cycle, and a G_2 inhibitor produced by the progenitor cells acts on cells entering mitosis from the G_2 phase. No work has been reported so far on whether circadian rhythms in cell cycle parameters are due to alterations in the production or the concentration of these two inhibitors. Variations in the relative sizes of progenitor cell and maturation compartments in an epithelium, could result in corresponding alterations in the inhibitor concentrations produced by each compartment. As a result of exfoliation of superficial cells, the maturation compartment could form a smaller proportion and the progenitor cell compartment a bigger proportion of a total lining epithelium, following maximum desquamation at the end of the day's activity and this could reduce the inhibitory effects of G_1 while increasing the effects of G_2 inhibitor (MacDonald, D.G., personal communication, 1973). Whether or not daily habit patterns could

incorporate feedback mechanisms for regulation of circadian rhythms in cell cycle parameters needs further detailed investigation.

1.7. SOME ASPECTS OF ALTERATION IN EPITHELIAL CELL KINETICS IN EXPERIMENTAL AND DISEASE STATES

1.7.1 Abnormal Growth - General Concepts

Quantitative abnormalities of cellular growth occur frequently. Depending on causative factors there may be excessive or decreased production of tissue. With an increased functional demand, permanent cell populations either show no morphological change (neurons) or else an increase in size of individual cells (cardiac muscle). Tissues composed of labile or renewing cell populations respond to stimuli by an increase in cell numbers. An increase in size of an organ or a tissue as a result of an increase in individual cell size is known as hypertrophy and when it is the result of increase in cell numbers it is called hyperplasia (Walter and Israël, 1974). In contrast to these two categories of growth disorders, abnormal new growths are referred to as neoplasms. By definition, a tumour or a neoplasm is an abnormal mass of tissue, the growth of which exceeds and is unco-ordinated with that of normal tissue and persists in the same excessive manner after cessation of the stimuli which evoked the change (Willis, 1967). One of the prerequisites for the transformation of a normal cell into a neoplastic cell is the ability to proliferate. More than 80 per cent of spontaneously arising malignant neoplasms are derived from renewing cell populations, such as tracheo-bronchial, gastro-intestinal, genito-urinary epithelia or epidermis (Oehlert, 1973).

Normally, thickness of surface epithelia is maintained within narrow limits by a process of continued cell production in the deepest cell layers (progenitor cell compartment) followed by maturation and

cell loss at the surface. It appears that local chemical mediators known as 'epidermal chalones' (Section 1.4.3) are operative in maintaining this balance in cell production and cell maturation. The characteristics of such renewal systems in normal health and maintenance of epithelial homeostasis were discussed in Section 1.4.

There is evidence to indicate that disease processes which manifest as atrophy, hyperplasia, pre-malignancy or carcinomas in covering epithelia basically arise as a result of a disturbance in epithelial homeostasis. The presence of increased numbers of cells in mitosis appears to be a common finding in hyperplastic and neoplastic lesions. It appears that this phenomenon is related in some way to a fall in chalone content in the tissue (Bullough, 1972).

1.7.2 Epithelial Cell Kinetics: Histogenesis of Epithelial Lesions

To understand the histogenesis of epithelial lesions, recently there has been an interest in relating the histological appearance and also the clinical morphology (Marks, 1975) of such lesions to the altered kinetics of cell production, cell maturation and cell loss. Quantitative analysis of the kinetic parameters should reveal the state of balance or imbalance existing in the tissue between cell production and cell loss. Under certain conditions altered cell production may be compensated by altered cell loss, establishing a new balanced state (Rytömaa, 1970). Epidermal hyperplasia appears to be an example of this situation. However, at the extreme end of the spectrum of proliferative disorders are the malignant neoplasms which are recognisable by the critical histological features which probably arise at least in part from a complete breakdown of this balance in cell production and loss.

The significance of studying cell kinetics in disease states in general has been looked at by various research workers and in particular by those involved in analysis of tumour growth. To this end two monographs, one on the cell cycle and cancer (Baserga, 1971) and another on human tumour cell kinetics (Perry, 1969) have already been published.

From the point of view of this present study it is pertinent to discuss the relationship between cell kinetics and the histogenesis and histology of epithelial lesions in order to understand the biological properties of growth of epithelial lesions. It is hoped that this may provide some information useful for the diagnosis and management of such lesions. In this section the relevant knowledge presently available from experimental studies on skin and oral mucosa will be reviewed.

Studies on experimental animal carcinogenesis appear to be the field in which most of the pioneering work has been carried out. Epidermal wounding, by application of friction or stripping of surface layers or by traumatisation also has been used as a model for such studies. Cytokinetic studies in diseases of epidermal hyperplasia, such as psoriasis and in cellular dysplasias, such as in carcinoma in situ in cervix and in skin tumours, have further contributed to the understanding of the subject.

1.7.3 Cell Kinetics in Experimental Carcinogenesis

For a long time it has been obvious that a disorder in regulation of cell production is an essential feature in carcinogenesis. The most important characteristic in growing carcinomas has been thought to be an increased rate of new cell production which exceeds the rate of cell loss or cell death.

From cell kinetic studies in tumours it has become apparent that increased cell proliferation in tumours could arise by alteration

in the following factors enumerated by Baserga (1971):-

1. by increasing the fraction of cells that go through the cycle,
2. by shortening of length of the cell cycle,

and 3. by decreasing cell loss.

Comparision of cell kinetic data of normal animal tissues with that following application of carcinogens has revealed an increased number of cells in mitosis and in DNA synthesis during carcinogenesis. This has been documented by Kiljunen (1956) in rat skin, by Iverson and Evensen (1962) in mouse skin, and Reiskin and Berry (1968) and Thilagaratnam and Main (1972b) in hamster cheek pouch. The latter authors noted in hamster cheek pouch a reduction in cell cycle time from a normal value of 163.9 hours to 90.8 in hyperplasia, 22.0 hours in premalignant epithelium and to 15.4 hours in squamous cell carcinomas. This reduction was found chiefly to be due to a shortening of the time spent by the cells in the G_1 phase. However, Baserga and Kisieleski (1962) and Baserga (1965) have demonstrated that the length of the cell cycle is shorter in certain cells of adult animals than in some of the fastest growing tumours. This led them to believe that tumour growth must involve alterations of other kinetic parameters besidesthe rate of cell proliferation. This could be a function of an increased number of proliferative cells which have been triggered to enter the dividing cell cycle from resting, non-proliferative phases, such as G_o phase. The proportion of proliferative cells in a total cell population was designated as "the growth fraction" by Mendelsohn (1960, 1962). He suggested a formula to calculate the growth fraction in breast cancer of C3H mouse. The concept of growth fraction of a cell population has been found to be of considerable value in the interpretation of proliferative parameters during carcinogenesis (Baserga, 1965; Rajewsky, 1972).

1.7.4 Kinetic Aspects of Epidermal Wound Repair

Following epidermal injury by application of friction (Mackenzie, 1974a and b), by stripping of superficial layers of epidermis using adhesive tape (Pinkus, 1951, 1952; Christophers, 1972a), by hair plucking of mouse skin (Hegazy and Fowler, 1973) and by inflicting wounds in mouse ear (Bullough and Laurence, 1960) and in guinea pig epidermis (Hell and Cruickshank, 1963) it has been established that high mitotic activity and increased numbers of cells in DNA replication are associated with the healing of these skin wounds. Mackenzie and Miles (1973) have suggested that the altered structure of hamster cheek pouch epithelium in the form of epithelial hyperplasia produced following application of chronic frictional stimulation is the result of increased cell proliferation and that this is a response to an increased functional demand.

1.7.5 Cytokinetics in Diseases of Epidermal Hyperplasia

Psoriasis is a chronic cutaneous disease characterised clinically by elevated white scales and histologically by the presence of epidermal hyperplasia. This is comparable to the experimental situation of epidermal hyperplasia produced by application of chronic friction.

Studies comparing cell proliferation data in normal skin and in the lesions of psoriasis have shown vastly increased numbers of mitoses in psoriasis. Van Scott and Ekel (1963) reported that mitotic activity in psoriasis was 27 times greater than that in uninvolved skin in patients with psoriasis vulgaris. Autoradiographic studies following administration of ^{3}H thymidine, also have shown increased numbers of cells in DNA replication (Weinstein and Frost, 1968; Hell and Hodgson, 1966). Turnover studies (see Section 2.6 for definitions) have shown that cell renewal occurs much faster in psoriatic epithelium. Normal skin has a turnover time of 27 days, but in psoriasis this was found to be

reduced to 4 - 7 days (Weinstein and Van Scott, 1965; Goodwin, Hamilton and Fry, 1974).

These cell kinetic observations have contributed vastly to the understanding of the pathophysiology of psoriasis and to some extent in therapy (Weinstein and McCullough, 1973) although the primary cause of the disease is still unknown (Fry, 1968).

1.7.6 Cytokinetics in Epithelial Premalignancy and Malignancy

Although only limited data is available on cell kinetics of human tumours, because of their superficial situation, skin tumours and malignancies of uterine cervix have recently been investigated in order to quantitate the rate of cell production. Langley and Crompton (1973) reviewed the kinetics of cellular proliferation in cervical epithelial abnormalities. Richart (1963) and Rubio and Langerlöf (1974) found that the number of cells in DNA synthesis in uterine cervix gradually increased with theseverity of the lesion from normal through epithelial dysplasia to carcinoma in situ.

Cell proliferation in human basal cell carcinoma was studied by Weinstein and Frost (1970) and Heenen, Achten and Galand (1973). It appears that there is more cell proliferative activity in basal cell carcinoma than is suggested by the slow growth of this tumour apparent clinically. This may be the result of rapid cell death which occurs concurrently, or due to failure of cells to complete the cell cycle resulting in death of cells during certain phases of the cycle.

Cell kinetic studies on squamous cell carcinoma in man (Bresciani et al, 1974) have shown that it grows faster because of the co-operative effect of a higher cell birth rate and smaller cell loss factors. Fabrikant (1971) reviewed the kinetics of cellular proliferation in normal and malignant tissues using data obtained from studies on larynx, trachea and bronchial tissues of man (Fabrikant and Wisseman, 1968; Fabrikant

and Cherry, 1969; Fabrikant, 1970). He indicated that much more work needs to be done on cell proliferation kinetics in human tumours for a better understanding of the growth characteristics of normal and diseased tissue and particularly neoplastic tissues in man.

Apart from the technical difficulties in assessing human tissues, malignant tumours have characteristics such as expanding growth, which differ from steady state systems, and thus impose further problems in the analysis of tumour growth (Mendelsohn, 1963). This is particularly so in the case of squamous cell carcinomas. However, in conditions such as reactive hyperplasia, seen following chronic friction, premalignant conditions and carcinoma in situ where alterations from normal are perhaps less dramatic, it is likely that the available methods of cell kinetic studies are still appropriate as these conditions may be characterised by only minor departures from steady state cell kinetics. Such studies may provide some insight into the origin of abnormalities of cellular growth.

1.7.7 Histopathology of Oral Epithelial Lesions and the Relationship to Altered Cell Kinetics

The histological features seen in oral epithelial lesions such as reactive hyperplasias and premalignancy (Cawson, 1969, 1975; Kramer, 1969; MacDonald, 1975) appear to be closely related to a state of imbalance in basic steady state epithelial cell kinetics described in Section 1.3.6. Although oral pathologists use these histological features to define criteria for diagnosis of such lesions, it appears that the relationship of these features to altered cell kinetics has not been evaluated. Furthermore, unrelated oral epithelial lesions have been found to share common histological diagnostic features (MacDonald and Rennie, 1975).

Apart from the critical feature of invasion seen in carcinomas,

other histological features associated with epithelial hyperplasia, premalignancy and carcinomas are now commonly grouped under the term epithelial dysplasia or cellular atypia. These histological features were described in detail by Smith and Pindborg (1969). Of the 13 atypical features described by these authors most of the abnormalities appear to be related to an alteration in cell production, migration or maturation (MacDonald, 1973, 1975). A presumptive correlation of these features needs discussion with reference to oral epithelium.

Basal cell hyperplasia describes an increase in progenitor cell compartment and the associated feature of drop shaped rete ridges may arise from downgrowths of newly formed daughter cells. The feature of lack of polarity of basal cells could arise as a result of increased (rapid) cell proliferation and therefore the build up of population pressure in this area.

Histological changes associated with mitosis or cell division were described by Smith and Pindborg (1969) as increased mitotic activity, mitosis in abnormally superficial sites and as the presence of bizarre mitoses. All these features suggest the presence of uninhibited cell production and therefore a probable lack of chalone action.

A disturbance in cell migration patterns could account for irregular stratification and probably also the loss of intercellular adherence seen in disturbed epithelia. Individual keratinization of cells, below the normal level of keratinization represents a disturbance in cell maturation. Nuclear hyperchromatism may arise as a result of an increased number of cells in DNA synthesis and in the G_2 phase of the

cell cycle, or may be due to the presence of abnormal nuclei.

In order to understand the cellular abnormalities which are present in epithelial premalignancy it seems proper that the dynamic alterations in cell division and maturation which give rise to these lesions should be looked at in detail.

CHAPTER TWO

METHODS OF STUDYING CELL RENEWAL

2.1 INTRODUCTION

Recently there has been a growing interest among research workers in several fields to investigate cell proliferation or renewal in various tissues. As a result numerous methods of collecting cell kinetic data have been evolved. These methods have been reviewed in general by Leblond (1959), Wimber (1963), Baserga and Wiebel (1969), Lehmiller (1971) and, with special reference to the oral epithelium, by Skougaard (1970) and MacDonald (1971a).

Of the methods available, some are useful only in particular instances. Depending on the nature of the study the investigator often has to modify these methods to suit a particular project.

The available methods for studying cellular renewal will be described in this section, so that the application of these in the present study can be presented more simply at a later stage. The relative merits of using the different methods to study human tissues will be stated and the parameters which are useful in expressing data on cell renewal will be highlighted. Where controversy exists, in the expression of these parameters, the different methods or index systems used will be reviewed.

2.2 METHODS IN GENERAL

Methods of studying cell renewal are based on estimations of the number of cells engaged in new cell production.

2.2.1 Counting of Mitoses and Mitotic Index

The earliest method for studying cell production and cell renewal was to estimate the number of mitoses present in a cell population. This can be carried out on conventional histological sections,

but the method is subject to a number of limitations and inaccuracies.

The major limitation is the relative infrequency of mitotic figures and therefore the need to examine a large number of preparations before a reliable estimate can be made. An inaccuracy involved in the method is the subjective error in detecting cells in mitosis. Various authors have used different features to identify cells in mitoses. The early stages of mitoses are not cytologically clear-cut. Depending on the criteria employed for recognition of prophases the count of mitoses may vary. It is sometimes difficult to distinguish an early prophase from clear cells, such as melanocytes which may be present in the area. The count may be considerably in error if these are included in the mitotic count. Another possible source of error is that the nuclei of dividing cells are often larger in size than the nuclei of cells in other stages of the cell cycle. Therefore counts of cells are likely to be biased in favour of mitotic figures.

In order to express the number of mitoses counted in a histological section meaningfully, it is necessary to relate this count to a reference unit in the epithelium. The commonly used method is to relate the mitotic count to 100 or 1,000 nucleated cells in an epithelium (Section 2.5.1). This involves counting the total number of viable cells in an area which can be a time consuming and elaborate procedure. In histological sections cell counts are usually carried out by counting the number of nuclei. The section thickness can considerably influence the nuclear count and therefore the estimated mitotic index. Agduhr (1941) pointed out the error involved in such estimates, and Abercrombie (1946) suggested a formula to correct the error arising from nuclear fragments which causesan over-estimation of observed cell numbers within the volume of the tissue section counted. The correction is a function of

nuclear diameter and section thickness. Marrable (1962) and Simnett (1968) have discussed the importance of applying appropriate corrections to cell counts. However, these corrections are often ignored in cell kinetic studies.

Another critical factor involved is that the number of mitoses in a section of a tissue is dependent on the time taken for completion of mitosis (mitotic duration) and therefore this additional information is required for an estimation of the cell renewal parameters. The mitotic duration cannot be measured from fixed preparations and an independent estimation of mitotic duration has to be made.

A large number of publications have appeared on mitotic activity of epithelia in animals based on mitotic counts in histological sections. Scheving (1959) and Fisher (1967, 1968b) have used this method for quantitative studies on human epidermis.

2.2.2 Mitotic Arresting Techniques

Some of the disadvantages of simple mitotic counting techniques can be overcome by the use of mitotic arresting methods. These allow the estimation of the mitotic index and the mitotic duration from one tissue sample. Pernice (1889) described the mitotic arresting properties of colchicine, and later Dustin (1936) reported on its use as a mitotic arresting agent. Colchicine is a plant alkaloid and has a specific action on the mitotic spindle, resulting in the arrest of cells in mitosis at the metaphase stage.

Initially mitotic arresting techniques were used to demonstrate sites of cell division, but Leblond and Stevens (1948), who investigated the intestinal epithelium of rat, suggested the use of colchicine for quantitation of cell production rates. This was achieved by calculating the

number of mitoses arrested during the period of action of the drug. This method was used later to study the rat epidermis by Storey and Leblond (1951). Hooper (1961) in an extensive study on the use of colchicine for measurements of mitotic rate in the intestinal epithelium of rat, established the criteria which should be fulfilled in a study of this nature.

Mitotic arresting agents were used to study cell renewal in the oral epithelium of the rabbit by Henry et al (1952). This appears to have been the first attempt to investigate methodically the rate of cell proliferation of oral mucous membrane. Karring and Löe (1972a) used colchicine to study diurnal and regional variations of mitotic activity in rat oral epithelium. In the Syrian hamster, where colchicine is not fully effective in arresting mitosis, Thilagaratnam and Main (1972a) used vinblastine sulphate to study the mitotic activity of cheek pouch, tongue and palatal epithelia.

Mitotic arresting agents have also been used by Kreyberg, Evensen and Iverson (1965), Bullough and Laurence (1966) and Tvermyr (1969) to study the diurnal mitotic rhythms and related control mechanisms in mouse epidermis.

The use of mitotic arresting agents to obtain cell kinetic data from human tissues was first attempted by Fisher (1967). He estimated the mitotic rate (as number of cells entering mitosis per 1, 000 epidermal cells for a period of five hours) and the mitotic duration in skin, using colcemid, which is a chemically purified product of colchicine. Fisher (1968a) applied this as a cream in a cetomacrogol base to the skin of the forearm.

Metaphase arresting agents have also been used in vitro, by incubating tissues in culture media to which these agents were added

in the desired concentration (Reaven and Cox, 1968; Hill, 1973). The techn ique of arresting mitoses in vitro could prove to be a useful method for human cell kinetic studies to estimate mitotic index, mitotic duration and the mitotic rate.

2.2.3 Radioactive Labelling Techniques

The ingenious idea of labelling cells in DNA synthesis, using radioactive labelled DNA precursors, stems from the work of Howard and Pelc (1951). This provides a method of identifying cells in the DNA replication phase (S phase) of the cell cycle. Under steady state conditions, the cells in S phase proceed to mitosis at a later time (Section 1.3.4) and therefore the method provides a sophisticated way of marking and measuring cells engaged in cell renewal.

The use of radioactive labelling techniques coupled with autoradiography, has produced rapid advances in the study of cell renewal in epithelial tissues. This is largely because it is a more versatile method than the mitotic counting and arresting techniques.

Following the report by Reichard and Estborn (1951) that the pyramidine base thymidine, is incorporated exclusively into DNA, a group of biologists at Brookhaven National Laboratory successfully tritiated the DNA precursor thymidine, in the 6th position (Taylor, Woods and Hughes, 1957). In 1958 Hughes et al reported on the application of tritiated (^{3}H) thymidine for the study of cellular proliferation in various mouse tissues. Friedkin, Tilson and Roberts (1956) reported on the suitability of an alternative isotope (^{14}C) to label thymidine for DNA replication studies.

^{3}H thymidine and ^{14}C thymidine are now widely used for

radioactive labelling of cells in DNA synthesis for light microscopic autoradiography.

2.2.4 Comparison of Mitotic and Labelling Techniques

Bertalanffy (1964) compared the colchicine method with the ^{3}H thymidine labelling method and there appear to be only mild discrepancies in data obtained by the two methods. However, the labelling techniques have advantages over the mitotic counting or arresting techniques in many ways (Bertalanffy, 1964; Iversen, 1967).

The identification of labelled cells by autoradiography is more precise than the recognition of mitoses or colchicine arrested metaphases. Further, the labelling technique allows the follow up of labelled cells by sequential studies unlike colchicine arrested mitoses which are destined to die. The size of a labelled cell in S phase is similar to the average size of progenitor cells and therefore the correction factors described in Section 2.2.1 are of less significance in labelling studies.

Since DNA synthesis occupies a greater proportion of the cell cycle time than mitosis, the labelled cells are more frequent than those in mitosis. Therefore the labelling index is likely to be more readily and more accurately measured.

At present DNA labelling techniques provide a versatile method for studying the cell kinetics of epithelial tissues. As this is the main method of investigation used in the present study, the principles and techniques of labelling, autoradiographic technique for identification of labelled cells and quantitative methods in histology will be described in detail in the sections to follow.

2.3 PRINCIPLES UNDERLYING DNA LABELLING FOR CELL KINETIC STUDIES

When labelled thymidine is made available to an organism in vivo or to a tissue in vitro, it mixes with the pool of natural precursors. The cells of renewing cell populations which are in the S phase incorporate the label during DNA replication into their cell nuclei. Cleaver (1967) and Feinendegen (1967) have discussed in depth the various aspects of many problems an investigator could encounter during the use of DNA labels for cell kinetic studies. There are several criteria which should be satisfied in a labelling procedure for cell kinetic estimations.

2.3.1 General Principles

The label must be incorporated into a specific chemical group in cells to result in permanent binding and not be removed by reagents used in later histological techniques. The unincorporated label and its catabolized products should be readily removable, ideally by being soluble in fixatives. The DNA should be metabolically inert in resting and mature cells so that labelled thymidine incorporated into DNA should have been utilised for replication only. Adrian (1971), in a review on this subject, has confirmed that there are firm grounds for believing that DNA is stable and constant for all practical purposes. Although minor exceptions to this rule were found these may be ignored in most cell kinetic studies.

2.3.2 Effects of Thymidine and Tritium

Labelling techniques have sometimes been criticised, in that tritiated thymidine might alter the rate of cell production. This could be due to an effect of thymidine, or be due to the radioactivity.

Exogenous thymidine, whether labelled or not, can stimulate

mitotic activity and this appears to be a dose related phenomenon. Blenkinsopp (1967) studied the effect of ^{3}H thymidine on the rate of cell entry into mitosis in mice and suggested a critical dose of thymidine above which alterations in cell kinetics could occur. He indicated that the amount of thymidine in a single injection should be less than 0.1 µg per g body weight for mice. Greulich, Cameron and Thrasher (1961) and Beagrie (1970) also proposed that free exogenous thymidine acted as a stimulusfor cells to enter mitosis but it seems that these authors used doses above the critical dose suggested by Blenkinsopp (1967).

Radiobiologic effects due to disintegrations of tritium within the nucleus could be present, but these probably do not add any error to cell kinetic data in short term experiments.

Therefore, it can be stated that in experimental studies using ^{3}H thymidine, its possible effects on DNA synthesis and other cell production parameters should be assessed and preferably be eliminated by using a dose below the critical level.

2.3.3 Labelling Techniques

For cell kinetic studies, numerous labelling techniques have been described (Wimber, 1963). These can be divided into three broad groups depending on the number of isotope treatments either as pulse labelling, double labelling or as continuous labelling. The principles underlying these different DNA labelling techniques need discussion.

2.3.4 Pulse Labelling

Pulse labelling or flash labelling involves incorporation of a labelled precursor into tissue in order to label all cells in one phase

at one moment in time. For this to be accomplished it is important that the label is rapidly cleared and that it is no longer present in the pool after a short period. In terms of epithelial cell kinetics this is so as to obtain labelling of only those cells in DNA synthesis at the time of administration of the labelled precursor.

Intravenous labelling results in true pulse labelling but, following intraperitoneal or intramuscular methods, tritiated thymidine may be available for longer periods and therefore Skougaard (1964), suggested a correction for this time discrepancy to be used in cell kinetic estimations. In vitro, the label is available only for the period the tissue is incubated with the isotope solution, and therefore this factor can be controlled by the investigator.

The time taken by the cells to incorporate the labelled precursor is short, and the number of cells labelled in a tissue is proportional to the compartment size of the cells engaged in reproduction and to the proportion of the time spent by these cells in S phase in comparison to the total cell cycle time.

The information which can be obtained from a pulse labelled tissue is related to the time interval between incorporation of the label and tissue sampling. The following three categories of sampling method provide useful information for cell kinetic studies.

Pulse Labelling and Sampling Within 1 - 3 Hours:

This allows an estimation of the number of labelled cells to be made using autoradiography. The usual method of expressing this count is to calculate a labelling index. This is most often expressed as a percentage of labelled cells in a given cell population. Alternatively, the index could

be expressed as labelled cells per unit surface length or basement membrane length of an epithelium. The relative merits of the different reference units used to express the labelling index are similar to those of mitotic index and these are discussed in Section 2.5.2.

Pulse Labelling and Sequential Sacrifice to Estimate Ts:

By observing the percentage of pulse labelled cells reaching a histologically identifiable stage later in the cell cycle an estimation of T_s and sometimes the total duration of cell cycle (T_c) can be obtained. Quastler and Sherman (1959) and Wimber (1960) utilised this principle in developing the percentage labelled mitoses technique to estimate T_s and T_c. They described how, following a pulse labelling procedure, the movement of a block of labelled cells could be watched as it passed through mitosis. The method involves incorporation of the label and sequential sacrifice of experimental animals or biopsy of tissue for counting labelled and unlabelled mitoses at increasing time intervals after labelling. The percentage labelled mitoses (PLM) graph which can be drawn using the above information (Quastler and Sherman, 1959) is diagrammatically illustrated in Figure 2.1. The duration of S phase (T_s) is the time interval between the 50 per cent levels of the ascending and the descending limbs of the graph.

For tissues with a short cell cycle time, such as the intestinal epithelium, a second wave of labelled mitoses can be seen by increasing the time duration between the injection and sampling, as the labelled daughter cells would undergo a second cell division. T_c can be estimated by measuring the time between two similar points in the two waves of the PLM graph. For tissues with a long generation time (T_c) this method of extrapolation is not possible and hence T_c has to be estimated using the formula given in Section 2.6.1.

Following Quastler and Sherman's report (1959), the percentage labelled mitoses method has been used in many studies to determine the duration of the S phase of many animal oral epithelia by pulse labelling in vivo (Dhawan and Toto, 1965; Toto and Dhawan, 1966; Brown and Berry, 1968; Thilagaratnam and Main, 1972b; Skougaard, 1970 ; Hamilton and Blackwood, 1974).

Cameron and Greulich (1963) using this method have presented evidence for a constant mean duration for DNA synthesis of about 7 hours for various epithelial tissues of the mouse. However, it appears that this method of estimation of Ts does not reveal any diurnal variation of this parameter, as only a 'mean' value over a period of several hours can be obtained from the graph. The method could be modified to test the possible variations in Ts by using more groups of animals and by incorporating the isotope into different groups of animals at different times of the day (Izquierdo and Gibbs, 1972, 1974; Moller, Larsen and Faber, 1974).

Pulse Labelling and Serial Biopsy or Sacrifice to Study Cell Migration:

This method involves observation of the location of labelled cells in autoradiographs prepared from pulse labelled tissues which have been sampled by serial biopsy or sequential sacrifice. By comparing the location of labelled progenitor cells which were in DNA synthesis at the time of incorporation of the label, with those of labelled daughter cells in autoradiographs prepared from tissues fixed at regular intervals from the time of labelling, the cell migration pattern can be plotted. Leblond, Greulich and Pereira (1964) and Pereira and Leblond (1965) used this method to study the cell migration pattern of labelled cells from basal to spinous cell layers in rat oesophagus. If the procedure is carried out for longer periods the

transit time (Section 2. 6. 2) of the entire viable cell population of an epithelium can be obtained as described by Epstein and Maibach (1965) in the human epidermis and Cutright and Bauer (1967) in rat oral epithelium.

As ^{3}H thymidine can only be used to study the renewal of the nucleated cell compartment, Susi (1968) incorporated ^{3}H cystine and ^{3}H thymidine into two groups of mice. The labelled protein precursor used, provided information on the passage of labelled cells through the stratum corneum. The method provided a means of estimating the transit time of the total thickness of the epithelium.

2. 3. 5 Double Labelling Method

A double labelling method using two isotope pulses separated by a known time interval to obtain cell kinetic data was proposed by Wimber and Quastler (1963) and independently by Maurer's group at the same period (Pilgrim and Maurer, 1962; Hilscher and Maurer, 1962).

The method is to pulse label a cell population with ^{3}H thymidine and then, following a period of time equal to or less than the duration of G_2, to pulse label again with ^{14}C thymidine. In autoradiographs prepared from ^{3}H and ^{14}C labelled material two labelled cell populations can be distinguished. The cells labelled with ^{3}H only are those which were in DNA synthesis at the time of incorporation of the first label and left this phase before the second label was given. Those labelled by ^{14}C are the cells which remained in the S phase and those which entered the S phase during the interval. Differentiation of cells labelled by ^{3}H only from those with ^{14}C or double labelled with $^{14}C + ^{3}H$ is possible because of the different energies of tritium and carbon (Section 2. 7. 3) which results in different grain distributions in the labelled cells in autoradiographs. ^{3}H label

appears over the cell nucleus only, while the ^{14}C label results in a halo of silver grains around the cell nucleus and the cell. It is not possible to differentiate between ^{14}C labelled cells from $^{3}H + ^{14}C$ labelled cells, but this is not necessary for the calculation, and all these are referred to as ^{14}C labelled cells. Using the counts of two types of labelled cells, ^{3}H and ^{14}C, and the time interval (t) between the two pulses, Ts can be estimated (Wimber and Quastler, 1963) by using the following formula:

$$\frac{Ts}{t} = \frac{^{14}C \text{ labelled cells}}{^{3}H \text{ labelled cells}}$$

Using this technique Skougaard (1965) determined the duration of the S phase to be 8 ± 1 hours for the attached gingival epithelium of marmosets.

The long path length of ^{14}C disintegrations sometimes results in a dispersed grain distribution in ^{14}C labelled cells that does not allow satisfactory light microscopic separation of these from any adjacent unlabelled or ^{3}H thymidine labelled cells, and therefore may hinder the identification of the latter in tissue sections for accurate counting. Therefore the use of ^{14}C as the second pulse label introduces the limitation that it can only be undertaken really satisfactorily on squash preparations.

Baserga and Lisco (1963) introduced the concept that double labelling could be achieved by two pulses of ^{3}H thymidine, spaced by a time interval. Galand et al (1968) used a double labelling method based on a similar principle by incorporating two pulses of ^{3}H thymidine of heavy and weak concentrations in vitro. In the auto radiographs prepared from this double labelled material, two groups of labelled cells were identified by the grain density over their nuclei. These were designated as heavily and weakly labelled cells. Galand et al (1968) assumed that

these corresponded to the two doses of isotope used, on the principle that the grain density on a nucleus is proportional to the amount of isotope incorporated. Using separate pulse labelling with the different doses used for the study, the expected grain densities corresponding to the two doses of the isotope were determined by these workers and this knowledge was used for distinguishing the heavily labelled from weakly labelled cells in double labelled material. The counts of heavily and weakly labelled cells (H. L. C. and W. L. C.) are used in the formula given below (modified from Wimber and Quastler, 1963) to estimate Ts where t is the time interval between the two pulses.

$$\frac{Ts}{t} = \frac{H.L.C.}{W.L.C.}$$

Apart from Ts, the double labelling technique also provides a method for estimating the rate of cell exit from the S phase, as the method yields counts of cells leaving this phase of the cell cycle during a known period of time.

2. 3. 6 Continuous Labelling Method

This method essentially consists of administering experimental animals with a labelled DNA precursor repeatedly at time intervals less than the duration of the S phase. An estimation of the increase in the percentage of labelled cells is made at sequential time intervals. Using these two sets of data, a graph can be drawn and this is called the continuous labelling curve. The primary aim is to obtain the labelling rate or flow rate of cells into the S phase and this can be calculated from the slope of the curve of the continuous labelling graph (Wimber, 1963).

This method also provides a means of estimating the proportion of cells engaged in cell production, in a heterogeneous cell population This proportion is known as the growth fraction.

In a cell renewal system with a homogeneous progenitor cell compartment, the method could be used to estimate the cell renewal time by estimating the time required to achieve 100 per cent labelling. To be able to apply the continuous labelling technique to extrapolate the above data, the progenitor cell compartment of the tissue should be clearly demarcated and the mature cells leaving the dividing cell cycle should not be contained within the progenitor cell compartment.

The main disadvantage in the continuous labelling method is that this can result in radiation damage to the cells incorporating the isotope and this therefore may alter their kinetic parameters.

2.4. ADMINISTRATION OF ^{3}H THYMIDINE FOR CELL RENEWAL STUDIES

^{3}H thymidine can be incorporated into tissues either in vivo or in vitro. For animal experiments the in vivo route has been used, but the in vitro method is generally preferred for labelling human tissues.

2.4.1 In Vivo Routes of Administration

Following intravenous administration, ^{3}H thymidine is rapidly cleared from plasma (Rubini et al, 1960) and labelling of bone marrow cells has been demonstrated within minutes of administration of the label into mice (Feinendegen, 1967). Therefore this appears to be an ideal method for pulse labelling tissues. Gillespie (1969) used intra-arterial and intravenous routes for administering ^{3}H thymidine into human subjects dying with terminal illnesses to study the cell renewal of the buccal epithelium.

The intraperitoneal route of injection to incorporate radioactive label in mice was used by Hughes et al (1958) and by Quastler and Sherman (1959) to study the intestinal epithelium. The latter authors

have shown that saturation of labelling was reached within 10 - 20 minutes by this route. This route has been used in many subsequent studies for DNA labelling of various epithelial tissues particularly in small animals. The advantages of the intraperitoneal route are the ease of administration and the reproducibility of the technique.

Petersen and Baserga (1964) found differences in the rate and efficiency of the incorporation of the label following intraperitoneal and intravenous injections of ^{3}H thymidine in different organs of mice.

Local in vivo labelling methods have been used recently (Johnson and Hopps, 1974) and some workers have utilised the intradermal route for incorporation of the ^{3}H thymidine into human subjects for cell kinetic studies of epidermis (Epstein and Maibach, 1965; Kahn et al, 1968: Weinstein and Frost, 1968).

2.4.2 In Vitro Methods of Incorporation

The method of in vitro incorporation of radioactive labels into tissues involves the use of small tissue fragments obtained at sacrifice or by surgical biopsy for incubation in culture media to which are added the radioactive precursor. Using the in vitro method either pulse labelling, double labelling or short term continuous labelling can be carried out.

The technique of in vitro organ culture was first described by Strangeways and Fell (1926) for embryonic tissue and this method was later adopted (Trowell, 1954; Sarkany, Grice and Caron, 1965) for culturing adult tissues. Application of this in vitro organ culture technique for radioactive labelling stems from the work of Lajtha, Oliver and Ellis (1954) who used an in vitro method for cell kinetic studies in human bone marrow cells with ^{32}P and ^{14}C adenine and from subsequent

organ culture studies using ^{3}H thymidine by Johnson and Bond (1961), Deschner, Lewis and Lipkin (1962) and Fabrikant and Wisseman (1968) in various other tissues.

Pulse labelling involves incubation with a labelled precursor for a short period of time which should be small in comparison with the duration of the S phase of the tissue investigated. Double labelling of tissues in vitro was first reported by Galand's group to study cell renewal of human epidermis and gastrointestinal tract tissues (Galand et al, 1968; Bleiberg et al, 1970; Heenan and Galand, 1971) and by Fabrikant (1970) for larynx.

The diffusion of the label into the tissue is obviously important and this appears to be mainly dependent on tissue block size and oxygen tension which can affect the uptake of thymidine by the tissue (Steel and Bensted, 1965; Rajewsky, 1965). Many investigators recommend the use of 1 mm thick slices of tissue (Lachapelle, 1969). Some found that the diffusion of the label into the centre of the tissue fragment was inadequate and therefore restricted the analysis to areas close to the edges of the specimen (Alvares et al, 1972). To increase the depth of labelling Fabrikant, Wisseman and Vitak (1969) used 100 per cent oxygen under high partial pressure. Lachapelle and Gillman (1969) made use of a continuous supply of a gas mixture containing 95 per cent oxygen and 5 per cent carbon dioxide.

2.4.3 Comparison of in vivo and in vitro Methods

The in vitro method has been reported to be reliable by comparison with in vivo labelling techniques, in human epidermis by Lachapelle and Gillman (1969) and Hell and Maibach (1972) and in gastrointestinal epithelium of dogs, by Willems, Galand and Chretien (1970).

2.4.4 Labelling of Human Tissues

Attempts at incorporation of ^{3}H thymidine into human subjects locally by intradermal injection or by other methods have been reported (Section 2.4.1). It is obvious that this is not the method of choice from the point of view of safety and for ethical reasons. The importance of using biopsy material for measuring cell kinetic parameters of human tissues, without the necessity of injecting ^{3}H thymidine or other isotopes into volunteer subjects or into patients is now becoming apparent.

Recently several tissues, both normal and pathological, have been invesitgated by in vitro labelling. Alvares et al (1972) used a single pulse label in vitro to obtain the labelling index of human buccal epithelium, and Toller (1971) used in vitro labelling to investigate cell proliferation in explants from odontogenic cysts.

2.5 REVIEW OF MITOTIC AND LABELLING INDEX SYSTEMS AND THEIR DISCREPANCIES

In most cell kinetic studies, in order to quantitate epithelial cell production, counts are made of cells engaged in mitoses or in DNA replication by the methods described in Sections 2.2. - 2.4. However, to express these counts of cells engaged in cell renewal with reference to an epithelium, it is necessary to relate this count to a reference unit in the epithelium. Using these reference units it is possible to express some features of cell proliferation as an index. Depending on the stage in the dividing cell cycle at which these proliferative cells are identified for counting, such an index is called either a mitotic index or a labelling index.

2.5.1 Index Systems

The index systems used to express mitotic activity are equally applicable to labelling index studies. In the following discussion, index

systems are described in relation to mitotic counts. When the labelled cells are counted the labelling index can be expressed using the same reference units. The systems presently in use are as follows:-

(a) Number of Mitoses per 100 or 1,000 Cells:

Minot introduced this system in 1908. It involves counting the total number of cells in an area of epithelium and relating the number of mitoses in the area to the total cell count. The mitotic index is expressed as the number of mitoses per 100 or 1,000 cells.

The total cell count is obtained by counting cell nuclei in histological sections. Usually only viable cells are counted by establishing criteria for excluding pyknotic nuclei from the count, so that the total cell count does not include epithelial squames or degenerating cells.

(b) Number of Mitosis per 100 Progenitor Cells:

Mitotic counts have also been related to the basal cell count of an epithelium. Alvares et al (1972), used an index relating labelled cells to 100 progenitor cells by counting cells in the three deepest cell layers and considering that these suprabasal cells in human buccal mucosa are also engaged in cell production.

(c) Number of Mitoses per Unit Epithelial Surface Length or Surface Area:

The number of mitoses under a measured surface length of an epithelium is counted and expressed as the number of mitoses per millimeter. By taking into account the section thickness to estimate the surface area, the count can also be related to unit surface area (Löe and Karring, 1969).

Methods of measuring surface length are reviewed in Section 2.8.

(d) Number of Mitoses per Unit Basement Membrane Length:

By counting mitoses along a measured length of basement membrane, the number of mitoses per millimeter of basement membrane can be estimated.

This method was used by Mühlemann, Ebneter and Rupf (1959), Renstrup (1963) and El-Labban, Lucas and Kramer (1971), for expressing the mitotic activity of oral epithelium.

2.5.2 Variability in Index Systems

The index systems mentioned above are each applicable for the estimation of mitotic activity of epithelium. However, when a comparison of mitotic activity is made between epithelia of different morphology, variations in the sizes of the reference units can introduce an appreciable error.

Karring and Löe (1972b) used three indices a, c and d (Section 2.5.1) in studies on cell renewal of ear epidermis and oral epithelium of rat and found a varying degree of correlation between the three index systems. They believed that this variation was related to the variations in epithelial cell density and to differences in basement membrane length to surface length relationships in the epithelia studied.

Since cell production and cell desquamation are related events, Löe and Karring (1969) suggested that a quantitative assessment of mitotic activity should be expressed in relation to the area occupied by the desquamating cells. An essential feature of this system is that it reflects the capacity of cell production in a specific area or volume of tissue irrespective of morphological, regional or pathological variations in the size of a cell population or basement membrane configuration.

The index system of expressing the number of cells in division in relation to the progenitor cell count appears to be more relevant than to the index using total cell count in some ways. An important feature in such an index is that the comparison made utilises a relatively homogeneous cell compartment in terms of cell size. However, a requirement for such an estimate is the need for identifying the distribution of the progenitor cell compartment. Studies which relate the dividing cells to a basal cell count, without considering the possibility of existence of progenitor cells in suprabasal layers, could be introducing a significant error in such estimations.

An ideal mitotic index for expressing the mitotic activity of human oral epithelium has not yet been established. It appears that a proper reference unit should be selected in estimating the mitotic index or the labelling index, depending on the nature of the study. When assessing mitotic variations between normal and pathological lesions, where epithelial thickness, cell size, cell density and epithelial-connective tissue interface may be altered to a considerable extent between any two samples, the effect of these variations on the reference unit should be taken into account.

2.6. MEASUREMENT OF CELL PRODUCTION AND RENEWAL

Different methods of studying cell production and cell renewal of epithelia have been discussed. As the parameters obtained by these techniques are different from one another, the data derived from such studies needs to be expressed in more closely related numerical units, for them to be of general use or for comparative purposes.

Mitotic index and labelling index, although relatively simple to obtain, have the apparent disadvantage that they both do not represent a

rate of cell production. This is because the time required for completion of each of these phases may considerably alter the actual number of cells seen in each phase. For example, where T_m is long more cells will be present in mitosis although cell production may not be high.

The rate of cell entry into mitosis (as measured by mitotic arresting techniques) or the rate of cell entry into DNA replication (as measured by double labelling techniques) can provide a measure of cell production which does not require knowledge of individual cell cycle phase durations. These two parameters can be used to estimate a daily growth rate (Section 2.6.2). However, the most consistently used measurement of cell renewal of epithelia by many workers is the renewal time or the turnover time.

2.6.1 Turnover Time

This yields information about the length of time required for renewal of a given cell population. Leblond and Walker (1956) defined the turnover time as the time taken for replacement of a number of cells equal to that in the whole population. Leblond, Greulich and Pereira (1964) remarked that the turnover time of an epithelium is influenced by rate of cell formation and the size of the cell population. Therefore they expressed it in terms of these two parameters:

$$T = \frac{N}{n_1} \qquad \text{---------} \quad \text{(Equation 1)}$$

(Where T = Turnover Time: N = Total number of cells in a population:

n_1 = numbers of cells added per unit time)

The actual calculation of the turnover time therefore involves estimation of the total cell population and the rate of cell production. The latter (n_1) can be measured either by estimating the rate of cell entry into mitosis or into DNA synthesis (assuming that all cells in DNA synthesis proceed

to mitosis at a later time).

Usually, the rate of cell entry into mitosis is measured by mitotic arresting techniques (Section 2.2.2), and n_1 is obtained by counting mitoses collected per unit time; $n_1 = \frac{n_c}{t_c}$

(Where n_c – numbers of mitoses arrested: t_c – effective time of collection).

Thus, $T = \frac{N \times t_c}{n_c}$ --------- (Equation 2)

Alternatively, this could be estimated using the mitotic index and the mitotic duration.

$$n_1 = \frac{Nm}{Tm}$$

(Where Nm - numbers of cells in mitoses: Tm = mitotic duration)

Therefore, Equation 1 could be rewritten as:-

$$T = \frac{N \times Tm}{Nm} \quad \text{--------- (Equation 3)}$$

On a similar principle n_1 could be obtained using parameters related to DNA synthesis phase, as follows:

$$n_1 = \frac{Ns}{Ts}$$

(Where Ns – number of cells in DNA synthesis:

Ts – duration of S phase)

Substituting n_1 obtained by such methods in Equation 1, the following equation is derived:

$$T = \frac{N \times Ts}{Ns} \quad \text{---------- (Equation 4)}$$

Equations 3 and 4 could be expressed using already discussed parameters as follows:

$$T = \frac{Ts \times 100}{\text{Labelling index}} \quad \text{or} \quad T = \frac{Tm \times 100}{\text{Mitotic index}}$$

Methods of measuring these parameters have already been discussed (Section 2.2. - 2.3).

For an epithelium consisting of two cell compartments, progenitor and maturation, the turnover time can be calculated separately for each compartment, or for the total cell population. Where a keratinized compartment is present, the turnover time of this has to be separately estimated (Section 2.3.4) to obtain the turnover time of the whole epithelium.

On the grounds that each dividing cell replaces two cells rather than one, MacDonald (1971a) postulated that the original definition of turnover time proposed by Leblond and Walker (1956) could be more correctly stated as the time required for addition to a cell population of a number of cells equal to that in the population.

Turnover time estimation is presently used to express the dynamic state of renewal systems. It is useful for comparison of cell renewal rates of epithelia of different body systems or for analysis of regional variations in the same epithelium. Turnover time may also be used for comparison of cell renewal patterns of an epithelium in normal and pathological states.

2.6.2 Transit Time

Transit time is defined as the time elapsed between a cell entering and leaving a compartment (Quastler, 1963). For the progenitor cell compartment this is not easily obtained as cells in this compartment in many cell renewal systems are not homogeneous and recycle before leaving the compartment . However, the mature spinous cells and

keratin cell layers are homogeneous in many cell renewal systems and therefore by incorporation of a proper label, a transit time close to the average life span of a cell in each of these compartments can be obtained. This is by estimating the time taken for the migration or disappearance of the first and the last labelled cells from one compartment to another, or through the whole epithelium.

2.6.3 Turnover Rate and Daily Growth Rate

An esti mation of daily cell production rate can be obtained by using a measure of cell entry into DNA synthesis or into mitosis. This is sometimes referred to as turnover rate. Meyer, Medak and Weinmann (1960) used a similar parameter of quantitation by expressing daily growth rate of rabbit oral epithelium in terms of epithelial thickness, renewed per day. This they found suitable for comparative cell kinetic studies.

2.7 AUTORADIOGRAPHY

2.7.1 Introduction

An autoradiograph is an image produced in a nuclear emulsion by the radiations from a radioactive substance contained in an object kept in close contact with the emulsion. The principle is similar to photography or radiography in that a sensitized film containing silver bromide crystals embedded in gelatin is exposed to a source of radiation, which forms a latent image in the film.

The principle of latent image formation depends on the fact that electrons released during disintegrations of ionizing particles are trapped in imperfections of silver halide crystals known as 'sensitivity specks'.

Collections of electrons at these sites render them negatively charged and this attracts positively charged silver atoms to migrate to these sites. The aggregation of silver atoms at sensitivity specks is referred to as the latent image formation.

The intensity of latent image is dependent on the amount of radioactivity present in the source, the sensitivity of the emulsion (Section 2.7.4), and the period of exposure of the emulsion to the radioactive source. Following exposure for a desired period in complete darkness the emulsion is developed.

During processing or development, the latent image is transformed into a visible image. A weak reducing agent is used for development and this causes reduction of silver halide crystals to metallic silver. This process is catalysed by silver atoms and therefore where aggregations of these exist (Latent images) more metallic silver is deposited. Unreduced silver bromide is removed during fixation. An autoradiograph therefore contains silver grains in places where radioactivity is present in the object.

2.7.2 History and Development of Presently Used Techniques

The history of autoradiography extends back to a little over 100 years. The first 'autoradiograph' was produced by Niepec de St. Victor in 1867, when he obtained blackening of silver chloride and iodide emulsion by a uranium salt. Later, in 1896, Hein Becquerel obtained similar results. In 1904, London of St. Petersburg, obtained an image of a frog exposed to radium and this was the first biological application of the method. In 1924 the technique was used by Lacassagne to study the distribution of polonium in biological material, using tissue sections. Lacassagne and his co-workers first used the term 'autoradiographie'.

These early attempts at autoradiography used photographic emulsions which were placed in contact with the specimen during the exposure period but were then separated for development. The next significant advance in the technique of light microscopic autoradiography was in the application of emulsion layers retained in contact with the specimen throughout the preparation procedure. This stems from the work of Belanger and Leblond (1946) who painted molten emulsions on histological sections, thus greatly improving the resolution. Two main techniques developed from this point were the dip-coating technique (Joftes and Warren, 1955, and Messier and Leblond, 1957) and the stripping film technique (Pelc, 1947). In the former the slides are dipped in liquid emulsion which is then allowed to dry over the section. In the stripping film technique small pieces of emulsion from photographic plates are floated in water and picked up on slides to cover the histological sections. Rogers (1967, 1973) and Baserga and Malamud(1969), in two excellent texts have described in detail these techniques and their application.

2.7.3 Autoradiography for Cell Kinetic Studies

The object of light microscopic autoradiography is that a radioactive isotope in tissue, either naturally occurring or introduced by the investigator, can be accurately located within the tissue. Many isotopes have been used for this purpose and it is not practicable to discuss them in detail. It is pertinent, however, to examine briefly the requirements for autoradiographic techniques as applied to cell kinetic studies.

The suitability of an autoradiograph prepared from radioactive labelled tissue, for cell kinetic estimation by light microscopy, depends to some extent on the properties of the isotope and the nuclear emulsion

used for the study. The desirable properties of an isotope are that

(1) it should allow the source to be detected and this is a function of energy of emission,

(2) it should remain detectable in tissue for a practicable period and this is function of the half life of the isotope.

The two isotopes most frequently used are ^{3}H and ^{14}C, and both of these emit Beta particles. Several investigators have studied the energy of emission (Langer and Moff t, 1952) and the average range or path length of Beta particles in tissues (Lajtha and Oliver, 1959, and Wimber, 1960) and in artificial materials with a density close to that of tissue specimens (Caro, 1962). These investigations have revealed that the use of ^{3}H which has an energy range of 0 - 18 keV and a path length of 1 - 2 μm in tissues provides a method for sufficiently accurate localization of the autoradiographic image to the source of emission, at a light microscopic level. Although ^{14}C has been used by some workers, due to its longer path length of about 10 - 60 μm, localization of the image to the source of emission is more difficult and therefore may require the use of squash preparations, in order to disperse cells to that nuclear labelling overlapping to adjacent cells can be prevented.

^{3}H thymidine is therefore preferred for use in cell kinetic analysis. It has a half life of $12\frac{1}{2}$ years which satisfies the second criterion stated above.

The choice of the nuclear emulsion is under the control of the investigator and the selection of this depends on the nature of the study. A considerable number of nuclear emulsions for dipping and stripping film techniques in autoradiography are now commercially available. Two important factors which guide the selection of an emulsion for an experiment are (1) sensitivity and (2) grain size.

Sensitivity refers to the potential for latent image formation in the emulsion and this is a function of 'sensitivity specks' in the emulsion particles. The more sensitive the emulsion the more rapid is this process of latent image formation and therefore a shorter exposure period is required for autoradiograph preparation. A consequence of high sensitivity is that it also results in the formation of latent images at points not sensitised by radiations and therefore results in 'false' silver grain production in autoradiographs contributing to the 'background'.

The microscopic appearance of silver grains in autoradiographs is related to the particle size of the silver halide crystals used for the preparation of the emulsion. It is possible to obtain fine grains or coarse lumps over the nuclei and therefore the selection of the emulsion depends on the type of autoradiographic image preferred. While an image appearing as a coarse lump helps in the easy recognition of labelled cells, fine grains are required if grain counting is to be carried out during the analysis.

The commercially available emulsions are graded on sensitivity and grain size by the manufacturer, to enable the investigator to choose any combinations of these two properties.

2.7.4 Results Obtainable from Autoradiographic Studies

The range of estimations which can be carried out on an autoradiograph depends to a greatextent on the aim and planning of the experiments and therefore is largely related to the labelling procedure and the method of autoradiograph preparation. Different labelling procedures useful for cell kinetic analysis were discussed in Section 2.3 and the choice of isotope and nuclear emulsion in the preceding Section 2.7.3.

One of the most frequent quantitations made is to estimate the number of labelled cells in a defined cell population and this is referred to as the labelling index (Section 2.3.4). This involves the autoradiographic identification of labelled nuclei. The correct identification of the labelled cells rests to some extent on establishing the degree of background labelling. Factors which contribute to increased background labelling and which may hinder the identification of labelled cells should be controlled and this subject was discussed by Caro and Van Tubergen (1962). The criteria for considering a cell nucleus to be labelled should be laid down. Factors which could erroneously result in positive or negative labelling should be borne in mind and causes for these such as positive and negative chemography and latent image fading should be carefully controlled. These control procedures are described in detail by Rogers (1973).

In some quantitative studies another feature taken into consideration during the analysis is the number of silver grains or the grain count over individual labelled cells. The grain density in a final image is a function of the number of disintegrations reaching the emulsion particles and is related to a large extent on the isotope concentration in the tissue. In a double labelling experiment (Section 2.3.5) using two doses of the same isotope, it is possible to identify two labelled cell populations by the differences in the grain counts over the labelled cells. Estimation of the number of grains over the labelled nucleus can be carried out visually and this is known as the visual grain counting method. Alternatively, photometric reflectance microscopy methods described by Rogers (1973) and Goldstein and Williams (1971) can be used. When only a relative quantitation, such as differentiation of heavy from light labelling, as opposed to an assessment of actual grain distribution is attempted, the visual grain counting method is adequate.

2.8 APPLICATION OF STEREOLOGY FOR MORPHOMETRIC ANALYSIS OF HISTOLOGICAL SECTIONS

2.8.1 Cell Kinetics and Morphometry

Preceding parts of this chapter have stressed the needs and methods of identifying and counting cells in S and M phases of the cell cycle for cell kinetic studies. In Section 2.5 the methods of relating these cell counts to epithelial structure were enumerated and index systems were discussed. In order to estimate these reference units in epithelia, a knowledge of morphometry, which is the use of quantitative data to describe structural features, is required. These measurements generally include estimation of surface length, basement membrane length and the size of tissue compartments. In practice it is difficult accurately to quantitate these parameters or to study the spatial distribution of tissues such as cell compartments. Until recently, apart from measurement of certain linear dimensions with micrometer graticules in light microscopic eyepieces, planimetry or use of map measurers (Marthaler, 1956; Renstrup, 1963; Main, 1970), little quantitative data on epithelial morphology has been available. However, stereology, which is a type of morphometry involving a geometrico-statistical analysis of structures, and includes methods which allow the derivation of three dimensional properties of structures from two dimensional sections (Weibel, 1969), can be employed for deriving quantitative data on epithelial structure.

2.8.2 Stereological Methods

The principle of stereology was first described by a French geologist and mathematician Delesse in 1847. While making a quantitative analysis of the mineral content of rock he suggested that "In a rock composed of a number of minerals the area occupied by any given mineral on the cut surface of a section of a rock is proportional to the volume of the mineral in the rock". Therefore, estimation of the area proportions

occupied by each component provides a direct method for volume estimations, provided an adequate sampling procedure is carried out. This geometric reasoning is equally applicable for morphometry of organs and tissues in biology and therefore for volumetric estimation of component elements in a tissue.

Area Measurement:

Two main methods have been described to carry out area measurements in stereology. Rosiwal (1898) estimated the area by a linear sampling method which involved measuring total fractional length of a line traversing each component of the section. Glagoleff (1933) described that area estimation could be obtained by superimposing a lattice with marked points and counting the number or proportion of points falling on each element to be quantitated.

The first application of Delesse's principle to tissue morphometry was by Chalkley (1943) who used a point counting method on histological sections with four point markers attached to the ocular of the microscope. Hennig (1958) further improved the method of sampling by using 25 points.

The point counting technique can be applied to analyse the size of progenitor, maturation and keratinized compartments of an epithelium, in quantitative studies.

Surface Length Estimation:

The length of a curved line can be estimated stereologically by the intercept point counting technique using a grid of parallel lines (Smith and Guttman, 1953). The surface length or basement membrane length of a stratified squamous epithelium can therefore be obtained by this method. The absolute length is a function of the number of intercept points counted (I) and the distance between the grid lines (d) and can be

obtained by using the formula

$$L = \frac{\Pi}{2} \times I \times d$$

This relationship is based on the solution of the Buffon needle problem (Buffon, 1777, quoted by Weibel, 1969).

2.8.3 Principles of Sampling

Stereology is based on geometric and statistical reasoning and therefore the sampling procedures are critical in that the areas quantitated should be representative of the internal architecture of the tissue. Weibel (1963) and Weibel, Kistler and Scherle (1966) discussed the principles involved in sampling of tissues in relation to lung and other organs. As the validity of the technique is dependent on random selection of areas to be quantitated, consideration of sampling is required at all levels of the study, and should be carried out through a bias free random process (Weibel, 1969). It is necessary to standardise the site of biopsy and from there on to carry out totally random or systematic random sampling.

Sample Size

Determination of sample size should be given an important consideration in any stereologic study. Obviously the larger the sample size the more likely it is that reliable information is obtained. However, the selection of a sample size for a particular study depends on the accuracy required, the time available for sampling, and the characteristics of the tissue (Weibel, 1969). One of these characteristics appears to be the proportion of components forming the tissue, and where one component is proportionately very much smaller than the rest of the components, a larger sample size may be required.

Random Sampling

In a tissue demonstrating random distribution of elements, a

random sampling procedure is carried out at all stages from the selection of tissue blocks up to the selection of the precise area of tissue to be used for quantitation (Weibel, 1969). In order to ensure adequate dispersion of the sample, stratified sampling by examining serial sections at various levels has also been recommended. Such a method is particularly required for analysing non-randomly distributed structures in tissues and organs. This is referred to as systematic random sampling if the first area is selected at random. However, if structural elements are orientated in preferential directions, further precautions are required in sampling such structures.

Sampling Anisotropic Tissue

In certain tissues the cellular components are orientated in preferential directions and this feature is known as anisotropy. Oral epithelium is an anisotropic structure in which the component cells and other elements are orientated with respect to the surface. Weibel (1969) recommends in general that sections examined should not be at random but the plane of section for anisotropic structures should include the axis of anisotropy. In surface epithelia, this axis is perpendicular to the surface. Such a plane of section would also allow identification of known histological strata, and contain all parts of the component elements in proportion to their frequency.

2.8.4 Practical Methods of Sampling

The application of intercept point counting for linear estimations, or point counting for area estimations, can be carried out either by using special graticules in light microscopic eyepieces, or by using transparent grids placed on projected images or photomicrographs.

For linear estimations by intercept point counting, a line grid with parallel test lines (Figure 2.2a) and for point counting regularly

arranged sets of points (Figure 2.2b) are used. Both test systems can be simultaneously arranged in a quadratic lattice of lines as described by Weibel (1969). Franz (1971) and Lennox (1975) have discussed the eyepiece graticule designs suitable for the point counting method.

Although these techniques appear simple, they are valid only if the requirements of the criteria mentioned in Section 2.8.3 are satisfied during the sampling and a reliable test system is utilised to obtain an acceptable degree of accuracy during the stereological analysis.

2.8.5 Application of Stereology to Studies in Oral Epithelia

Schroeder and Munzel -Pedrazzoli (1970) were the first to apply stereological methods to study oral epithelia and this was to estimate the volume density of the constituent elements in gingival mucosa. In 1973, they discussed the sampling techniques and test procedures suitable for the study of gingival mucosa. MacDonald (1971b) described the application of these methods to the study of oral epithelium in general and discussed the test systems suitable for intercept and point counting techniques. He used stereological methods to estimate the cell compartments in photomicrographs of normal and carcinogen treated hamster cheek pouch at light microscopic level and also to estimate the proportional length of hemidesmosomes in the basal lamina in electronmicrographs of similarly treated tissues (MacDonald, 1973). Alvares et al (1973) used stereological methods to estimate the nucleolar and nuclear volumes in normal and leukoplakia lesions of human oral mucosa. Franklin (1974) reported on the application of a stereological technique for morphometric quantitation of nuclear-cytoplasmic ratios of epithelial cells during experimental carcinogenesis in hamster cheek pouch.

CHAPTER THREE

PRELIMINARY STUDIES ON TISSUE LABELLING AND AUTORADIOGRAPHY: A COMPARISON OF IN VIVO AND IN VITRO ^{3}H THYMIDINE LABELLING TECHNIQUES IN HAMSTER TONGUE EPITHELIUM

3.1 INTRODUCTION

Preliminary experiments were carried out in order to gain familiarity with in vivo and in vitro methods of ^{3}H thymidine labelling of tissues and the preparation of autoradiographs.

In order to evaluate the reliability of the in vitro technique developed, before using this method in humans, an experimental study was designed to compare the in vitro method with in vivo labelling in experimental animals. Although such comparisons of in vivo and in vitro labelling have been documented (Section 2.4.3), these reports deviated in some ways to the exact technique selected (from preliminary studies) for the present study and also did not deal with oral epithelium.

As it is known that diurnal variations exist in several cell kinetic parameters (Section 1.5), a further part of this study was to make a preliminary assessment of diurnal variations and, in particular, to assess whether an in vitro labelling technique would quantify these variations in the manner demonstrated by in vivo labelling.

3.2 MATERIALS AND METHODS

3.2.1 Experimental Animals

Black eared, lemon yellow hamsters from a line bred strain were used as the experimental animal for the study. The hamster was

chosen as it is a convenient laboratory animal for handling during research work involving oral mucosa and it is known that hamsters are particularly well suited for the study of diurnal rhythms (Aschoff and Meyer-Lohmann, 1954); some aspects of cell kinetics of oral epithelium of the Syrian hamster have already been documented (Brown and Berry, 1968; Thilagaratnam, 1969). The black eared, lemon yellow strain was chosen as its body weight range is less than that of the golden hamster and hence the quantity of isotope needed per animal for the in vivo study was less, thus reducing the cost of experiment. Male hamsters were used to avoid any effect of sex hormone variations in female animals.

All hamsters were 8 - 10 weeks of age on arrival from the supplier* and were housed two in a cage and maintained in a regular dark-light cycle with artificial lighting from 0700 to 1900 hours. They were fed with water and standard laboratory pellets ad libitum and were standardised in this regular environment for four weeks before experiments were begun.

Animals within the weight range 78 - 100 g were used for the study and were randomly divided into two groups for the comparison of in vitro and in vivo labelling techniques.

3.2.2 Selection of Experimental Site

The tissue investigated was the ventral surface of the tongue (see Figure 3.2). This consists of a sharply demarcated area of lining epithelium. Although the cheek pouch epithelium has been the site of choice for many earlier studies on cell kinetics in the hamster, it was not selected for the present study as preliminary work proved that, due to the gelatinous mucoid layer in the corium, the handling of the cheek pouch epithelium during organ culture was difficult. As a result of curling of the specimens, the orientation at the blocking out stage was

* Coombehurst Breeding Establishment, Basingstoke, Hampshire.

much more difficult using the cheek pouch. These practical problems were not encountered while using ventral tongue mucosa. This site has the added advantage in that it has suprabasal dividing cells in the progenitor cell compartment and is similar in this respect to human oral epithelium.

The dorsal tongue and palate were not selected as these are more specialised epithelia and therefore the control of cell proliferation in such sites could be more involved than in the lining epithelium of ventral tongue.

3.2.3 Isotope for Labelling

Tritiated (^{3}H) thymidine was used as the radioactive marker for labelling cells in DNA synthesis. The isotope was obtained from the Radiochemical Centre, Amersham, in the form of 6 - ^{3}H thymidine in aqueous solution. A label with a specific activity 22 - 26 Ci/m mole was used. 1 ml of the compound containing 1 mCi was diluted 1 : 10 with sterile distilled water to obtain a concentration of 100 μCi/ml which was found to be a convenient dilution for use. The necessary safety precautions were taken in the handling of the isotope.

3.2.4 In Vivo Method (Experiment 1)

The method used for the in vivo study was pulse labelling and sequential sacrifice (Section 2.3.4). This was used primarily to estimate the labelling index and the T_s. The intraperitoneal route was used for injecting the animals. Each animal was given 1 μCi/g body weight. To study the effect of the diurnal variation on the labelling index, animals selected for the in vivo study were divided into three groups and were pulse labelled at different times of the day; Group I (animal numbers 1 - 13) between 0925 - 0950, Group II (animal numbers 14 - 16) at 1230, and Group III (animal numbers 17 and 18) at 1530. In addition to the

labelling index, the T_s was estimated by the percentage labelled mitoses method of Quastler and Sherman (1959) using material from the animals in Group I, injected between 0925 - 0950, and sacrificed sequentially over a period of 12 hours.

3.2.5 Procedures for Injection and Sacrifice of Animals

The animals were weighed and the volume of the isotope to be injected to give a dose of 1 μCi/g body weight was calculated. As the diluted compound contained 100 μCi/ml this was 0·70 - 1·0 ml depending on the body weight of each animal. Disposable sterile syringes with disposable sterile needles were used for the intraperitoneal injections. The injection times are detailed in Table 3.1. The animals were returned to their respective cages after the injection.

Within 30 minutes of the injection, animals were found to be resting and later observations, made without disturbing the animals, revealed that they were asleep most of the day, until disturbed for sacrifice.

All animals were sacrificed at appropriate time intervals following the injection, by cervical dislocation following light ether anaesthesia. Of animals in Group I, the first injected was sacrificed after half an hour and the rest at hourly intervals from their respective times of injection. Animals in Groups II and III were sacrificed at hourly intervals up to three hours. The actual times of sacrifice are shown in Table 3.1.

3.2.6 Specimen Collection, Trimming and Fixation

After sacrifice the animals were held in the restraining apparatus illustrated in Figure 3.1 which is a modification of that described by Moss, Collins and Cole (1965). The mouth was opened using elastic bands around the central incisors. The tongue was grasped

by flat bladed tweezers and was held in a vertical position by the operator using his left hand. Using a scalpel with a number 11 blade, two vertical parallel incisions were made on the ventral surface of the tongue (Figure 3.2) and, using a pair of pointed scissors, the edges and the base of this area was dissected out to obtain a specimen of about 6 x 3 x 3 mm as illustrated in Figure 3.3. The site of biopsy was chosen with reference to the mid-line raphae thus ensuring that exactly comparable sites were examined in different animals. A technique comparable to a 'biopsy procedure' was used in specimen collection so that the tissue could be stretched consistently during removal, but then allowed to contract following dissection. This method was considered to be superior to a procedure of dissecting out the tongue first and then trimming, which makes it more difficult to obtain specimens of a comparable size from a defined site under an equal tension.

The specimens obtained were trimmed 1 x 3 x 3 mm pieces along the short axis of the tongue and were fixed in Bouin's fluid (appendix 1). After two to three hours of fixation the tissues were transferred to 70% methylated spirit.

3.2.7 In Vitro Method (Experiment 2)

The method used for the in vitro study was modified from the techniques described by Heenan and Galand (1971) and Lachapelle and Gillman (1969). Essentially the method involved using biopsy material for labelling in vitro, by incubating small pieces in tissue culture medium, to which was added the ^{3}H thymidine.

Animals were maintained under the same environmental conditions as those used for the in vivo study. At different times of the day between 1000 and 1538 hours (Table 3.2), animals were sacrificed by the technique described in Section 3.2.5 and were quickly 'biopsied'

as described in Section 3.2.6. Tissues were trimmed to 1 mm thick slices for in vitro labelling and were placed in tissue culture medium 199* in sterile Bijou bottles. These were transferred to the laboratory in vacuum flasks containing crushed ice. Incubation with ^{3}H thymidine was begun within one hour of each biopsy.

3.2.8 Incubation and Labelling Procedures

5 ml quantities of Medium 199 were pipetted out into sterile universal containers and brought to 37°C by being placed in an incubator about one hour prior to the experiment. The biopsy tissue was added to these containers which held a measured quantity of medium at pH 7·2 - 7·8.

The universal containers were closed with sterile plastic tops which had two holes, through one of which a glass tube bent at 90° was passed. The containers were placed in the water bath maintained at 37°C - 37·5°C and the glass tubes were connected by rubber tubing to a gas cylinder containing 95 per cent oxygen and 5 per cent carbon dioxide. A continuous supply of the gas was passed through the incubating medium at a rate of approximately one bubble per second. Figure 3.4 illustrates the experimental set up used for in vitro incubation.

To each universal container with biopsy tissue, 5 µCi of ^{3}H thymidine was added to give a concentration of 1 µCi/ml of the medium. Under the conditions mentioned above, the incubation was carried out for 20 minutes. This was the first pulse label with the "weak dose" of the isotope. The tissues were randomly divided into two lots after this pulse label. One group was transferred for a rinse in label free medium for 10 minutes before fixation and these tissues will be referred to as single pulse labelled material.

*Gibco Bio-Cult, Paisley, Scotland.

The rest of the tissue was returned to the incubator in universal containers with label free Medium 199, and gassed as before for a period of one hour.

At the end of the incubation for one hour in label free medium, tissues in this second group were transferred to containers with 5 ml of Medium 199 to which was added 50 μCi of ^{3}H thymidine to obtain a concentration of 10 μCi/ml of the medium. This being the 'heavy dose'. At the end of 20 minutes' incubation with this heavy dose these tissues were transferred to label free medium for a rinse of about 10 minutes before being fixed in Bouin's fluid. The tissues in this second group which received two labels will be referred to as double labelled material.

3.2.9 Thymidine Control Study (Experiment 3)

When using ^{3}H thymidine, both thymidine and tritium could alter the cell proliferation parameters of tissues either by stimulation or by inhibition (see Section 2.3.2). A control study was required to assess this possibility. As the only identifiable stage of the cell cycle of dividing cells in untreated animals is mitosis, it was decided to compare the mitotic index of ^{3}H thymidine injected animals with untreated controls sacrificed at corresponding times of day. The assumption was made that all labelled cells following ^{3}H thymidine injection would proceed normally to mitosis. This is a reasonable assumption to make with reference to normal tissues.

The untreated tissue for this control study was obtained from hamsters (numbers 14, 16, 17 and 18) which were sacrificed for the in vitro labelling study. The last animal in this series was sacrificed at 1538. This gave a period of 6 hours to observe any change in mitotic index following the injection in the treated group. In order to assess any possible effects of thymidine manifesting later on in the treated group,

one further (control) animal (number 24) was sacrificed at 1830, thus prolonging the time interval between the injection and sacrifice to nine hours. Biopsies of the ventral surface of tongue used for this study were immediately fixed in Bouin's fluid. The animal numbers used for obtaining these tissues and the respective times of their sacrifice are shown in Table 3.2.

3.2.10 Processing, Embedding and Section Cutting

The tissues from all three experiments were fixed in Bouin's fluid for 2 - 3 hours. The processing was carried out in a Histokine and the processing cycle used is detailed in appendix 2. All tissues were then paraffin embedded.

The face of each paraffin block was reduced in size to 4 x 4 mm by removing excess wax and sections were cut on a rotary microtome at 3 μm thickness with the connective tissue and muscle side of the tissue blocks meeting the knife-edge first.

Preliminary work showed that the most suitable section thickness for a study of this nature was 3 μm. At this thickness a satisfactory section could be obtained which, under the objectives 25, 40 or 100 (oil) gave the best resolution for counting cell nuclei. Below this thickness section quality was very variable and increasing the section thickness beyond 3 μm gave rise to problems in counting cell nuclei due to superimposition.

Sections were mounted on subbed slides (Section 3.2.11) by floating in a water bath maintained around 50°C. Four short ribbons of sections from each block were mounted across the slides for autoradiography. These were serial sections at levels 1 - 4, 13 - 16, 25 - 28 and 37 - 40. Sections at levels in between these were mounted separately for

haematoxylin and eosin staining for the purpose of utilising them in Experiment 3 (Section 3.2.9).

3.2.11 Glass Slide Cleaning and Subbing

As the slides should be completely dust free to obtain good results from autoradiography, a meticulous technique for cleaning was adopted. The slides were immersed for 24 - 48 hours in a 5 per cent solution of the biological cleansing agent 'Alconox'* using staining dishes with slides arranged in racks. The slides were then washed in running tap water for 4 - 8 hours and left in distilled water overnight. The slides were rinsed in two changes of distilled water and were wiped dry with a 'J' cloth. Finally, the slides were wiped with 'selvyt' polishing cloth.

In order to obtain good adherence of the section and the autoradiographic emulsion, the slides were coated with a subbing solution made up as follows:

Gelatin	0·625 g
Chrome alum	0·0625 g
Distilled water	125 ml

The subbing solution was freshly prepared and filtered before use.

The pre-cleaned slides were dipped once in this solution at room temperature. The backs of the slides were wiped and the film of gelatin was allowed to drain and dry, in a dust free atmosphere. The 'microflow' laminar flow cabinet was found useful for this purpose. The slides when dry were kept in covered plastic containers.

3.2.12 Autoradiographic Technique

The liquid emulsion dipping technique (Section 2.7) was used

* Alconox Inc., New York, NY 10003, U.S.A.

for preparing the autoradiographs. This method was found to be easier for preparing a large number of autoradiographs when compared with the stripping film technique (Section 2.7.2). Of the many emulsions available for the dipping method Ilford K5 emulsion* supplied in gel form was used. This was selected after conducting preliminary studies with Ilford G5, K2, K5 and L4 emulsions to evaluate which emulsion was providing autoradiographs most suitable for visual grain counting (Sections 2.3.5 and 2.7.4). It was possible to obtain silver grains which were countable with a x40 and x100 (oil) objective, using autoradiographs prepared with K5 emulsion. The G5 emulsion was found to be unsuitable for visual grain counting as labelling often appeared as blobs and the individual silver grains were not sufficiently resolvable for counting. Due to its high sensitivity the exposure time required with the K5 emulsion was short and was found to be more practically suitable than K2. Although L4 emulsion provided the smallest grains, the results obtained with light microscopic autoradiographs were less consistent with this emulsion. Therefore Ilford K5 was selected as the most suitable liquid emulsion from the Ilford range for dip coating autoradiographs.

The method used for dip coating the autoradiographs was modified from Messier and Leblond (1957), Kopriwa and Leblond (1962), and Rogers (1967 and 1973). The paraffin sections were dewaxed completely in two changes of xylene, as traces of wax interfere with the uniform spreading of the emulsion. The sections were brought to water and all subsequent steps were carried out in the darkroom under safelight conditions. The safelight (Ilford S 902) was placed about 3 - 4 metres away from the working bench and was directed away from the work bench to avoid direct lighting.

* Ilford Ltd., Essex, England.

The equipment set-up used for dip coating autoradiographs is illustrated in Figure 3. 5. A Coplin jar was filled with 30 ml of distilled water and 0·6 ml of glycerol and was kept in the water bath maintained at a temperature range of 43° - 47°C. In the two graduated measuring cylinders, lines were drawn, at the 30 ml mark in one and the 60 ml mark in the other. Shreds of Ilford K5 emulsion gel, from the stock bottle, were transferred up to the 60 ml mark in the measuring cylinder (A) and this was allowed to stand in the water bath with the empty cylinder (B) for about 20 minutes. Once the emulsion was molten, gentle stirring was carried out without incorporating any air bubbles. 30 ml of molten emulsion was measured out using cylinder (B) and this quantity was poured into the Coplin jar to obtain a 1 : 1 dilution with distilled water. This was allowed to stand in the water bath for a further 5 - 10 minutes and was gently stirred. Any air bubbles appearing on the surface were removed with a spatula. In order to check the uniformity of the emulsion and whether any air bubbles were still present, a clean slide was dipped into the emulsion and this was taken close to the safelight and viewed directly against it. When a uniformly thin layer without air bubbles was obtained, the emulsion mixture was considered to be ready for dipping.

The slides were dipped vertically into the emulsion and were withdrawn gradually to obtain an even layer. The back of each slide was wiped and the slides were placed on the horizontal metal plate with their coated side facing up. Once all slides had been dipped and transferred on to the metal plate, they were kept there until absolutely dry, in complete darkness for about $1\frac{1}{2}$ - 2 hours.

The controls required for assessing the background silver grains, positive and negative chemography (Section 2. 7. 4) were also prepared.

This consisted of two steps. A non-radioactive specimen was coated with emulsion to assess the background and to reveal any positive chemography; this will be referred to later as the 'cold control'. After dip coating, a slide mounted with a radioactive specimen was exposed to white light by bringing this slide out of the darkroom, and this was later used to assess any negative chemography present in the specimen.

All slides were packed into light proof boxes* and were wrapped in black envelopes. The slide boxes were kept in a refrigerator at 4°C for the desired period of exposure. The exposure period was critical for the double labelled material, as heavily and weakly labelled cells had to be distinguished by their grain density. Autoradiographs from single pulse labelled material were used as a guide to determine the exposure period. Trial autoradiographs, exposed for 4, 8, 10 and 14 days using single pulse labelled material, were developed under the conditions mentioned later in this section. From these preliminary studies 8 days was found to be a suitable exposure period

After the exposure period the slides were transferred to glass racks under safe light conditions. The reagents used for the processing of autoradiographs were:

Kodak D19 Developer** (diluted 1 : 1 with distilled water)

Distilled water stop bath

Kodafix** (diluted 1 : 3 with distilled water)

These reagents were kept in staining dishes in that order and were maintained at 18° - 20°C using a water bath cooled if required with crushed ice. The slides were developed for two and a half minutes, rinsed in distilled water stop bath for half a minute and were fixed for five minutes.

* Camlab, Cambridge.

** Kodak Ltd., Hemel Hampstead, England.

Slides were poststained with haematoxylin and eosin. In preliminary studies it was found that poststaining was preferrable to the prestaining method. This was mainly because in prestained slides, the photographic processing altered the stain and the application of an impermeable membrane (Rogers, 1967) was found to be a time consuming procedure. The actual staining procedure used was to overstain the specimens with Meyer's haematoxylin, followed by differentiation in acid alcohol and blueing in Scot's tap water substitute. To avoid deep staining with eosin, due to the gelatin layer in autoradiographs, the staining time in eosin was restricted to a half to one minute. After rinsing in methylated spirits and absolute alcohol, the slides were mounted in HSR mounting medium.

3.2.13 Procedure for Counting Autoradiographs from In Vivo Study (Experiment 1)

In the autoradiographs, cells which showed four or more silver grains over their nuclei were considered as labelled (Figure 3.6). This criterion was established by examining the autoradiographs prepared from 'cold controls' (Section 3.2.12) as these revealed that the maximal background labelling (Section 2.7.4) over the cell nuclei was not above two silver grains.

The total labelled cells in autoradiographs from hamsters number 1 - 3 (from Group I), 14 - 16 (Group II), 17 and 18 (Group III) were counted under x100 (oil) objective. The total cell count was also obtained for the same areas of epithelium in which labelled cells were counted by counting nucleated viable cells under a x25 objective. Squames in the stratum corneum were not included in the total cell count and degenerating cells close to the cornified layer were also excluded from this count, by identifying degenerating cell nuclei using

the following cytological characteristics:-

(1) Pyknosis - considered as shrinking of the nucleus into a hyperchromatic mass.

(2) Flattening of the nucleus to the extent that no nuclear material was observable within the condensed 'nuclear membrane'.

(3) Fragmentation of nuclear membrane.

The labelled mitoses and unlabelled mitoses (Figure 3.7.) were counted from the autoradiographs prepared from hamster numbers 1 - 13 (Group I) under x100 oil objective. A record was not made of the stage of mitoses as this was not required for the estimations attempted. However, the characteristics used for identifying mitoses were based on the cytological features seen in different mitotic phases and therefore these characteristics are enumerated below under various mitotic phases:-

(i)	In Prophase	(a) condensation of chromatin threads
		(b) increased basophilia
		(c) relative increase in nuclear size and spherical form
		(d) loss of nuclear membrane
(ii)	In Metaphase-Anaphase	separation of chromatids
(iii)	In Telophase	two daughter cells with increased basophilia lying adjacent to each other.

3.2.14 Procedure for Counting Autoradiographs from In Vitro Study (Experiment 2)

Cold controls processed with in vitro labelled batches, revealed that maximal background labelling was less than two grains per cell nucleus. Therefore the criteria for identifying labelled cells in autoradiographs from in vitro labelled material was similar to in vivo material in that cell nuclei demonstrating 4 or more silver grains were considered as labelled.

For all counting, areas of epithelium were selected randomly. This was achieved by moving the specimen by a fixed distance from the edge of the section before commencing the count. This also eliminated the edges which were damaged during specimen collection. Using autoradiographs prepared from single pulse labelled material, labelled cells (Figure 3.8) were counted under x100 oil objective. The total count of nucleated viable cells in the same areas of epithelium was recorded under x25 objective. The characteristics used for identifying degenerating superficial cells to exclude them from the total cell count were similar to that described earlier, in Section 3.2.13.

In the autoradiographs prepared from double labelled material it was necessary to identify two labelled cell populations (heavy and weak) corresponding to the 1 μCi/ml or 10 μCi/ml doses of ^{3}H thymidine. For the differentiation of these two categories (Figure 3.9) the expected range of grain counts for the two pulse labels required to be established. Using in vitro pulse labelled material with a single dose of 1 μCi/ml, the average grain counts over the labelled nuclei in these autoradiographs were assessed. A cell with 4 - 25 silver grains was considered to be labelled with the 1 μCi/ml dose and this criterion was used to distinguish weakly labelled cells from heavily labelled cells. In double labelled material the heavily labelled cells had a grain count distribution much above this range and usually labelling in these cells appeared as a confluence of silver grains.

In each area of epithelium counted, a record was made of heavily and weakly labelled cells and the total count of nucleated viable cells.

3.2.15 Procedure for Counting Mitoses in Experiment 3

Material for counting mitoses in the ^{3}H thymidine treated group was obtained by utilising haematoxylin and eosin stained spare sections

from Group I hamsters, number 3, 5, 6, 7 and 10 (Section 3.2.10). Haematoxylin and eosin stained sections from hamsters, number 14, 16, 17, 18 and 24 were used as control slides for this experiment.

Under x25 objective, the number of mitoses recognisable by earlier mentioned criteria (Section 3.2.13), were recorded from each specimen. This mitotic count was related to two reference units in the epithelium (Section 2.5.1), these being the total nucleated viable cell count and the surface length. To obtain the surface length of the epithelium, a graticule was used in the light microscopic eyepiece and the number of graticule lengths of specimen examined was noted. The upper edge of the square of graticule was kept horizontally along the keratin surface. Using the magnification factor and the length of the graticule the absolute surface length of each specimen examined was estimated.

3.3. RESULTS

3.3.1 Histology

Examination of the ventral surface of the hamster tongue by light microscopy showed that it consists of three cell compartments which could be identified as progenitor, maturation and cornified cell compartments (Figure 3.10). The progenitor cell compartment was not limited to the basal cell layer, as suprabasal dividing cells were readily identifiable.

The fixative used - Bouin's fluid - was found to be satisfactory with good preservation of nuclear detail. Mitoses could be readily seen in haematoxylin and eosin stained sections.

3.3.2 Autoradiography

Systematic study of all batches of autoradiographs and simultaneously processed 'cold controls' revealed neither unacceptable background labelling nor positive chemographic effects. Control slides which were exposed to white light showed absence of any negative chemography. The K5 emulsion, exposure period and photographic processing technique used were found to be yielding consistent results.

All animals which were given the intraperitoneal injection of ^{3}H thymidine (Experiment 1), showed nuclear labelling in the progenitor cell compartment. Labelling of suprabasal cells was evident in pulse labelled material from animals sacrificed within one to three hours.

Autoradiographs from tissues labelled in vitro (Experiment 2) also showed uniform labelling. Careful examination of all sections revealed that ^{3}H thymidine labelling was not limited to the periphery of tissue blocks. However, the two edges of histological sections (approximately 0·3 mm) showed dense labelling and this was attributable to the effects of injury to the periphery of the tissue blocks during specimen collection. These edges were not utilised in labelled cell count estimations in Experiment 2. It was evident that the pattern of labelling in the central areas of in vitro labelled specimens was similar to that seen in autoradiographs prepared from in vivo labelled tissues.

3.3.3 Labelling Index

In vivo

The labelling indices were estimated, using the counts of labelled cells and total cell counts for each animal by substituting these data in the formula given below:-

$$\text{Labelling index} = \frac{\text{Labelled cells}}{\text{Total cells}} \times 100$$

The results of labelling indices for animals in the 3 groups injected at different times of the day are shown in Table 3.3. The mean labelling index for each group appeared to show the expected diurnal variation in this parameter and this is illustrated graphically in Figure 3.11.

In vitro

The labelling indices calculated from the data derived from autoradiographs of single pulse labelled material are shown in Table 3.4. Labelling indices were also calculated from double labelled material, using counts of heavily labelled cells (Table 3.5), considering that this group of cells was labelled by a single pulse of 10 μCi/ml (Section 2.3.5). Figure 3.12 and Figure 3.13 illustrate these labelling indices obtained in vitro, against the time of the day biopsies were obtained. The diurnal variation in the labelling index is again apparent. Figure 3.14 illustrates the comparison of labelling indices estimated in vitro, either by a single pulse label of 1 μCi/ml or by counting heavily labelled cells by a pulse of 10 μCi/ml in the double labelled material. The labelling indices of hamster ventral tongue epithelium, when measured by in vitro labelling, using these two different doses, appear to be closely similar.

Comparison of in vivo and in vitro labelling:

Figure 3.15 illustrates the comparison of labelling indices obtained by in vivo and in vitro labelling techniques and the results appear to be closely similar. A Kolmogorov-Smirnov Test (Siegel, 1956) indicated that the minor differences are not statistically significant

3.3.4 $\hat{T}_s$ Estimation

In vivo

The counts of labelled and unlabelled mitoses in ventral tongue epithelium following ^3H thymidine injection for animals in Group I (Experiment 1) are shown in Table 3.6. The percentage of labelled

mitoses at hourly intervals following the injection was calculated. The percentage labelled mitoses graph (Section 2.3.4) drawn using these data is illustrated in Figure 3.16.

No labelled mitoses were present in tissues obtained from animal number 1 sacrificed half an hour following the injection. This suggests that the G_2 period of this epithelium is longer than half an hour.

The graph illustrates that the 50 per cent labelling of mitoses was reached in 2·2 hours. This is the time taken by the cells to complete $T_{g2} + \frac{1}{2}T_m$. 100 per cent labelling of mitoses was reached and then the percentage fell to the 50 per cent level 11·2 hours after the injection. T_s was estimated as the time distance between the 50 per cent levels of ascending and descending limbs of this graph (Section 2.3.4) and this was found to be 9·0 hours (Figure 3.16).

In Vitro

The counts of heavily and weakly labelled cells from double labelled tissues are shown in Table 3.5. For T_s estimation the following formula (Section 2.3.5) was used:

$$\frac{Ts}{t} = \frac{\text{Heavily labelled cells}}{\text{Weakly labelled cells}}$$

As the time interval (t) between the two pulses was 1 hour, T_s was directly estimated from the proportion of heavily to weakly labelled cells. T_s estimation in vitro is shown in Table 3.5 and a range of values from 5·5 hours to 7·36 hours was obtained. Figure 3.17 illustrates the T_s variation for animals sacrificed at different times of day and shows a reduction in T_s when the labelled index was found to be decreased.

3.3.5 Mitotic Indices for ^3H Thymidine Treated and Untreated Control Groups

Using mitotic counts for treated and untreated animal groups,

the mitotic index for each was calculated using the total cell count and the surface length estimations. The formulae used for this estimation were:-

1. $\frac{\text{Number of mitoses}}{\text{Total cell count}} \times 1000$

2. $\frac{\text{Number of mitoses}}{\text{Unit surface length}}$

The results are tabulated in Table 3.7 for the treated group and in Table 3.8 for the untreated group.

Comparison of mitotic indices in treated and untreated groups:

When examined by both index systems the mitotic curves for animals injected with ^{3}H thymidine and the controls sacrificed at comparable times of the day appear to be closely similar except for animal number 1, and this is illustrated in Figure 3.18 and Figure 3.19. The first animal sacrificed half an hour following injection appears to show a mitotic inhibition.

The Kolmogorov-Smirnov test was applied to test the total mitotic indices in the two groups and these were found not to differ significantly.

3.3.6 Turnover Time Estimation

The turnover time of the nucleated cell compartment of ventral tongue epithelium of hamster was calculated using labelling index data and T_s measurements obtained by in vivo and in vitro methods. The formula used is given below:-

$$\text{Turnover Time} = \frac{Ts \times 100}{\text{Labelling Index}}$$

In order to estimate the magnitude of variations that can occur in the turnover time calculation as a result of diurnal changes in the two observed parameters, the labelling index and T_s, the values obtained for each time period were substituted in the above formula (see Table 3.9). Using in

vivo labelling index data, which yielded three values for the three time periods of the day and a mean T_s obtained by the percentage labelled mitoses method, the estimated turnover time was found to vary from 6·4 - 19·7 days with a mean value of 10·93 days. Using the in vitro method for estimating labelling index and T_s on five occasions between 1000 and 1538 hours the estimated turnover time varied between 4·8 and 19·4 days with a mean value of 12·5 days.

3.4. DISCUSSION

3.4.1 Comparison of In Vivo and In Vitro Labelling Techniques

The results from Experiments 1 and 2 appear to provide direct evidence that satisfactory and uniform labelling of ventral tongue epithelium can be obtained by both the in vivo and in vitro labelling methods described in this chapter. Furthermore, the two techniques give closely similar results.

3.4.2 Diurnal Variation in the Labelling Index

A diurnal variation is apparent in the labelling index data derived from animals which received the isotope at varying times of the day. The invivo and in vitro methods of estimation both show this variation clearly. During the observed six hour period of the light cycle (0930 - 1530 hours) there appears to be nearly a three fold variation in the labelling index of hamster ventral tongue epithelium. Such an observation was not unexpected as many other animal tissues have been shown to exhibit a large range of diurnal periodicity in this parameter (Section 1. 6. 4).

As labelling index data for ventral tongue epithelium has not been reported in earlier studies, it is not possible to compare the results of the present study with any published work. However, the labelling index curves for cheek pouch epithelium of the Syrian and the Chinese

hamster have been reported (Brown and Berry, 1968; Izquierdo and Gibbs, 1974). On comparing the present data with the representative areas of the labelling index curves during the light cycle of these published works, it does indicate that although the absolute labelling index values are different (probably due to variations in animal strains, experimental site and environmental conditions) the patternsof the curves are similar, in that the labelling index value drops as the light cycle proceeds reaching minimum values in the evening.

A crucial factor arising from the finding of diurnal variations in the labelling index estimation, is that the estimated values in any study of this nature are related to the time of the day they are measured To be able to use this parameter as a measure of cell production either the range of variation should be known or the 24 hour mean value for labelling index should be stated for a particular cell renewal system.

3.4.3 Comparison of T_s Estimation by Percentage Labelled Mitoses Method and Double Labelling Method

Results from the in vivo experiment showed that the 'mean' T_s as estimated by the percentage labelled mitoses graph was 9 hours. However, the double labelling method which measured the T_s at different times of the day, did show a range of values for T_s from 7·2 to 5·5 hours.

The duration of the S phase has been regarded by some as being a constant for mammalian tissues and evidence for this was presented by Cameron and Gruelich (1963). Recently a few publications have indicated the presence of diurnal variations in the S phase. Most of these observations have been made by groups of workers using methods based on techniques other than the percentage labelled mitoses method. Scheving and Pauly (1967b) used the mean grain counts of corneal epithelium

of the rat, and Tvermyr (1972) employed a double labelling technique in mouse epidermis to arrive at such conclusions. Using the percentage labelled mitoses method, Moller, Larsen and Faber (1974) were able to show a marked circadian variation of T_s in hamster cheek pouch by using four groups of animals and injecting these at four different times of the day.

The discrepancy in the T_s values obtained by the in vivo and in vitro methods in the present study may be partly explained by the presence of a diurnal variation in the labelling index. Cell renewal systems which demonstrate labelling index and mitotic index rhythms have been described as showing partial synchronisation of cells in these phases (Section 1. 6. 6), mainly due to variations in the rate of entry of cells into these phases in the cell cycle (Pilgrim, Erb and Maurer, 1963; Brown and Berry, 1968; Izquierdo and Gibbs, 1974). The present study was not designed to verify the idea of partial synchrony of cells in the S phase, but it might be that a partial synchronisation of S phase cells was operating in this system, as there were more cells in DNA synthesis at 0930 - 1000 hours than at later times of the day. Therefore, after a period of $T_s + T_{g2} + \frac{1}{2}T_m$ a wave of labelled cells will enter mitosis increasing the percentage of labelled mitoses. Using a value of 7·0 hours (in vitro estimation) for T_s and a value of 2·2 hours for $T_{g2} + \frac{1}{2}T_m$ (in vivo PLM graph), this period of $S + T_{g2} + \frac{1}{2}T_m$ would have been about 9·2 hours. Therefore an increased proportion of labelled cells would have reached mitosis 9·2 hours after the injection, which was the time the PLM graph was descending (Figure 3. 15). From this inference it is logical to assume that these labelled mitoses disproportionately increased the percentage of labelled mitoses and produced a shift in the PLM curve to the right, to result in an over-estimated T_s by the in vivo method.

Apart from variations in the rate of entry of cells into the S phase, diurnal variations in the duration of the S phase can also lead to observed

variations in the labelling index (Section 1.6.6). Results from the in vitro study indicated that at times of the day when the T_s period was least, that the labelling index was also drastically reduced. However, the degree of T_s variations observed by the double labelling method was not sufficient to explain the fluctuation of the labelling index as the degree of reduction of this parameter was much more than the observed reductions in the S phase. Therefore at present it is not clear to what extent any form of partial synchronisation of rates of cell entry to the S phase and the variations in T_s account for the diurnal rhythm in labelling index.

The disadvantages of the percentage labelled mitoses technique are many. It is necessary to count mitoses and this is a tedious procedure, especially in tissues where mitoses are infrequent. Large numbers of animals are required and the T_s estimation obtained is a mean value for the period between injection and sacrifice. Thus any diurnal variations present in this parameter are masked. In estimating the T_s by this method, it is also assumed that T_{g2} is a constant and it is not known whether marked variations are present in this parameter. Gelfant (1963) has presented evidence to show that the G_2 phase is variable.

The main advantage of the double labelling technique is that it is not necessary to identify and count mitoses and therefore the disadvantages of a mitotic counting technique (Section 2.2.1) are not encountered. The number of animals required for this method is much less than that required for the PLM method. By double labelling, T_s is estimated for a particular time of day and therefore the method allows diurnal variations to be assessed.

Although the double labelling method using two doses of the same isotope is a reasonable method for obtaining T_s, it also has limitations.

The method is totally dependent on identification of labelled cell nuclei corresponding to the two pulses. A large range of variable factors encountered during the labelling and autoradiographic process could obviously affect the final image. A meticulous technique was adopted to control these variables and the autoradiographic method used appeared to give reliable results. A criticism of the experimental method used is that, although the grain count yield corresponding to (1 μCi/ml) single weak pulse was analysed, this was not carried out for a single heavy pulse of 10 μCi/ml at the time of these experiments. A possible minor error arising as a result of this is discussed in Chapter 7.

3.4.4 The Dose of ^{3}H Thymidine

The results from Experiment 3 do provide evidence that the dose of ^{3}H thymidine of 1 μCi/g body weight (specific activity 22 - 26 Ci/m mole) used for in vivo labelling did not have any apparent stimulatory or inhibitory effects on the cell production parameters in this system.

Only the first animal which was sacrificed half an hour after the injection showed an increased mitotic index (Section 3.5.5). This could be due to some stimulation caused as a result of the intraperitoneal injection and handling. It is possible that the half hour interval this animal had for its recovery between the injection and the sacrifice was not sufficient. It is not possible to draw firm conclusions on this point as a sham treated control (e.g. by injecting saline) was not used. However, the remainder of the animals receiving ^{3}H thymidine injections appear to be having mitotic indices not statistically different from untreated animals sacrificed at corresponding times. The amount of thymidine contained in each dose was 0·011 μg/g body weight. This dose level is below the critical dose level suggested by Blenkinsopp (1967) for cell kinetic studies in mice (Section 2.3.2) and appears to be suitable for cell kinetic analysis of the hamster in vivo.

It was not possible directly to establish the suitability of the dose of ^{3}H thymidine used for the in vitro study. However, indirect evidence is available to exclude the possibility that the doses used in vitro caused any harmful effects. The amounts of thymidine added in the weak and heavy pulses were in the order of 0·011 µg per ml and 0·11 µg per ml of the culture medium. As the labelling index values obtained in vitro for each animal by the weak and the heavy pulses were closely similar (Figure 3.14), there was no reason to assume that increasing the thymidine dose in the heavy pulse by ten times affected cell kinetics of this epithelium. Further, as it could be shown that in vitro labelling index was not statistically different from the in vivo values obtained with a satisfactory in vivo dose, it does confirm that the the in vitro doses used were not altering the cell kinetic parameters of hamster tongue epithelium to any considerable extent.

3.4.5 Turnover Estimation

The turnover time estimation for the ventral tongue epithelium was made using the labelling index and T_s parameters measured by both in vitro and in vivo methods. The importance of measuring these at different times of the day in order to obtain mean values of these parameters to calculate the turnover time of the epithelium is apparent from the results presented in Section 3.3.6. A 3·4 fold variation in turnover time was found depending on the time of the day the labelling index value was obtained. In many studies the labelling index has been measured at one time of the day and this has been used to calculate the turnover time. This practice appears to give erroneous results unless the circadian rhythms in those parameters are limited in a particular tissue. The need for measuring the labelling index parameter over a 24 hour period, to estimate turnover time of cell renewal systems showing circadian rhythms is apparent in this study. This conclusion is also supported by recent studies reported by Izquierdo and Gibbs (1974).

The turnover time estimated in the present study is valid for the renewal of nucleated cell compartments of ventral tongue epithelium of the hamster. However, it does not relate to the whole epithelium as renewal of the keratin layer was not measured (Section 2. 6. 1). As separate estimations of the sizes of the progenitor and mature cell compartments were not made,the compartmental turnover times cannot be estimated from the present study. However, a turnover time of 10 - 12 days for the total cell population appears to demonstrate the dynamic nature of this stratified squamous epithelium.

3. 5. CONCLUSIONS

In vitro and in vivo labelling techniques were found to give closely similar results. A diurnal variation in the labelling index was observed in the ventral tongue epithelium of hamsters maintained in a 12 hour dark-light cycle. Estimation of the T_s by the in vitro method showed variations in this parameter. Although earlier workers believed T_s to be a temporal constant, evidence is accumulating to support the view that variations in T_s are also present in some renewal systems. T_s was found to be low when the labelling index was at minimum but this reduction was not sufficient to explain the three fold variation in the labelling index parameter. The limitations of the percentage labelled mitoses method in estimation of T_s are discussed.

The mean turnover time for the nucleated cell compartment of ventral tongue epithelium was found to be 10 - 12 days by the two labelling methods. Data derived in this study appears to be comparable to the turnover times of tongue and other oral epithelia of the rat, the mouse and the rabbit, described in the literature.

The importance of obtaining mean values for the labelling index and the T_s before using these parameters to estimate the turnover time

of a renewal system was found to be crucial. The in vitro method described can be recommended for measuring cell kinetic parameters of renewing epithelia and appears to be a safe, reliable and a simple technique for use in human studies.

CHAPTER FOUR

AN EXPERIMENTAL STUDY OF THE CELL KINETICS OF NORMAL HUMAN BUCCAL EPITHELIUM BY IN VITRO LABELLING WITH ^{3}H THYMIDINE

4.1. INTRODUCTION

Our present knowledge of the cell renewal of oral epithelium stems largely from data derived from studies carried out in animals. A few attempts have been made to study cell kinetics in human oral epithelium by counting progenitor cells in mitosis (Meyer, Daftary and Pindborg, 1967; El-Labban, Lucas and Kramer, 1971) or in the S phase of the cell cycle as demonstrated by radioactive thymidine uptake (Kaidbey and Kurban, 1971; Alvares et al 1972). From these preliminary studies mitotic index and labelling index values for buccal mucosa have been reported.

It is not possible to proceed from the information gathered by these studies to estimate the cell production rate or turnover without a knowledge of the duration of the S or M phases of the cell cycle or the rate of entry of cells into one of these phases. This information has not previously been available as it cannot be derived from single pulse labelling or by counting mitoses in fixed preparations. Kaidbey and Kurban (1971) and Alvares et al (1972) used assumed values for the S phase duration obtained from data reported for the epidermis by other workers to estimate the turnover time.

Gillespie (1969) used intra-arterial and intravenous injection of ^{3}H thymidine in patients with terminal illnesses to estimate the time taken for exfoliation of labelled cells in buccal epithelium. There are obvious limitations in this in vivo method and its unsuitability for general use in cell kinetic studies in man was discussed in Section 2.4.3.

The magnitude of any diurnal variations in cell kinetic parameters in human oral epithelium is not known. Any marked variation might invalidate turnover estimation using data obtained for only one particular time of the day (Section 1.6.5).

The aims of the study described in this chapter were:-

1. to investigate the cell production of human oral epithelium by counting cells in the S phase and estimating T_s simultaneously by in vitro labelling, in order to obtain a measurement of cell renewal.

2. to quantitate any diurnal variations in labelling index and T_s by a 24 hour sampling procedure. This was to establish in particular whether diurnal variations during daytime show fluctuations of a magnitude which might preclude the use of labelling studies as a diagnostic tool.

3. to assess the suitability of presently available reference units in the expression of the labelling index.

4.2. MATERIALS AND METHODS

The basic design of the study was to obtain biopsies from normal individuals and, following in vitro labelling with ^3H thymidine, to prepare autoradiographs for identification and counting of cells in DNA replication (S phase). To quantitate the diurnal variations in cell kinetic parameters biopsies were obtained at six different times of the day.

Experiment 4, to be described in this chapter, was carried out in two parts. In the first instance biopsies from three subjects at six times of the day were studied. Subsequently additional biopsies were obtained at some of the times of the day from eight more individuals. For reasons to be discussed, these two groups examined will be reported in two parts of the same experiment, as Experiment 4A and 4B.

The techniques of tissue labelling and preparation of the autoradiographs were similar to the techniques described in Chapter 3, and therefore these technical aspects will not be described in detail in this chapter. Some modifications were made in the autoradiographic technique and in tissue morphometry and these will be mentioned.

4.2.1. Experiment 4A - Tissue Sampling and Biopsy Procedure

Biopsies for this experiment were obtained from three adult males who were 26, 28 and 32 years old. Two of these were non-smokers. One was a light pipe smoker, but refrained from smoking for several hours before each biopsy. The site investigated was the buccal mucosa below the occlusal line opposite the first molar and second premolar teeth.

Biopsies were taken from each subject at scheduled times four hours apart at 1000, 1400, 1800, 2200, 0200 and 0600 hours. For each individual time period, all three subjects were biopsied at one sitting within the course of 30 minutes. As it was not practicable to obtain all six biopsies during a single 24 hour cycle, the sampling was carried out on three occasions. The actual procedure was to obtain the first biopsy from the right buccal mucosa and four hours later to biopsy the other side. After at least two weeks' healing period, two further biopsies were obtained and the final two were taken at a third occasion. On the second and third occasions the wound sites of earlier biopsies were not apparent in most instances. When any evidence of scar tissue was present biopsies were taken from adjacent normal looking areas. For the overnight biopsies the subjects were encouraged to maintain, as far as possible, their normal nightly sleeping habits in order to avoid any additional disturbances to their individual biological rhythms (Section 1.5.2).

Biopsies were taken under local regional anaesthesia using

lignocaine hydrochloride 2 per cent without a vasoconstrictor (Lignostab*). Local infiltration immediately around the site of biopsy was avoided as it was thought that this might change the tissue architecture and tension. The use of a vasoconstrictor in the anaesthetic agent was avoided to eliminate any possible effects of agents such as adrenaline on cell kinetics (Section 1.5.1). The method of obtaining anaesthesia was to infiltrate the long buccal nerve in the second molar region at the sulcus and the mental nerve in the first premolar region. A 3 mm trephine type punch was used to make a circular incision in the buccal mucosa as illustrated in Figures 4.1 and 4.2. Care was taken to avoid rubbing the surface of the epithelium and thus causing loss of superficial cells. The base of the punched mucosa was then cut with a pair of fine scissors (Figure 4.3). A 'punch biopsy' of about 3 mm diameter was obtained by this method (Figure 4.4). The wound at the biopsy site was found to heal rapidly without the necessity for suturing. None of the individuals experienced other than mild discomfort immediately after the biopsy procedure or during the healing phase.

Biopsies were transferred in tissue culture medium M199 at room temperature to the cell kinetic laboratory and labelling experiments were begun within 10 minutes of the time at which the biopsy of the last of the three subjects was carried out.

4.2.2 In Vitro Labelling

The biopsies were trimmed into 1 mm slices by cutting perpendicular to the epithelial surface to obtain three tissue blocks from each biopsy. All three pieces were then incubated with 1 μCi/ml of ^{3}H thymidine (specific activity 22/26 Ci/m mole) for 15 minutes under the incubation conditions stipulated in Section 3.2.8. Two of the specimens

* The Boots Company Limited, Nottingham.

were removed after this single label and rinsed in label free medium for 10 minutes prior to fixation. The remaining block from each case, after being in label free medium for one hour, was given a second pulse label of 10 μCi/ml of ^{3}H thymidine of the same specific activity for 15 minutes. These specimens were then rinsed in label free medium and fixed. All tissues were fixed in Bouin's fluid (Appendix 1) for approximately two hours and then processed and paraffin embedded. Due to the small size of blocks, it was necessary to use a dissecting microscope to obtain satisfactory orientation of blocks, at the time of embedding in wax.

4.2.3 Section Cutting, Mounting and Autoradiography

3 μm serial sections were cut with a rotary microtome and were mounted on subbed slides (Section 3.2.11). The arrangement of serial sections followed during mounting is illustrated in Figure 4.5 and this enabled every eighth section to be utilised for the study by dip coating two slides A and C, as indicated in the figure. The other two slides B and D were kept as spares. On occasions, when the eighth section was not suitable, due to technical errors in cutting, mounting or autoradiography, the next closest serial number was used for examination.

The autoradiographic technique was similar to that described in Chapter 3 in that the same emulsion (Ilford K5) and same dilution of 1:1 in distilled water with 0·4 ml of glycerol was used. A dipping jar was made by cutting a 100 ml glass measuring cylinder to the level of 42 ml so that 20 ml of distilled water and 20 ml of molten emulsion could be used with ease for the dipping procedure in this modified jar (Figure 4.6). The methods and times of exposure, developing and fixation were similar to the procedures described in Chapter 3.

4.2.4 Analysis of Autoradiographs: Single Labelled Tissue

Autoradiographs prepared from single labelled tissue (1 μCi/ml) were utilised to obtain the labelling index using all four reference units

described in Section 2. 5. 1. As quantitation of cell production in oral buccal epithelium has not been systematically investigated, use of all four reference units in this study was considered a prerequisite to decide on the suitability of the available index methods for further work.

Selection of Area of Specimen for Counting:

The area of each histological section used for counting was selected before each specimen was analysed by the different reference units. A revolving circular stage was used to orientate the slide so that the epithelial surface of the specimen was aligned horizontally. Under x25 objective, a column of epithelium at right angles of the mean epithelial surface was defined using an eyepiece graticule. Systematic random sampling was used (Section 2. 8. 3). Bias in selecting the first microscopic field for analysis was avoided by moving the specimen by half a graticule width from the right hand edge in every case. This also allowed for elimination of damaged tissues at margins where a falsely high labelling may be seen (Section 3. 2. 14).

Depending on the availibility of the length of sections, one, one and a half, or two graticule widths from consecutive fields were analysed.

Counting of Total Nucleated Cells:

The total nucleated cells in a column of epithelium (defined by the graticule in the eyepiece as described earlier) was counted using x25 objective. This objective was selected as it was possible to count cell nuclei at this magnification without changing the focal plane once it was set for an individual section. This allowed cell nuclei to appear as a monolayer in 3 μm sections and overlapping of cells was infrequent.

Twenty five smaller squares in the graticule, as illustrated in Figure 4. 7, were used to delineate small areas of epithelium which

grouped cell nuclei into smaller units. This helped the counting procedure, simulating the principle of counting blood cells in a haemocytometer. Where the thickness of the epithelium was greater than the height of the graticule, the specimen was moved up to a second position to complete the counting of a column of cells through the full epithelial thickness.

The total count was grouped into two categories by identifying the epithelial cells as progenitor or mature cells to enable the labelling index of progenitor cells to be estimated. The criteria used for the differentiation of progenitor and mature cells will be described fully in Chapter 5, which deals with a more elaborate cell compartment analysis.

Counting of Labelled Cells:

Cell nuclei over which lay more than four silver grains, were considered as labelled. This figure was arrived at after examining the background labelling in autoradiographs prepared from control unlabelled tissue.

The number of labelled cells along the same width of epithelial column used for the total cell count was counted under x100 (oil) objective. This magnification was found desirable for identification of labelled cells in human buccal mucosa.

At the same time a record was also kept of individual grain counts of each labelled cell by visual grain counting. This information was required for identifying 1 μCi/ml labelled cells later on when double labelled material would be analysed (Section 4. 2. 6).

Measurement of Surface Length and Basement Membrane Length:

The absolute surface length and the basement membrane length of

the same area of specimen selected for the analysis were measured stereologically. This was done by projecting the sections on to a Leitz projection head (Figure 4. 8). Two vertical lines were drawn on the projection screen to correspond to the horizontal width of the cell column examined during cell counting. In the projected image, due to a magnification factor of x12·5 in the projection head attachment, this same width of cell column was obtained using a x16 objective rather than a x25 objective which was used while viewing sections through x10 ocular during cell counting.

The intercept point counting along the surface and the basement membrane of the epithelium, was carried out using a circular transparent perspex grid with parallel lines 10 mm apart, superimposed on the projected image (see illustration in Figure 2. 2a). A system of parallel lines is anisotropic and the number of intercept points will vary depending on how the grid is presented to the section. In order to eliminate this, six counts per field were made by rotating the grid to six positions. This had the added advantage that it overcame any possibility of the periodicity of the grid lines corresponding to some periodic distribution of elements in the tissue. The six grid positions used for analysis were assigned by reference points on the rim of the projection head. The angular distance between counting positions was 30^{o} in five of the positions, and 20^{o} between the centre two positions. This was to avoid overlapping of grid positions which would have occurred at the sixth count if a grid of parallel lines were moved by 30^{o} to each counting position.

4. 2. 5 Corrections to Cell Counts

As cell counts were carried out by counting nuclei in histological sections, corrections were required to account for nuclear fragments which can result in an overestimate of cell numbers. Two main

methods have been described in the literature for this purpose. Abercrombie (1946) suggested the use of a formula to correct for the errors arising from nuclear fragments (Section 2.2.1). Weibel (1962) utilising stereological principles suggested a formula involving a shape co-efficient and an estimate of the volume density of the structure being measured. Abercrombie's method was found to be more appropriate and easily applicable for correcting light microscopic cell counts in the oral epithelium.

As Abercrombie's correction is a function of nuclear diameter and section thickness, such a correction was found not to be required for correcting progenitor labelling index as the labelled cells are of similar nuclear diameter to the rest of the progenitor cells in the oral epithelium. However, it was necessary to apply the correction factor for correcting the labelling index estimated by total cell count. The reason for this was that mature cells have a larger nuclear diameter than progenitor cells (or labelled cells) and therefore may have relatively more nuclear fragments in a given volume of section.

In order to apply the Abercrombie's correction to the total cell count, it was necessary to measure the nuclear diameter and the section thickness.

Measurement of Nuclear Diameter:

The nuclear diameter of individual epithelial cells in a column of epithelium was measured using a standard eyepiece measuring graticule in a light microscopic eyepiece at a magnification of x1000 using x100 (oil) objective. Two autoradiographs at two levels of each biopsy utilised for Experiment 4A were studied to obtain nuclear measurements.

For measurement of nuclear diameter Abercrombie (1946) suggested that the block should be recut at right angles to the original axis to estimate the cross section or length of each cell nucleus at right

angles to the plane of section utilised for cell counting. This was not practicable for the present study due to limitations in the size of the tissue blocks used for organ culture. For the purposes of this light microscopic study it was assumed that the horizontal width of a nucleus would approximate closely to the required measurement at right angles to the plane of section.

The widest horizontal distance of each nucleus in a randomly selected cell column was measured in μm using a micrometer graticule in the eyepiece. The graticule also delineated approximately the cell column examined in each specimen. In making the actual measurement, the distance between two vertical imaginary lines touching the widest points of the nucleus was recorded. The case for measuring all cell nuclei in a column of epithelium was considered to be superior to the customary procedure of measuring selected nuclei (Dunhill, 1968; MacKenzie, 1970), based on criteria to include only those which demonstrate the presence of central or widest diameter. Such a procedure was considered to cause some bias in favour of measuring large cells.

Records of measurements belonging to the progenitor and mature cells were kept separately to obtain the mean nuclear diameter of cells belonging to each compartment. At least 25 cells of each compartment in an individual autoradiograph were utilised in this measurement.

Measurment of Section Thickness:

All sections were cut at the 3 μm setting on a rotary microtome, specially maintained for research work, as routinely used microtomes in the laboratory are generally regarded as less reliable for cutting

sections of constant thickness. However, even then it was necessary to standardise or make a random check on the thickness of sections. Although methods of measuring section thickness have been described (Marengo, 1944), these were found to be extremely elaborate. Rogers (1973) considered that measurements made by focussing the upper and lower levels of a specimen and reading the difference in height from the calibration of the fine focus control of a microscope are not accurate below a thickness of 20 μm. However, this error in measurement basically arises from differences in the power of accommodation of human eye and due to the depth of focus of the objective used. The larger the depth of focus the bigger the range of measurement obtained. In order to eliminate the human factor, a method was devised using photomicrography. This was based on the reasoning that the camera would record only one plane at a time and therefore any difference in accommodation becomes a factor of the depth of focus of the objective which is a known parameter.

A through focus series of photomicrographs was taken at 1 μm apart using the graduated fine focus on a good quality Leitz Orthoplan microscope with a sturdy base to minimise vibrations which could be transmitted to the camera. As autoradiographs were utilised for this estimation, the focal plane of silver grains was assumed to be 1 μm above the focal plane of the cellular layer in the histological section. Starting at this plane of silver grains (illustrated in Figure 4.9a) and by step-wisely changing the fine focus by 1 μm to go through the complete cellular layer, photomicrographs were taken until the eye could not accommodate the cells. Six randomly selected specimens were utilised to analyse the consistency of section thickness. Although the human eye did accommodate up to about 6 - 8 μm of depth below the silver grain focal plane, the photomicrographs revealed that beyond 3 - 4 μm changes in focus the camera could not register any cellular detail (Figure 4.9).

As the depth of focus of x100 (oil) objective is 1·0 μm (Drury and Wallington, 1967) it was reasonably assumed that the section thickness was close to 3·5 μm. This was taken as the mean section thickness for the cell count correction.

Application of the Correction Factor:

Using this mean section thickness and the mean nuclear diameters estimated for progenitor and mature cells of individual biopsies, corrections to cell counts were made using the formula given below (Abercrombie, 1946): -

$$N = n \times \frac{T}{T + D}$$

where N - corrected cell count

n - crude cell count

T - section thickness

D - nuclear diameter

4.2.6 Analysis of Double Labelled Autoradiographs

Two labelled cell populations were identified and counted as weakly and heavily labelled cells in autoradiographs prepared from double labelled material. Nuclei with 4 - 25 silver grains were grouped as weakly labelled cells as this was the grain distribution seen in single pulse labelled material with the 1 μCi/ml dose (Section 4.2.4). Heavily labelled nuclei which usually showed a large cluster of silver grains over the nucleus were considered as labelled by the second pulse of 10 μCi/ml (Figure 4.17). As previously described, two graticule lengths of epithelium under x25 objective were examined and the total number of nucleated cells in that part of the specimen was counted. The same length of epithelium was examined under x100 (oil) objective for differentiation of labelled nuclei into heavily and weakly labelled cells.

4.2.7 Experiment 4B

This study consisted of eight other young adult males (number 4 - 11). Their ages varied from 21 - 32 years. Each individual volunteered to be biopsied twice at four hours apart on the same day. The times of biopsy of each subject are shown in Table 4.1. The procedure adopted was to biopsy two subjects at one sitting.

The site and method of biopsy was exactly similar to those described in Section 4.2.1 for the three subjects. The labelling technique, autoradiography and counting procedure were also similar to those of Experiment 4A.

4.3 RESULTS - EXPERIMENT 4A.

Tissue preservation following in vitro labelling was found to be satisfactory (Figure 4.10) and uniform labelling of cells was obtained in the autoradiographs except for tissue samples obtained at 0200 and 0600 hours from all three subjects. These autoradiographs were found to be poorly labelled even after repeating the autoradiographic procedures utilising the spare sections. As no apparent cause for this was detected, this part of Experiment 4A was repeated on a later occasion and satisfactory labelling was obtained.

In the serial sections examined, a proportion of suprabasal cells were labelled, thus confirming the original belief that the progenitor cell compartment is multilayered in human buccal mucosa.

4.3.1 Estimation of Reference Units and Labelled Cell Count

In Table 4.2 are listed the total counts of nucleated cells grouped into progenitor and mature cells and the labelled cells counted in each biopsy. The values of cell counts are not corrected (Section 4.3.4). The table also shows the intercept point counts along the epithelial

surface and the basement membrane. The absolute values for the surface length and the basement membrane length were calculated by substituting the intercept point counts, the magnification factor, and the distance between the grid lines in the formula given in Section 2.8. These estimations are tabulated in Table 4.2.

4.3.2 Labelling Index and Diurnal Variation

The labelling index for each individual biopsy at the six different times was calculated asa percentage of total cells and of progenitor cells and also per unit length of epithelial surface and of basement membrane. These labelling indices are tabulated in Table 4.2.

The labelling index values, estimated by all four index systems for each biopsy period are shown graphically in Figures 4.11 - 4.14. All three subjects show a rise in the evening with peak values at 2200 hours and a rapid fall in the early hours of the morning. The pattern is most clear-cut and consistent in the labelling index curve expressed in terms of surface length.

Figure 4.15 shows the mean values of labelling index for the three subjects at six periods of biopsy illustrated by the total cell count index. At 2200 hours, which was the peak labelling period, 3·18 per cent of total cells were in the S phase as compared with 2 per cent seen at 0600 hours. The 24 hour mean values obtained for each subject by total cell indices were 2·42, 2·57 and 2·42 per cent.

Statistical Analysis:

The existence of similar patterns of variations in labelling indices in all three individuals at different times of day is strong suggestive evidence of a circadian rhythm. It was desirable to attempt to substantiate this conclusion by statistical tests. Because of the small

number of observations made at each time period nonparametric statistical methods were employed.

Initially it was necessary to test whether the three individuals differed significantly in their labelling indices expressed by the four reference units. A Kruskall-Wallis one way analysis of variance was used. The analysis relating to labelling index per 100 total cells is shown in Table 4.3. Tests of all labelling indices gave a probability of $P>0.10$ indicating that there was no significant difference between individuals.

Since the individuals did not differ significantly it was felt reasonable to assume that any variation in labelling index at different time periods was not a function of the individual from whom the biopsy was obtained, but was related to the time of the biopsy. A Kruskall-Wallis one way analysis of variance was used to examine this possibility. The null hypothesis was that the 18 values obtained for the labelling index at six times of the day were drawn from a single population. Table 4.4 shows the test applied to the data of the labelling index expressed by total nucleated cells. This gave a value of $P<0.05$ indicating a significant variation of labelling index with time. The labelling index expressed in terms of the progenitor cell count again showed the variation with time to be significant ($P<0.05$).

When the Kruskall-Wallis test was applied to the labelling index data expressed with reference to surface length and basement membrane length, the variation with time was found not to be significant. Inspection of the graph shown in Figure 4.13 shows that the values in the 2200 hour biopsies were higher than at other times, but that at the five other times sampled the values obtained were closely similar. It is possible that due

to similarities in data obtained at these five periods, the Kruskall-Wallis test fails to reveal a significant difference at a single time period. Accordingly the question was asked "do the values obtained at 2200 hours differ significantly from labelling index values obtained at other times?" The appropriate test to answer this question is the Mann-Whitney U Test. Table 4.5 shows this test applied to the data from the labelling index expressed in terms of surface length. This gave a value of $P = 0.002$ indicating that the labelling index at 2200 hours was significantly higher than at other times when the index per unit surface length was used. Similar results were obtained when the Mann-Whitney U Test was applied to the labelling index values expressed in terms of basement membrane length ($P = 0.002$).

4.3.3 Correlation and Variability in Index Systems

The labelling indices expressed in the four reference units do not demonstrate identical patterns of diurnal variation. This variability in the expression of the labelling index may be related to variations in the reference units. Some of these variations, such as cell density, appear to be due to diurnal variations in biopsies taken at different time periods, while others, such as variations in the ratio of basement membrane to epithelial surface, are probably caused by minor site differences within the cheek.

In order to assess the variability and correlation of the four index systems, the co-efficient of correlation of each index to the surface length index was analysed. The surface length was chosen for this purpose as it is probably the most constant reference unit. The co-efficient of correlation was close to 1 for the basement membrane index ($r = \cdot 97$), $r = \cdot 76$ for the total cell count index and $r = \cdot 70$ for the progenitor cell index. The significance of these correlations in the interpretation of labelling index data is discussed in Section 4.5.2.

4.3.4 Corrections to Total Cell Count and Labelling Index

Table 4.6 shows the mean nuclear diameter of progenitor and mature cells for the 18 biopsies obtained by direct measurements with the eyepiece micrometer graticule (Section 4.2.5).

Using these nuclear diameters and a mean section thickness of 3·5 μm (Section 4.2.5) for all cases, correction factors for progenitor cells and mature cells were estimated using Abercrombie's formula (see Section 4.2.5). These correction factors estimated for each biopsy are shown in Table 4.6.

The corrected total cell and labelled cell counts are tabulated in Table 4.6. The estimated labelling index following this correction is also shown in this Table. Figure 4.16 shows the labelling index by corrected total cell index for the three subjects at the six time periods.

This correction does not appear to make any significant difference to the labelling index data presented in Table 4.2 obtained using uncorrected cell counts. This is due to the fact that the differences in mean nuclear diameters in progenitor and mature cells are limited to 1 μm in most cases.

4.3.5 T_s Estimation

An autoradiograph prepared from double labelled material is illustrated in Figure 4.17. The counts of heavily and weakly labelled cells are tabulated in Table 4.7. T_s was estimated using the proportion of heavily to weakly labelled cells for biopsies taken between 1000 and 2200 hours and these values are also presented in Table 4.7. These estimations of T_s for each subject are graphically illustrated against the time of biopsy in Figure 4.18. The range of T_s variation found between 1000 and 2200 hours was 5·1 to 6·9 hours and no consistent pattern was seen in this variation.

T_s could not be estimated in the autoradiographs prepared from biopsies taken at 0200 and 0600 hours as it was not possible to differentiate heavily from weakly labelled nuclei from these autoradiographs (see Section 4. 5. 3). This may be related to, or arise as a result of, a lack of cell movement in the dividing cell cycle at this time period and this feature is further discussed in Section 4. 5. 3.

4. 3. 6 Rate of Cell Entry into the S Phase

The rate of cell entry into the S phase was calculated by comparison of the percentage of labelled cells counted in the single and the double labelled material. The increase in the number of labelled cells in double labelled material (Table 4. 8) was assumed to be due to additional cells which had moved into the S phase during the interval between the pulses or during the second pulse labelling period, totalling 75 minutes in all. The rates of cell entry/100 cells/hour for the three subjects at each biopsy period are shown in Table 4. 8 and illustrated graphically in Figure 4. 19.

Although there were marked fluctuations during daytime (at 1000 and 1400 hours) a consistent high rate of cell entry was observed at 1800 hours and this dropped to very low rates at 0200 hours. Differences in the rate of cell entry at different time periods of the day were found to be not statistically significant (Kruskal-Wallis one way analysis of variance; Siegel, 1956). For the three subjects investigated the mean rates of cell entry into the S phase over a 24 hour period were 0·72, 0·52 and 0·55 cells/100 cells/hour. The use of this parameter to estimate the daily cell production rate is discussed in Section 4. 5. 5.

4. 3. 7 Turnover Time

Turnover time was calculated using the corrected mean labelling index over a 24 hour period and mean T_s values for four of the six time

periods. The turnover times of the total nucleated cell layers of the buccal epithelium of the three individuals were 10·16, 9·11 and 10·16 days. However, if cell movement into and out of the S phase does not occur between 0200 and 0600 hours, as suggested by the low rate of cell entry into the S phase at 0200 hours and minimum labelling at 0600 hours, the actual turnover times could be up to a third longer than the estimated values presented here.

The mean turnover time of the progenitor cell compartment was also obtained using the labelling index values estimated by the progenitor cell index for the three subjects. The estimated turnover times of progenitor cell compartment was 5·76, 4·85 and 5·60 days.

4.4 RESULTS OF EXPERIMENT 4B

The results of Experiment 4B are recorded in Table 4.9 and Table 4.10. The labelling index range for these additional eight subjects was found to be closely similar to the three subjects already described in Experiment 4A. However, when the diurnal variation in labelling index with time was analysed, subject number 4 appeared to show consistently high values for his two biopsies which were taken at 1000 and 1400 hours. This subject was different from the rest of the subjects in the group in that he smoked about six cigars a day. He was excluded from further analysis. The labelling index curve shown in Figure 4.20 was drawn using the mean values obtained from the rest of the subjects (numbers 5 - 11). It was not possible to record the labelling index for subject number 6 at 1400 as the autoradiographs prepared showed inadequate nuclear labelling. For purposes of comparison Figure 4.20 includes the graph drawn from the mean labelling indices of subjects 1 - 3 in Experiment 4A (same as Figure 4.15).

Table 4.10 shows the T_s estimation and the rate of cell entry into the S phase for seven of the subjects in Experiment 4B. Material from subject number 10 was not utilised due to technical errors during wax embedding.

4.4.1 Comparison of Results of Experiment 4A and Experiment 4B

The 24 hour mean labelling index for all 18 biopsies taken from the three subjects in Experiment 4A was 2·48 per cent and the mean value for the 13 biopsies taken during a 12 hour period from the 7 subjects included in Experiment 4B was 2·62 per cent by the total cell count index.

The range of values obtained for the labelling index T_s and rate of cell entry into DNA synthesis in both groups of subjects in Experiment 4A and 4B were close.

4.5 DISCUSSION

This appears to be the first study in which the labelling index of human buccal mucosa has been studied over a 24 hour period. The values obtained compare quite well with labelling indices of normal buccal epithelium reported by Alvares et al (1972) for 1100 - 1230 time period.

4.5.1 Labelling Index and Diurnal Variations

The consistent diurnal pattern observed in the three subjects in Experiment 4A and the fact that a reproducible pattern was seen in the additional biopsies studied, by increasing the number of subjects (Experiment 4B), is strong presumptive evidence that there is a diurnal variation in the labelling index. A peak labelling index was observed at 2200 hours and minimum labelling at 0600 hours.

Two of the samples were taken during the eight hour period suitable

for any routine clinical investigation. The magnitude of variation in the labelling index in these 1000 and 1400 hour biopsies was limited and the mean labelling index value of 2.37 per cent for the six samples during this period is close to the 24 hour mean value of 2.48 per cent.

The only other comparable study which has reported a 24 hour analysis of labelling index in human tissue is that by Kahn et al (1968) by in vivo labelling of epidermis. They showed a slightly increased labelling index at midnight by a six hour sampling procedure. This two hour difference in the time of peak labelling as noted by Kahn et al (1968), and that found in the present study is probably related to the differences in the time schedules used for sampling tissues.

4.5.2 Assessment of Index Systems

The analysis of labelling index by four different reference units allowed several comparisons to be carried out. As the labelling index data measured by all reference units delineated the rhythm in labelling index more or less in the same fashion as illustrated graphically in Figures 4.11 - 4.14, it can be reasonably assumed that the variations demonstrated were not relative variations in the reference units, but that this was an absolute variation in the number of cells in the S phase at different times of the day.

However, the degree of diurnal variation exhibited was not similar in all four reference units when examined graphically. Following the suggestion of Löe and Karring (1969) that epithelial surface index is the least variable, the data derived using surface length index was used as a reference guide to estimate the co-efficient of correlation of the other three index systems. The data by the progenitor cell index was found to be the most variable (co-efficient of correlation 0·70). This

may have arisen as a result of subjective errors in differentiation of progenitor cells from mature cells during counting and thereby in the estimation of cell numbers. A further and a more important possibility is that this reference unit is variable due to a diurnal variation in the proportion of epithelium formed by the progenitor compartment. If such a variation is present in this reference unit it may not be a suitable index for examining diurnal variations in labelling index as the alteration in the reference unit itself may add undesirable variations to the estimated labelling index. This probability will be analysed in the next chapter which deals with a detailed cell compartment analysis.

The surface length was found to be the most convenient and practicable method for use, as the estimation of surface length was less time consuming than cell counting. However, cell counts are required to obtain a labelling index in a form which allows the calculation of turnover times.

4.5.3 T_s Rate of Cell Entry into S Phase and Labelling Index

In this study, the variations in T_s observed at the four time periods could not be related to any diurnal pattern. The rate of cell entry into the S phase did show variations which, when analysed with reference to the 24 hour cycle, demonstrated peaks at 1800 hours and at 0600 hours. A rapid fall in the rate of entry of cells into the S phase was observed at 0200 hours.

The low grain counts seen in the autoradiographs prepared from tissues labelled at 0200 and 0600 hours which even masked the identification of heavily labelled cells could also be related to this low entry of cells into DNA synthesis at this time of the morning. This, therefore, may represent a lack of cell movement into the S phase around 0200 hours.

Differences in the rate of cell entry at the different time periods were found to be not statistically significant (Section 4.3.6). This may be due to the small number of subjects included in the study and also due to relatively large fluctuations in the rate of cell entry obtained around 1000 and 1400 hours (Figure 4.19). The mean rates of cell entry into the S phase for the three subjects were graphically analysed (Figure 4.21) by correlating with the mean labelling index curve (Figure 4.15). The peak labelling index at 2200 hours can be considered as a reflection of the high rate of cell entry four hours earlier. The low rate of cell entry at 0200 hours appeared to cause the low labelling index at 0600 hours. This suggested the possibility of uneven rates of cell entry into the S phase resulting in the apparent alteration in the number of cells in S phase at a given time, in the absence of significant changes in T_s. Grube, Auerbach and Brues (1970) reported that flow rates into S phase varied rhythmically throughout the day and that these paralleled changes in labelling index in mouse epidermis. This provided experimental proof for the concept of a partial synchronisation of S phase cells first suggested by Pilgrim, Erb and Maurer in 1963 in mouse tissues. The changes in the rate of cell entry into S phase and parallel changes in labelling index observed in the present study could be regarded as preliminary evidence for the existence of a partial synchronisation in S phase cells in human buccal epithelium.

4.5.4 Turnover Time of Human Buccal Epithelium

It was possible to obtain turnover time estimations for the total cell layers and the progenitor cell layers separately. Assuming that all labelled cells will proceed to mitosis at a later time, the total cells were found to renew in approximately 10 days, and the progenitor cells in approximately 5 days. From this it may be assumed that the transit time for cells leaving the progenitor layers to the surface would again be 5 days. These estimated parameters reflect an equal

distribution of cell numbers in progenitor and mature cell compartments which was observed during cell counting.

4.5.5 Daily Cell Production Rate

Using the values obtained for the mean rates of cell entry into DNA synthesis, an estimate was made of daily cell production rate

For the three subjects the 24 hour mean rates of cell entry into DNA synthesis were found to be 0·72, 0·52 and 0·55 per cent of total cells in an hour. This suggested that approximately 0·6 per cent of total cells in human buccal epithelium enter DNA synthesis in an hour and therefore 14·3 per cent of total cells would enter DNA synthesis in one day. As about 50 per cent of the total cells form the progenitor cell compartment at this site, one fourth to one third of progenitor cells appear to be engaged in cell production on each day. As the growth fraction of this epithelium has not been estimated it is not possible to evaluate the turnover from the daily cell production rate.

Assuming that 100 per cent of progenitor cells are engaged in cell production, the time taken for complete renewal of this compartment would be about 3·5 days. This value is lower than the turnover estimation obtained using progenitor cell labelling index and T_s (Section 4.3.7). Although such a difference could arise as a result of methodological errors, it is more probable that 100 per cent of progenitor cells are not engaged in cell production and that cells which have left the dividing cell cycle are included in this compartment. Therefore the assumption that 100 per cent of progenitor cells may enter DNA synthesis is in error and this may have resulted in an underestimation of renewal time of progenitor compartment by this method.

4.6 CONCLUSIONS

The in vitro ^{3}H thymidine labelling method described here for studying cell kinetics appears to be a suitable method for the quantitation of cell production of human buccal mucosa.

The human buccal epithelium having a turnover time of 10 days appears to be renewing faster than the human epidermis with a renewal time of 27 days (Section 1.7.5), but slower than the gastro-intestinal epithelium which is replaced in 3 - 6 days (Lipkin , 1965). This finding supports the existing knowledge derived from animal studies.

The reference index systems used in the present study appear to be equally applicable for a study of this nature when comparing normal tissues of a particular anatomical site. The estimation of the surface length was found to be less time consuming and more practicable. However, to estimate turnover data, an index related to a count of progenitor cells or total cells is required.

Diurnal variations in the labelling index were observed. It was possible to relate this rhythm to possible changes in the rate of cell entry into the S phase suggesting the presence of a partial synchronisation in the flow of cells through the dividing cell cycle. However, the range in variations in the labelling index during mid-morning and mid-afternoon was limited. This is an interesting finding. As the magnitude of fluctuation was limited, it appears that labelling index data and turnover times could be used as diagnostic indices to investigate and quantitate cell production in normal and to compare with pathological states where cell kinetics appear to be altered. The data obtained in the present study could be used as base line parameters for further studies in diseased oral mucosa.

CHAPTER FIVE

CELL COMPARTMENT ANALYSIS OF NORMAL HUMAN BUCCAL MUCOSA WITH PARTICULAR REFERENCE TO DIURNAL VARIATIONS IN CELL PRODUCTION PARAMETERS

5.1 INTRODUCTION, DEFINITIONS AND AIMS

Normal human buccal mucosa consists of two compartments of cells; a progenitor cell compartment and a mature cell compartment, which are distinguishable morphologically (Section 1.3.2). By definition, a compartmental system is one which is made up of a finite number of sub-systems, called compartments or pools which interact by exchanging material. The mathematical theory of the behaviour of such systems is called a compartmental analysis or the theory of compartmental systems. Although such an analysis is generally undertaken at a macroscopical level, in biological studies cell compartments may be identified at a microscopical level based on anatomical and histological features.

Quastler and Sherman (1959), and Quastler (1960) introduced the basic concept of compartmental analysis into the study of cell kinetics of renewal systems. Some theoretical applications of data derived from the study of cell compartments for analysis of cell renewal systems were discussed in Section 1.3. A standard diagrammatic representation of a two compartment system using a box to represent a compartment and arrows to represent transfer of cells into and out of compartments as applicable to the buccal epithelium was presented as Figure 1.1. There may be input into one or more compartments and output from one compartment to another or to the environment. If exchange of material occurs with the environment, as in buccal mucosa where cell loss occurs at the epithelial surface, such a system is called an open system.

The concept of analysis of oral epithelium on a cell compartment basis is a new approach to the study of its morphological and kinetic features. MacDonald (1973) used such an analysis in hamster cheek pouch to quantitate changes during experimental oral carcinogenesis. In the present context it has obvious advantages over the division of epithelium into histological strata, as such a division does not relate the morphological distribution of cells to cell production parameters.

The aims of the present study (Experiment 5) were:-

1. to develop a suitable technique to quantitate the size of epithelial cell compartments in normal human buccal mucosa.
2. to study the proportions formed by each compartment in order to understand and evaluate their inter-relationships by integrating this analysis with the labelling index and turnover data reported in Chapter 4.
3. to assess any diurnal variations in cell compartments.

This information was thought to be of importance in assessing the role played by the sizes of cell compartments in the control of circadian rhythms of epithelial cell production and cell maturation as outlined in the scheme suggested in Section 1.6.7. Figure 5.1 illustrates such a possible control mechanism involving two inhibitors, controlling entry of cells into the S phase and into mitosis, modified from the ideas reported by Elgjo, Laerum and Edgehill (1971, 1972) in mouse epidermis. As a direct extension of this idea of control of cell production by G_1 and G_2 inhibitors discussed in Section 1.6.7 it was postulated that variations in the concentration of these two factors may arise as a result of diurnal variations in the sizes of the compartments producing each inhibitor. The attempt to analyse the size of these two compartments over a 24 hour period in the present study may throw some light on the possible existence

of any such rhythms. As the variations in the number of cells in S phase and the rate of entry of cells into the S phase were quantitated in the experiment previously described, the results from the analysis of compartment sizes can be integrated with these to discover any inter-relation with the cell kinetic parameters.

5.2. MATERIALS AND METHODS

Using the material obtained for Experiment 4A a morphometric study was made of compartment sizes and the sizes of their constituent cells in the biopsies of buccal epithelium taken from the three subjects at six times of the day.

Autoradiographs prepared from material labelled with 1 μCi/ml (15 minutes, single pulse) and utilised for Experiment 4A, were used for this analysis. For the integration of cell compartment estimations with cell kinetic data it was necessary to use the same sections for this analysis. Because of the small size of biopsy specimens immediately fixed tissues at corresponding times during a 24 hour cycle to the cultured tissues were not available and it was therefore not possible directly to compare the specimens used with fresh fixed material. However, specimens from other experiments which had been in tissue culture medium for 15 minutes before fixation when compared subjectively with corresponding fresh fixed material did not show any significant difference in the histological appearances of the epithelium.

Basically, the compartment analysis was carried out by two methods. One was by counting the constituent cells belonging to each compartment and this data was already available from the results of Experiment 4A (Section 4.3.1) where cell counts were estimated as a part of the labelling index calculation. The other method was to estimate

the size of epithelial compartments by stereological point counting techniques applied to the histological sections. As this part of the study was strictly stereological in nature, the principles of sampling already discussed in Section 2. 8. 3 were followed. The material utilised was that prepared for Experiment 4A and, as mentioned in Section 4. 2. 4, random selection of blocks and stratified random sampling of sections for counting had already been carried out.

5. 2. 1 Identification of Constituent Cells Belonging to Each Compartment

Ideally the differentiation of constituents in a stereological study should be by the application of small numbers of precisely defined criteria. In the distinction between progenitor and mature cell compartments it was not possible to establish such criteria and the distinction had to be made subjectively to include a number of variable characteristics. These were features such as cell size, nucleo-cytoplasmic ratio, cell orientation, location and staining characteristics exhibited following haematoxylin and eosin staining. Progenitor cells were found to be smaller in size, darkly staining and subjectively appeared to have a larger nucleo-cytoplasmic ratio. Most progenitor cells were limited to the deepest three cell layers and the presence of mitoses and labelled cells in the autoradiographs confirmed this distribution. The shape and orientation of individual cells were also found to be reliable features for differentiation of progenitor from mature cells. While progenitor cells were elongated at right angles to the basement membrane and frequently had a pallisaded arrangement, mature cells demonstrated flattening and were orientated in general, with the longer axis at right angles to that of progenitor cells and parallel to the surface. This gave the impression that in histological sections taken perpendicular to the surface, the progenitor cells were smaller along their horizontal width than mature cells. Figure 5. 2 illustrates some of these features described as used for the differentiation of cell compartments.

In order to ensure a consistent interpretation of the boundary between the two compartments was being made, a small number of sections were studied on several occasions with an interval of a few days between individual examinations. A high degree of consistency and reliability of interpretation was obtained.

5.2.2 Stereological Analysis of Compartment Sizes

Two histological sections (autoradiographs) at 48 μm apart from each biopsy were utilised for this study. In order that the area of the specimen examined was selected without bias, the section was moved 0.21 mm (Section 4.2.4) from the right hand edge of the epithelium in all cases. This distance was determined by the use of an eyepiece graticule. A column of epithelium projected in between the two vertical lines drawn on the screen of the Leitz Ortholux microscope (Figure 4.8) was used for the cell compartment analysis. The analysis was carried out using a x16 objective which gave a magnification of x200 on the projection screen.

In order to eliminate possible observer errors in the distinction of compartments during counting, it was found valuable to draw a line of demarcation between the two compartments on the image in the projection screen for each area of section before counting was begun.

Stereologic point counting was carried out using a transparent perspex grid with squares made by scoring two sets of parallel lines perpendicular to each other on the grid. The intercepts of these lines were used as points during the stereological analysis. By superimposing this grid on the projected image (Figure 5.3) the points falling on each cell compartment were counted. As discussed earlier (Section 4.2.4), the number of counts obtained may vary depending on how the grid is presented to the projected image. Therefore six counts on each section were made by rotating the grid into six positions where the angle between

grid positions was 30^{o}, except for one instance where a 20^{o} angle was used. This was to avoid overlapping of grid positions which would occur at the third and the sixth count if a grid with squares is moved by 30^{o} to each original grid position (Section 4.2.4). By placing the grid in six positions the number of points counted per biopsy was raised to over 350. A laboratory counter was used to record the points counted for each cell compartment.

5.3 RESULTS

5.3.1 Compartment Sizes (Area Measurements)

The number of points counted per cell compartment for each biopsy is shown in Table 5.1. The number of points (P) falling on each cell compartment is proportional to the area (A) of the compartment on the section (see Section 2.8.2 for stereological principles). On this basis the areas of progenitor and mature cell compartments (A_p and A_m) were estimated (Tables 5.2 and 5.3) in columns of buccal epithelium, including the full epithelial thickness at right angles to the mean epithelial surface.

As sections were taken perpendicular to the surface and in each specimen an equivalent column of epithelium was examined, the area measurements were proportional to the mean thickness of each cell compartment. Area estimations were converted to mean thickness measurements by dividing the area by the column width.

It was evident that in normal human buccal epithelium the overall epithelial thickness varied greatly from one biopsy to another, even in the same individual (Table 5.4). Therefore it was felt probable that the absolute size of a cell compartment was not a meaningful estimate for the purpose of a comparison of the sizes at different times of the day. It was

decided as an alternative to investigate the proportions of total thickness formed by each cell compartment.

Since thickness was proportional to area and since the stereological point counts were also proportional to the area, it was possible to estimate the ratio, thickness of progenitor compartment (T_p) divided by total epithelial thickness (T_t) directly from the ratio P_p/P_t. This proportion was expressed as a percentage of total epithelial thickness formed by the progenitor compartment at each time interval.

Figure 5.4 illustrates the proportion of total epithelial thickness formed by the progenitor compartment at different times of the day for the three individuals studied. This graph demonstrates broadly similar patterns for the three subjects. A graph drawn with mean values of the three subjects at each time interval is shown in Figure 5.5. This shows a reduction in the proportion of the epithelium formed by the progenitor cell compartment at 2200 and 0200 hours in comparison with other times of the day.

The question was then asked "Do the proportions of epithelium formed by the progenitor cell compartment show significant variations with time?" A Friedman two-way analysis of variance was used for this comparison and the compartment sizes were found not to be significantly different ($P > 0{\cdot}5$). This may be due to the small number of subjects utilised for the study. Alternatively, the relatively crude technique used for the size measurements might not have registered comparatively minor variations within four-hour samples. Therefore the biopsies were re-grouped into eight-hour samples by pooling measurements into morning samples (0600 and 1000 hours), afternoon samples (1400 and 1800 hours) and night samples (2200 and 0200 hours) as it was apparent in Table 5.1

that there are larger variations between samples at eight hour intervals. The statistical validity of this might be questionable if there was a difference between the three individuals. A Kruskall-Wallis one-way analysis of variance test was used to compare the data from the three subjects and this showed that there was no significant difference between individuals. The biopsy data, therefore, was cast into three columns as shown in Table 5. 5 and the Friedman two-way analysis of variance test was applied to the ranks in the three columns which were found to be significantly different at a level $P < 0{\cdot}05$. It could perhaps be construed that the statistics were being manipulated in an attempt to prove a particular hypothesis. Accordingly the suggestion that there is a diurnal variation in the proportion of epithelial thickness formed by the progenitor cell compartment is presented as a possibility with some tentative supportive evidence acknowledging that more extensive experimental data are required before it can be regarded as proven.

5. 3. 2 Compartment Sizes (Cell Counts)

Using the results presented in Section 4. 3. 4 a compartmental analysis was carried out using the cell counts in the two compartments after correction factors had been applied to correct for nuclear fragments in each cell compartment. Table 5. 6 tabulates the results of the progenitor cell counts and mature cell counts already presented in Table 4. 6 and now being utilised for the compartmental analysis for estimating the percentage of progenitor cells in each biopsy.

The graph drawn using these results (Figure 5. 6) did not show a simple pattern of variation of cell numbers with time. However, the mean values for the three individuals (Figure 5. 7) were analysed graphically and this indicated that the proportion of total cells in the progenitor compartment was greatly increased at the times when the thickness of progenitor compartment was reduced in size.

5.3.3 Comparison of Progenitor Cell Compartment Size by Area Measurements and Cell Numbers

Results obtained by the two methods utilised for cell compartment analysis were compared to detect whether variations in the size of the progenitor compartment during a 24 hour period were related to parallel variations in cell numbers in this compartment. A linear relationship could not be established and paradoxically Figures 5.5 and 5.7 illustrated an inverse relation, especially at 2200 and 0200 hours. As such a discrepancy could arise as a result of variations in cell sizes at different times of the day it was decided to estimate mean size of progenitor cells at different time periods.

5.3.4 Progenitor Cell Sizes

The mean area of a progenitor cell was estimated using the data already available on progenitor cell numbers under a unit length of surface and the absolute area of progenitor cell compartment measured under a unit width in each biopsy. The value of cell numbers per mm surface length was derived from results presented in Table 4.2 from Experiment 4A in which progenitor cells were counted and the surface length of epithelium was measured stereologically by intercept point counting (Section 4.2.4). The area of the progenitor cell compartment in each biopsy per mean epithelial surface length, as indicated by the counting column width, was obtained by converting the stereological point counts presented in Table 5.1 to an area estimation using the magnification factor involved in the projection (Table 5.2). Each point of the counting grid represented a 100 mm^2 area on the projected image. As the projection magnification was x200, the surface area of each compartment was obtained in mm^2 by multiplying the point counts by a factor of 0·0025. The estimated areas of progenitor cells in each biopsy are tabulated in Table 5.2 and graphically presented to illustrate the variation of cell size at different time periods in Figure 5.8. The mean values for the three

subjects are illustrated in Figure 5.9.

The mean area of a progenitor cell estimated in a histological section is proportional to the cell volume and therefore is referred to as cell size. The progenitor cells appeared to decrease in size at 2200 and 0200 hours.

Unfortunately the unit of length used in the cell count study was an actual measure of epithelial surface length, whereas that used in the compartment area estimation part of the study was the width of the counting column. Because of surface irregularities the actual surface length was greater than the column width, probably by not more than 10 per cent in most extreme cases. The calculated mean cell area in section may thus be a slight underestimation, but it is unlikely that the conclusions about comparative changes in cell size at different times would be affected by this error.

5.4 DISCUSSION

Although the analysis described in this chapter was designed to estimate epithelial cell compartment sizes, before discussing these results the morphometric data derived from Experiments 4 and 5 can be examined to describe certain quantitative morphological features of normal human buccal epithelium.

5.4.1 Total Epithelial Thickness of Buccal Mucosa

Tissue shrinkage which may occur during fixation and processing, and tissue compression which may take place during microtomy will result in underestimation of the actual thickness of an epithelium when routine histological sections are used for such measurements. Therefore absolute estimations are not meaningful unless a factor of shrinkage and compression is estimated for a particular study. However, in identically

treated tissues relative changes evident in direct comparisons of morphometric data may yield valuable information.

During the stereological point counting of projected images it was evident that the epithelial thickness of human buccal mucosa varied even though these biopsies were taken from a standardised site (Section 4.2.1) and were treated in an identical way. The epithelial thickness observed in the 18 biopsies ranged from 174 μm to 730 μm with a mean value of 390 ± 143 μm. Meyer and Gerson (1964) in a quantitative study on human oral mucosa estimated the thickness of buccal epithelium in 15 biopsies by planimetry and direct microscopic measurements. They derived a value of 450 ± 46 μm. The differences may be due to variations in the technical aspects of tissue preparation and morphometric techniques.

5.4.2 Cell Sizes

Average sizes of progenitor cells of the buccal epithelium were calculated in terms of the mean area occupied in histological sections. Subjectively these progenitor cells appeared smaller than mature epithelial cells. The average area of a progenitor cell was estimated to be 118 ± 34 μm^2. The mature cells occupied 955·1 ± 285·0 μm^2 on average. These results on cell sizes may be slightly underestimations due to the minor error in surface length estimation mentioned in Section 5.3.4.

These quantitations reflect to a degree some of the cytological features observed in human buccal epithelium during this study where it was seen that as the cells migrated from progenitor layers they gradually increased in size in the area immediately superficial to the progenitor cell compartment and thereafter demonstrated a rapid increase in cell area to give a ballooned out appearance in the middle third of the epithelium. Thereafter the mature cells progressively flattened in shape even though they were not progressing to produce a stratum corneum in this non-keratinized site.

5.4.3 An Interpretation of the Relationship of Cell Compartment Sizes to Epithelial Cell Kinetics

One of the aims of this study was to assess diurnal variations in cell compartment sizes in order to relate the size of cell compartments to any intrinsic regulatory mechanism in cell production and cell loss in human buccal epithelium. Having carried out a labelling index estimation and a cell compartment analysis at six scheduled times of the day an attempt was made to discover any relation between these two parameters.

The following questions were asked:-

1. Do the 24 hour variations in the size of cell compartments reflect to some degree a pattern of the kinetics of cell production and cell movement?
2. Do the variations in proportional sizes of each cell compartment reflect any relationship to a possible cell desquamation pattern during a 24 hour cycle?
3. Are the controls in a 24 hour balance of cell production, and the regular circadian rhythms observed in the numbers of cells in S and M phases, imposed by the variations of the size proportions of different compartments during a 24 hour cycle and thereby related to the alterations in concentrations of G_1 and G_2 inhibitors produced by each cell compartment?

In a preliminary attempt to answer these questions the mean values of the three subjects were utilised. To present a comprehensive picture, a composite graph was drawn (Figure 5.10) assembling these mean values to illustrate the inter-relationships of cell compartment analysis by thickness measurements and by cell numbers, to the size of the individual progenitor and mature cells and also to the labelling index of human buccal epithelium (Table 5.7).

It was evident that the data were not sufficiently large to arrive at firm conclusions, but it was possible to derive some ideas from these observations.

The proportion of total thickness formed by the progenitor compartment increased during the morning and afternoon and was significantly reduced in the night biopsies at 2200 and 0200 hours. However, in these night biopsies progenitor cells formed an increased proportion of cell numbers. Such an increase in cell numbers can be related to the high labelling index seen at 2200 hours and the higher numbers of mitotic figures observed in biopsies taken at 0200 hours (Subjective evaluation; no quantitative data are available). The cell area estimations do indicate that the mean progenitor cell size in the night biopsies was smaller and therefore probably reflected an increased proportion of small daughter cells produced at this time of the night as an outcome from the burst of mitosis. Probably migration of larger cells from the progenitor compartment may also contribute to the reduction in progenitor cell size. Both factors, migration of larger cells and production of smaller daughter cells contribute to the reduced area size of progenitor compartment, even in the presence of a relative increase in cell numbers in this compartment at 2200 and 0200 hours.

The increase in cell size of progenitor cells during morning and afternoon could be related to the continued protein and other synthetic phenomena in these newly produced daughter cells born around 0200 - 0600 hours. A rapid increase in the cell size of mature cells at 1000 - 1400 hours resulting in ballooned out mature cells in the epi-progenitor area may arise due to a burst of cell maturation following cell migration from progenitor to mature compartment around 0600 hours. This cell migration probably follows a rapid increase in the population pressure in the progenitor compartment at early morning hours following the peak mitosis. The reduction in the proportion of total epithelium formed by progenitor

compartment in area as well as cell numbers at 1000 hours may arise from this burst of maturation occurring earlier to this time of the day due to migration of cells from progenitor to mature compartment.

In answer to the second question posed, it is probable that the gradual increase in the proportion of total thickness formed by progenitor cell compartment after 1000 hours, up to about 1800 hours, is a reflection to some extent of a reduction in thickness of the maturation compartment as a result of desquamation at the epithelial surface which obviously takes place at the expense of the size of the mature compartment. Even though the newly produced mature cells in the early morning would be individually increasing in size throughout this period, this factor still does not appear to compensate for the reduction of the total size of the maturation compartment occurring probably as a result of cell loss.

These arguments suggest a relationship of cell desquamation to cell production occurring during a 24 hour cycle. The reduction in the size of the maturation compartment throughout the active period probably arises as a result of cell loss at the epithelial surface. This proportional reduction of the size of maturation compartment at the end of the day suggests the probability of a reduction in the output of G_1 inhibitor as the day proceeds. An absolute reduction in G_1 inhibitor concentration at the end of daytime would lead to a burst of activity of progenitor cells in G_1 phase and thereby provoke entry of cells from G_1 to S phase in the dividing cell cycle. Such a hypothesis is substantiated by the high labelling index seen at 2200 hours. Once these stimulated cells go through the dividing cell cycle from S to G_2 to M phase, this would result in an absolute increase in the number of cells in the progenitor compartment from 0600 hours onwards. Such an increase of progenitor cell numbers would account for a high concentration of G_2 inhibitor developing in the progenitor compartment, resulting in a lack of mitoses for the rest of the day until this increase is

compensated for by the migration of progenitor cells from the progenitor to the mature compartment.

These inferences further substantiate the hypothesis put forward in Section 1.6.7 that cell production, cell maturation, cell migration and cell loss at the epithelial surface are closely related events which appear to occur in peaks at different time periods throughout a 24 hour cycle and may be regulated by changes in the proportions of the epithelium formed by the two cell compartments. Most investigators limit their studies to identifying and counting cells in S or M phases and therefore the most commonly observed diurnal variations are the mitotic index and the labelling index rhythms. There appears to be a diurnal or circadian variation in most other cell kinetic parameters and these in turn result in a dynamic balance of the total epithelium over a 24 hour cycle.

5.5 CONCLUSIONS

The cell compartment analysis described in this chapter constitutes a preliminary attempt and a new approach to obtain information on diurnal cell kinetic features. The number of subjects utilised for the study and methods of 24 hour sampling employed were not entirely satisfactory, but on the whole was a practicable approach to obtain optimum results required for the analysis, in a study based on human volunteer subjects.

The study has yielded some important new data on the two cell compartments of human buccal epithelium with regard to relative sizes and size of constituent cells at different times of the day.

Peak activities in cell production and the related events of cell migration, cell maturation and subsequent cell loss appear to conform

to a regular pattern with individual features showing peak values at different periods during a 24 hour cycle. One event appears to contribute to or regulate another, resulting in the movement of blocks of cells along the dividing cell cycle, and also leading to maturation of G_1 cells in turn at particular times of the day. Such a phenomenon is consistent with the concept of a partial synchronisation which has been described in some renewal tissues in respect to the M and S phases of the cell cycle.

More direct experimental evidence is required to confirm whether the synchroniser (or zeitgeber) is related to the primary event of cell desquamation during daytime leading to a reduction in G_1 inhibitor concentration, thus taking away the inhibition on G_1 phase cells in the dividing cell cycle around 2200 hours.

The inferences drawn about diurnal variations in the size of cell compartments and their regulatory role in epithelial homeostasis are not conclusive at this stage but are reasonably substantiated by the findings discussed in this chapter. Further controlled studies are needed to evaluate the present findings and the lines to be pursued are discussed briefly in Chapter 7.

CHAPTER SIX

EPITHELIAL CELL KINETICS IN LEUKOPLAKIA OF HUMAN BUCCAL MUCOSA

6.1 INTRODUCTION

In the preceding parts of this thesis methods of measuring epithelial cell proliferation have been discussed and the application of in vitro ^{3}H thymidine labelling for quantitation of cell production of normal human buccal epithelium has been assessed. The morphology of epithelia is related to controlled cell production, cell maturation and cell loss. Therefore quantitation of these features is particularly useful in investigating and understanding the pathogenesis of epithelial lesions which demonstrate altered morphology. In Section 1.8 data reported by earlier workers from investigations on epithelial kinetics in disease states such as wound healing, epidermal hyperplasia, experimental carcinogenesis, premalignancy and in tumours were reviewed.

In oral epithelium most pathological lesions which arise as a result of altered cell production and cell maturation manifest as epithelial atrophy or hyperplasia. In these lesions an increased cell production may be suggested by the presence of increased numbers of cells in mitosis or by mitoses appearing in abnormally superficial sites in an epithelium. These features are sometimes associated with other abnormalities in cell morphology, staining characteristics and keratinization. To describe this type of structural abnormality in epithelia, the terms dyskeratosis, dysplasia or epithelial atypia are used. Lesions which demonstrate these cellular abnormalities in epithelia, in the absence of histological characteristics of invasion of the underlying tissue, are recognised as premalignant on histological grounds. It is known that 4 - 6 per cent of oral epithelial lesions which demonstrate these histological features of epithelial atypia and are

therefore grouped as oral premalignant lesions, later progress to malignancy (Einhorn and Wersall, 1967; Pindborg et al, 1968; Silverman and Rozen, 1968, and Banoczy and Sugar, 1972). Although clinically such premalignant lesions in the oral cavity may manifest in various forms which are well described (Cawson, 1969, and MacDonald, 1975) a majority of these lesions present as keratosis or hyperkeratosis. The term "leukoplakia" is often used to describe these keratotic lesions. Leukoplakia is used here to describe a firm adherent white patch that cannot be rubbed off and cannot be assigned to any other diagnostic category on the basis of clinical and microscopic features (Kramer, 1969). As only a minority of these lesions which are diagnosed as premalignant will actually undergo malignancy, it is important to establish the histological features which are most useful in predicting cases of leukoplakia that subsequently develop into a carcinoma. The histological and cytological features which are presently in routine use for evaluation of atypia in oral epithelial premalignant lesions were reviewed in Section 1.8. In the same section a case was presented for relating the histogenesis of these morphological features to a basic alteration in cell kinetics.

A survey of the dental literature indicates that studies of the relationship of altered clinical morphology in keratotic oral lesions to cell kinetics are limited. A few investigators have attempted to quantitate epithelial cell production by counting mitoses in histological specimens from keratosis, hyperkeratosis or leukoplakic lesions (Renstrup, 1963; Main, 1965; Meyer, Daftary and Pindborg, 1967; El-Labban, Lucas and Kramer, 1971) and have reported an increased cell production. Alvares et al (1972) using an in vitro labelling technique, found a significant increase in the number of cells in S phase in homogeneous leukoplakia. These studies did not analyse the duration of the M or S phases, nor did they include a correlation of epithelial cell production

and turnover or the maturation kinetics to determine the relationship of altered cell production to epithelial structure.

Experiment 6 described in this chapter is an attempt to apply the techniques of in vitro ^{3}H thymidine labelling of buccal epithelium to investigate cell production and turnover in oral leukoplakia and to obtain morphometric data on proliferative and mature cell compartments in order to relate the findings on cell proliferation to possible alterations in cell maturation.

6.2 MATERIALS AND METHODS

6.2.1 Patient Selection

Material for this study was obtained from patients who attended the consultant clinics of the Department of Oral Medicine, Glasgow Dental Hospital, with the complaint of persistent white lesions in the oral cavity. Ten cases were selected on the basis that the lesions were classified as leukoplakia by the definition set out in Section 6.1. White patches in two of these cases, although clinically appearing as homogenous white lesions without any linear or lacy pattern on histology, proved to be lichen planus and therefore were excluded from further analysis.

Of the eight patients included in the study three were males and five were females and belonged to an age group of 41 - 77 years with a mean age of 58 years (Table 6.1).

6.2.2 Clinical Considerations

All eight patients, on clinical examination, revealed white lesions of more than 2 x 2 cm in extension and, in most cases, involving both sides of the cheek. Patient number 1 demonstrated some degree of redness and ulceration, especially at the angles of the mouth and the lesion was diagnosed clinically as speckled leukoplakia (Figure 6.1). The white patches in the other seven subjects on clinical examination appeared to be homogeneous leukoplakias (Figures 6.2 and 6.3 illustrate

two of these cases).

All eight patients, until the time of presentation, had been cigarette smokers; some smoking 40 cigarettes per day. In addition, in patient number 2 there was direct evidence of chronic frictional irritation from ill-fitting full dentures, and in patient number 8 from broken down crowns of molar and premolar teeth. Both smoking and chronic friction are known aetiological factors in oral leukoplakia (Cawson, 1969).

Apart from oral keratosis there was no clinical evidence of any other local abnormality or of any general medical conditions which might complicate the oral epithelial changes. Subject number 7 had previously suffered from pernicious anaemia, but the condition was controlled by monthly supplements of vitamin B_{12}.

6.2.3 Biopsy Procedure

The method of tissue sampling was to biopsy a representative area of the white lesion in buccal mucosa and also to obtain a biopsy from a clinically normal area of cheek at least 2 cm away from the main lesion to provide a control specimen.

Biopsies were taken under regional local anaesthesia by infiltrating the long buccal and mental nerves using lignocaine with 1:80000 adrenaline. Adrenaline was not used in the experiments described in Chapter 4 in order to eliminate any possible effects on cell kinetics. However, it was decided to use a vasoconstrictor in the local anaesthetic agent during specimen collection for this study as the biopsy technique was modified to obtain larger specimens upon which routine diagnostic histopathological investigations, as well as kinetic studies, could be performed. Use of a vasoconstrictor in the anaesthetic agent

was considered to be a requirement for reducing bleeding during the biopsy procedure and post-operatively.

Four of the subjects were being seen for the first time in the Department and therefore needed a biopsy of the lesion to confirm the clinical diagnosis. Two others had been previously examined but needed a further biopsy to evaluate the progress of lesions. In all six cases an incisional biopsy of the lesion was carried out by taking a mucosal ellipse and a part of this was utilised for the cell kinetic study while the other part was fixed for histopathological examination. The control biopsies from a clinically normal area of cheek were taken with a trephine type biopsy punch of 3 mm diameter (Section 4.2.1). All procedures were undertaken with the informed consent of the patients.

Two of the cases did not require a routine diagnostic biopsy and the nature of the study having been explained, biopsies were taken for this particular experiment with the informed consent of the patients. In these patients, in order to avoid the discomfort of a large mucosal biopsy requiring a further visit to the hospital for removal of sutures, two punch biopsies were taken, one from a clinically affected area (Figure 6.3) and one from a normal area of cheek.

As it was evident from the results of Experiment 4 that diurnal variations in the labelling index were minimal between 1000 and 1800 hours the patients were seen at times suitable to individual subjects during clinic hours. Four of the patients were biopsied between 1000 and 1130 hours and the other four between 1430 and 1630 hours. Table 6.1 shows the times of biopsy of individual cases.

6.2.4 In Vitro ^{3}H Thymidine Labelling

The specimens were trimmed perpendicular to the epithelial surface into 1 mm slices to obtain tissue blocks of 2 - 3 mm^2 surface

area and having about 1 - 2 mm thickness of connective tissue. These were transferred to the laboratory in Medium 199. All labelling experiments were started within half an hour of each biopsy.

Tissues from the control sites and leukoplakia lesions of individual patients were labelled with ^{3}H thymidine simultaneously under the same incubation conditions in one water bath with specimens in separate containers. Each specimen received a single pulse of 1 μCi/ml of incubation medium and two blocks from each container were removed after an incubation of 15 minutes. These were rinsed in label free medium for 10 minutes and fixed in Bouin's fluid. The remaining blocks from each case, after being incubated in label free medium for one hour, were given a second pulse label of 10 μCi/ml of medium for 15 minutes. In two cases where additional fresh material was available a single pulse of 10 μCi/ml of medium was also used.

The techniques of tissue processing and preparation of autoradiographs were those described in Experiment 4A.

6.2.5 Analysis of Tissue

Autoradiographs of single weakly labelled material from control specimens and leukoplakia were analysed to obtain the labelling index by all four reference units as used in Experiment 4A. Four serial sections of each case were utilised and two to three consecutive microscopic fields were examined in each section.

By comparison with cold controls, the autoradiographic background level was assessed. These control preparations exhibited one to two grains over the area occupied by an epithelial cell and very rarely showed three silver grains overlying a single cell. Therefore nuclei over which four or more silver grains were deposited were identified as labelled.

To quantitate the four labelling index reference units, viable keratinocyte counts and estimates of surface length and basement membrane length were required. All cell counts were carried out under a x25 objective and a record was kept of progenitor and mature cell counts separately. In the same areas of epithelium in which cell counts were made the surface length and the basement membrane length were measured stereologically by intercept point counting. This was carried out on projected images using the Ortholux microscope (see Section 4.2.4).

The labelled cells were counted under x100 (oil) objective in the same area of epithelium in which the reference units were quantitated.

The grain count overlying each labelled nucleus was noted to obtain the range of grain counts in 1 μCi/ml labelled cells in order to allow identification of the cells labelled by the weak pulse in double labelled material. The great majority of single labelled cells were found to have a grain count of 4 - 5 silver grains over the nuclei. An occasional cell was noted to have over 25 silver grains.

The position of labelled cells relative to the basal cell layer was noted in order to obtain a preliminary assessment of the extent of the progenitor cell compartment in control tissues and in leukoplakia.

In order to quantitate the total epithelial thickness and to analyse the size of individual cell compartments in oral leukoplakia and in control normal sites, a morphometric study was carried out using the stereological methods described in Chapter 5. The sections used were the single labelled material discussed above.

T_S was estimated from double labelled material by obtaining the proportions of heavily and weakly labelled cells. The two cell

populations were identified by the grain counts over the nuclei. Examination of single pulse labelled material with either the weak dose or the heavy dose enabled grain count distributions to be predicted for cells labelled with each dose. Cells with 4 - 25 grains were considered to be labelled by the first pulse of 1 μCi/ml as this was the grain count range observed in the single labelled material with the weak dose. The cells which were identified as heavily labelled demonstrated silver grains in clusters which usually occupied the whole area of each labelled nucleus. Examination of material labelled with a single dose of 10 μCi/ml (Section 6.2.4) showed a pattern of grain yield closely corresponding to this labelled cell population identified as heavily labelled cells in double labelled material where the heavy dose was 10 μCi/ml. Occasionally both single pulse labelled material with either 1 μCi/ml or 10 μCi/ml showed labelling with grain counts close to 25 grains which was the level used for the separation of the two labelled cell populations. As a result misidentification may occur of a small minority of labelled cells but it was felt that the errors involved in each group would have probably been balanced and thus cancelled during allocation of heavily and weakly labelled cells.

In order to estimate the rate of cell entry into S phase, the total labelling index (LI_2) was obtained from double labelled material by counting total nucleated cells and using records of heavily and weakly labelled cells in the same area of each specimen. By comparing this total labelling index (LI_2) obtained by both pulses with the single labelling index (LI_1) obtained from the first pulse, a rate of cell entry into the S phase was obtained as a percentage of total cells per hour.

Corrections to cell counts were required to account for nuclear fragments which result in an overestimate of cell numbers in histological sections. In the study reported in Chapter 4, the variations in nuclear

diameter in progenitor and mature cells of normal human buccal epithelium were found to be small and therefore the application of correction factors made no significant change to the estimated labelling index. The sizes of cells and nuclei of control tissues and leukoplakia lesions were observed subjectively to differ. This could lead to a varying magnitude of overestimation in the nuclear count. Therefore it was necessary to apply Abercrombie's correction to the cell counts and the method described in Section 4.2.5 was used. The mean nuclear diameters of progenitor and mature cells in individual specimens were obtained using an eyepiece micrometer graticule and the mean section thickness was obtained by through focus photomicrographs taken at 1 μm intervals (Section 4.2.5).

6.2.6 Histological Assessment: Special Stains for Keratinization and Candida

As it is known that the type of keratinization in pathological specimens may be correlated with epithelial cell kinetics (Section 1.5.3) the material from the present study was assessed with modified Mallory stains in order to examine the pattern and degree of keratinization in control and leukoplakia specimens. As the number of subjects examined was limited the specimens were grouped into four broad categories on the characteristics revealed by the modified Mallory stain. These were orthokeratinization, parakeratinization, a mixed group showing both types of keratinization, and a non-keratinized group.

The presence of candidal invasion of the epithelium is associated with some leukoplakias (Cawson, 1969) and it is suspected that such infection with candida produces epithelial hyperplasia. PAS stained material from control and leukoplakia biopsies was examined for the presence of candida hyphae in the epithelium.

6.2.7 Epithelial Atypia

In order to assess any correlation of the cell kinetic measurements with the histological characteristics of epithelial atypia in the leukoplakias, an objective atypia scoring technique described by Smith and Pindborg (1969) was adopted. An estimation was made of the degree of severity of atypia in each biopsy from both control and leukoplakia lesions, taking into consideration the 13 histocytological characteristics described in the monograph on epithelial atypia scoring (Smith and Pindborg, 1969). Photomicrographs in this monograph were used as photographic standards for estimating the grade of severity of atypia. The sections were allocated random numbers and were scored blind. The four sections used for cell counting were examined, except in two of the cases where only three sections were available. For each of the 13 features the highest scores obtained in any of the four sections of each case was noted and the sum of these gave the atypia score or index for the case.

6.3 RESULTS

6.3.1 Microscopical Evaluation

Histological examination of haematoxylin and eosin stained sections revealed that all leukoplakia specimens demonstrated varying degrees of keratosis (Figure 6.4) while control sites were non-keratinized (Figure 6.5). Assessment of haematoxylin and eosin stained sections at four levels from each tissue block and a modified Mallory stain at one further level revealed that the epithelium in the leukoplakia lesions of six of the patients showed evidence of orthokeratosis, one showed parakeratosis and the other demonstrated mixed areas of orthokeratinization and parakeratinization. These features are recorded in Table 6.2.

Subjectively the epithelium in the leukoplakic areas appeared

to be thin in the majority of cases and the results of morphometric measurements of mean epithelial thickness in the two groups are presented in Section 6.3.8.

Table 6.2 also tabulates the histological assessment for candidal hyphae in the epithelium using the PAS technique. Only one of the patients (number P7) demonstrated candida organisms in the epithelium.

All leukoplakia lesions showed varying degrees of infiltration of plasma cells and lymphocytes in the lamina propria.

The atypia scores for control and leukoplakia biopsies are shown in Table 6.2. These are the total scores out of a maximum of 75 for the 13 features examined. All control biopsies did show some degree of atypia. The total score ranged from 2 to 16 in the control biopsies. The leukoplakia lesions, except in one case, had higher atypia scores than their matched controls. The atypia scores in the leukoplakic group ranged from 9 to 32. A Wilcoxon matched-pairs signed-rank test was used to assess the significance of the differences in atypia scores in the two groups and it was found that the atypia scores in the leukoplakia group were significantly higher ($P < 0{\cdot}02$).

6.3.2 Labelling Index Estimations

An autoradiograph prepared from in vitro single labelled material is shown in Figure 6.7. The observed data for the biopsies from control sites are tabulated in Table 6.3. The uncorrected counts of progenitor cells, mature cells, total nucleated cells and labelled cells are shown in this table. The intercept points counted along the epithelial surface and the basement membrane of each specimen are tabulated and, using these data estimations of the surface length (mm) and basement

membrane length (mm) examined in each specimen are recorded. In Table 6.4 the comparable data for leukoplakia lesions are shown.

The four reference index systems already utilised for analysing the labelling index of normal buccal mucosa in Experiment 4A were used to express the labelling index for control and leukoplakia lesions in this study. The estimated labelling indices by total cell index (LC/TNC %), by progenitor cell index (LC/PC %), by surface length index (LC/SL) and by the basement membrane index (LC/BML) are tabulated in Table 6.5. In leukoplakia 5·7 per cent of total cells were labelled while only 3·2 per cent of cells were in S phase in control biopsies.

As was discussed in Section 4.2.5, the calculation of the labelling index by total cell counts can be affected by differences in nuclear size of progenitor and mature cells. The measurements of nuclear diameters of progenitor cells and mature cells in control and experimental sites are shown in Tables 6.6 and 6.7. The mean section thickness was estimated as 3·5 μm. Using the nuclear diameters and this mean section thickness a correction factor (C.F.) was derived from Abercrombie's formula (Section 4.2.5) and all crude cell counts presented in Tables 6.3 and 6.4 were corrected individually. The corrected labelling indices are shown in Table 6.8. Following this correction the calculated mean labelling indices for the control specimens altered little - from 3·2 to 3·39 labelled cells per 100 total nucleated cells. The labelling index for the leukoplakias was more obviously affected and rose from 5·7 to 6·0 labelled cells per 100 total nucleated cells.

6.3.3 Comparison of Labelling Indices of Control Biopsies and Leukoplakia

The labelling indices were found to be increased in leukoplakic

lesions. Wilcoxon matched-pairs signed-ranks tests were used to ascertain whether or not the differences in the labelling indices were statistically significant. The test applied to the labelling index expressed in terms of the total cell count is shown in Table 6.9 and yields a value of $P < 0{\cdot}005$. The same test applied to the labelling index per 100 progenitor cells gave a value of $P < 0{\cdot}005$ and for the labelling index per unit surface length and by unit basement membrane length, values of $P < 0{\cdot}025$ were obtained. Thus the number of cells in the S phase in the leukoplakia group was significantly higher than the controls with all four systems of expressing the labelling index.

6.3.4 Distribution of Labelled Cells in Progenitor Cell Layers

In the autoradiographs prepared from single labelled material the positions occupied by the labelled cells in relation to the basal cell layer are shown in Table 6.10. The sites of labelled cells are recorded as basal and suprabasal and the suprabasal labelled cells are further grouped by their position into two columns as those in cell layers two and three, and as those above the third cell layer. Such a grouping was adopted for the analysis of the site of labelling, because data derived from autoradiographs of normal human buccal epithelium prepared for Experiment 4 showed that labelled cells were confined to the deepest three cell layers. The differences in the site of labelling of control and leukoplakia specimens were then analysed. The total labelled cells in each category and their proportions expressed as a percentage of total labelled cells for the control and leukoplakic groups are shown in Table 6.11. In leukoplakia 39·6 per cent of labelled cells were in the basal cell layer, while only 25·7 per cent were in the basal layer in control biopsies. A Chi square test was used to examine the statistical significance of this difference. This is shown in Table 6.11, where the total numbers of basal and suprabasal labelled cells in the two samples

are contrasted. The increased proportion of basal labelled cells in the leukoplakia group is significant at the level P = 0·016.

It is known that cell production occurs in the deepest three cell layers in normal human buccal epithelium (results of Experiment 4; Alvares et al, 1972), and that epithelia with increased cell production demonstrate dividing cells in abnormally superficial sites (Bullough, 1972). Therefore it was decided to analyse whether the number of labelled cells in sites superficial to the deepest three cell layers in leukoplakia was different to the distribution seen in control specimens. In the control specimens 6·7 per cent of labelled cells were found to be superficial to the deepest three cell layers, but in leukoplakia the percentage of labelled cells in this category was 7·7 per cent. This difference is not statistically significant (Chi square test - Table 6.11).

6.3.5 T_s Estimation

An autoradiograph prepared from double labelled material is shown in Figure 6.8. The numbers of heavily and weakly labelled cells counted in the double labelled specimens are shown in Table 6.12. The T_s estimated by this technique was 6.0 hours for both control and leukoplakia lesions.

6.3.6 Rate of Cell Entry into S Phase

The rate of cell entry into the S phase was estimated using the difference between the labelling index obtained by the first 15 minute pulse and labelling index obtained by the two pulses given during the 75 minutes period. The rate of cell entry into S phase expressed as cells per 100 cells per hour is shown in Tables 6.13 and 6.14. Within the control and leukoplakia groups there is a large variation in the rate of cell entry to the S phase and in one of the control biopsies and four of

the leukoplakias a negative value was obtained. It is not clear from this experiment whether this apparent rate of cell entry into the S phase is a true reflection of the in vivo state, or whether it is an artefact created by the in vitro labelling procedure.

While some dubiety may exist about the absolute values for the rate of cell entry into S phase shown in Table 6.14, it is still pertinent to compare the ratios in control and leukoplakic biopsies from the same patient and handled in an identical manner. A Wilcoxon matched-pairs and signed-ranks test (Table 6.15) indicates that the reduction in the rate of cell entry into the S phase in the leukoplakia specimens is significant ($P < 0{\cdot}05$).

6.3.7 Turnover Estimation

The turnover time for the control and leukoplakia biopsies was estimated using the corrected labelling index and the calculated T_s. The estimated turnover times of the total viable cell population for the two groups were 7·37 days for the control group and 4·16 days for the leukoplakias. This value for the leukoplakias does not include the stratum corneum. For the turnover of the progenitor compartment the estimated times were 3·65 days for the control specimens and 2·24 days for the leukoplakia group.

6.3.8 Epithelial Thickness

Using the total stereological point counts (P_t) obtained for each specimen, the section area (A_t) of the epithelium examined in each biopsy was estimated. As the counting procedure used columns of known width extending perpendicular to the surface through the full thickness of the epithelium it was possible to derive from the section area measurement an estimate of the mean thickness of the epithelium as described in Section 5.3.1.

The values for the mean thickness of the control and leukoplakia specimens are shown in Table 6.16. In six of the eight patients the leukoplakic specimens were thinner. In one patient both specimens showed the same epithelial thickness, whereas in one case the leukoplakic specimen was substantially thicker than the corresponding control. A Wilcoxon matched-pairs signed-ranks test give a value of $P > 0{\cdot}05$ indicating that the differences between the leukoplakias and controls were not significant. This was due to the leukoplakic lesion in patient number 7. This leukoplakic lesion was the only one in which candidal hyphae were demonstrated. If the biopsies from this patient are excluded the leukoplakia lesions are significantly thinner than the matched controls (Wilcoxon matched-pairs signed-ranks test, $P < 0{\cdot}05$).

6.3.9 Cell Compartment Analysis

The number of points counted for progenitor and mature compartments in control specimens and for progenitor, mature and keratin compartments in leukoplakia lesions are presented in Table 6.17. The proportion of epithelium formed by each compartment was estimated. 16·0 per cent of epithelium was found to be formed by the progenitor cell compartment in the control biopsies and 17·5 per cent in leukoplakia. The difference in proportions in the two groups was not statistically significant (Wilcoxon matched-pairs signed-ranks test).

The maturation compartment in the control specimens formed the same proportion of the total epithelium as the combined maturation and keratinized compartments in the leukoplakia group. However, the proportion of the total thickness formed by the maturation compartment alone showed a significant reduction in the leukoplakia group (Wilcoxon matched-pairs signed-ranks test, $P < 0{\cdot}001$).

6.3.10 Comparison of Control Biopsies and Normal Buccal Epithelium

The normal tissues studied in Experiment 4 were not age and sex matched to the controls in the experiment reported in this Chapter and it was therefore not possible to make a detailed comparison of these control tissues with normal tissues. However, several factors suggest that the control specimens used in this experiment were not in fact normal. Histologically the control specimens were thinner than normal epithelium and mature cells showed little of the ballooning seen in the normal buccal mucosa. Epithelial atypia ranging from scores of 2 to 16 was present in the control biopsies.

Comparison of the labelling index data with that for normal buccal epithelium for corresponding time periods showed higher values in the Experiment 6 controls. For normal buccal epithelium it was observed that the labelling index between 1000 and 1800 hours ranged from 2·02 per cent to 2·95 per cent by the corrected total cell index (Table 4.16) whereas in these control clinically normal specimens from leukoplakia patients the labelling index ranged from 1·87 per cent to 5·01 per cent with a mean of 3·39 per cent.

Comparison of labelling index data of the two groups using a Kolmogorov-Smirnov two sample test gave a value of $P < 0{\cdot}05$. Factors which probably give rise to this difference are discussed in Section 6.4.

6.3.11 Correlation of Epithelial Atypia and Labelling Index

The epithelial atypia score is indicative of the severity of tissue changes in premalignant lesions. It was therefore decided to examine whether the labelling index values were correlated with the atypia scores. In order to test this the Spearman rank correlation co-efficient test was applied when the ranks of atypia scores for the control and leukoplakia

groups separately were assessed. The Spearman rank correlation co-efficients (r_s) were 0·51 and - 0·27 respectively, giving values of $P > 0·05$. However, when the control and leukoplakia groups were combined the correlation of atypia scores and labelling index data gave a value of $r_s = 0·50$ which, for this larger number of cases, gives a value of $P = 0·019$ (Table 6.18). This indicates a highly significant correlation between increased labelling index and increasing severity of epithelial atypia.

6.4 DISCUSSION

The results of the labelling index study can be compared with the data presented by Alvares et al (1972) who first reported a preliminary comparison of the labelling index of homogeneous leukoplakia with control buccal mucosa. The rise in labelling index observed in this study from 3·20 per cent in the clinically normal control sites to 5·7 per cent in leukoplakias is comparable to the labelling index values documented by these authors (2·72 per cent in normal, 4·74 per cent in leukoplakia). The increase in the number of cells engaged in DNA synthesis at a given time does suggest an increased cell proliferation in leukoplakia. The degree of increase in cell proliferation observed (1·72 fold) is similar to that which can be estimated from the results of Alvares et al (1972) (1.78 fold). It is also interesting to note that Meyer, Daftary and Pindborg (1967) by quantifying the number of mitoses observed in a similar range of increase in the number of dividing cells (1·37 fold rise by surface length index) in a comparable leukoplakia group with an atrophic epithelium, to that found in the present study (1·49 fold rise by surface length index).

It is pertinent to discuss briefly how this increased cell production occurs in leukoplakia by relating the labelling index to the other cell kinetic parameters studied. An increase in labelling index may be

apparent in a tissue as a result of:-

1. more time spent in the S phase by individual cells,
2. increased rate of cell movement in the dividing cell cycle which may manifest as a high rate of cell entry into any of the phases,
3. an increase in the size of the progenitor compartment,
4. an increase in the proportion of progenitor cells active in the dividing cell cycle. This would be an increase in the growth fraction.

The T_s obtained, being six hours for both groups (Section 6.3.6) does not disclose any difference in the time spent by DNA synthesising cells in the S phase in leukoplakia in comparison with control epithelium. The rate of cell entry data (Section 6.3.6) does not support an increased or rapid cell movement in the dividing cell cycle as it was found that the entry rate was in fact reduced in leukoplakia as opposed to control specimens.

The proportion of the total thicknessof the epithelium formed by the progenitor cell compartment was the same in the leukoplakia and control groups. When taken in conjunction with the evidence that the leukoplakias were in the main thinner than controls, this suggested that the progenitor cell compartment was in fact small in the leukoplakia group. The paucity of labelled cells observed in superficial sites supported the view that the increased labelling index was not a function of a larger progenitor compartment.

It appears that the main difference which has taken place in leukoplakia contributing to the increased numbers of labelled cells is that a greater proportion of the cells in the progenitor cell compartment are taking part in preparation for cell division. This constitutes a probable increase in the growth fraction of the epithelium. The results

indicate that this increase in growth fraction must be of the order of 1·7 fold to account for the magnitude of the rise in labelling index. It was not possible to estimate the growth fraction of the epithelium by the experimental techniques utilised in this study. Therefore direct evidence is lacking to confirm the possibility of an increased growth fraction operating in leukoplakia to account for the increased cell production. However, the preliminary investigation on the distribution of labelled cells indicates an increased proportion of labelled cells present in the basal cell layer in leukoplakia. This leads to the suggestion that any increase in the number of cells in the dividing cell cycle primarily takes place by more cells in the basal cell layer re-cycling before leaving the progenitor cell compartment to become mature cells. In oral lichen planus, Walker and Dolby (1974) reported a significant increase in basal labelling index and suggested an altered distribution of mitotic activity. The present finding of an increased proportion of basal cells engaged in DNA synthesis appears to reflect a similar alteration.

In this study the comparison of data from leukoplakic lesions was made with control specimens of clinically normal looking areas from sites close to the white lesions in the same individual.

Such control samples have the advantage of providing exact age and sex matched controls. Furthermore, both biopsies can be obtained at the same time of the day. However, in this study it was observed that these control specimens were different to the normal human buccal epithelium of young adults in many ways.(Section 6. 3. 10). As these biopsies from clinically normal sites of leukoplakia patients were not compared with exact matched controls it is not possible to examine whether these differences are related to any field change in this clinically normal looking mucosa. Another probable factor which could

have altered the epithelium in the patients in Experiment 6 was that they were all smokers.

6.5 CONCLUSIONS

The study described in this chapter attempted quantitation of cell proliferation in a disease state in oral epithelium. By estimating the number of cells in the S phase using in vitro ^{3}H thymidine labelling, cell proliferation was found to be increased by 1·72 fold in leukoplakias. This increase was found to be related to an increase in the proportion of cells in the progenitor compartment engaged in cell production. Both the control and leukoplakia specimens showed epithelial atypia, although in most cases this was only mild. A significant positive correlation was found between the epithelial atypia score and the labelling index.

CHAPTER SEVEN

A DISCUSSION OF THE EXPERIMENTAL FINDINGS AND OF SOME PRACTICAL PROBLEMS AND METHODOLOGICAL ERRORS INVOLVED IN THE STUDY OF CELL KINETICS

7.1 INTRODUCTION

The main object of the study reported in this Thesis was to develop a suitable technique for the estimation of cell production in human oral epithelium and to establish the range of variation in normal tissues with particular reference to diurnal effects. As the methods of expressing cell renewal in studies reported by other workers were found to be variable, an evaluation of presently used index systems was also carried out. With the knowledge of normal values for cell production parameters a preliminary study was undertaken to investigate the use of labelling index in assessing cell proliferation in oral luekoplakia. In this chapter a general discussion of experimental findings is presented.

During the later parts of this experimental study some practical problems were encountered mostly due to inherent limitations and errors involved in the methods of application of quantitative techniques to derive information from histological material. In order to eliminate and standardise these sources of uncertainty several corrective methods were adopted in this study. In this chapter it is intended to discuss some of these factors in the light of experience gained during the present study.

7.2 DISCUSSION OF EXPERIMENTAL FINDINGS

In the preliminary study described in Chapter 3, an in vitro ^{3}H thymidine labelling technique using small biopsy specimens was described and this provided a means of estimating cell production. The method was found to give reliable results comparable with in vivo labelling in hamster tongue epithelium. The in vitro results depicted

the diurnal variation in labelling index in a closely similar fashion to in vivo labelling results. It was observed that for a renewal cell population, such as hamster tongue epithelium, demonstrating a circadian rhythm in cell kinetic parameters, 24 hour mean values for the labelling index and T_s are required to use these data in the calculation of the turnover time.

Using this in vitro technique, pulse labelling of human buccal mucosa was carried out and by autoradiography cell proliferation at this site was quantitated. Using double labelling with two doses of ^3H thymidine, T_s and rate of cell entry into the S phase was also estimated.

In order to improve objective analysis of human pathology, the importance of quantitation of microscopical histopathological features is now becoming apparent in many fields. A prerequisite for such analysis is a knowledge of normal parameters. One of the objects of this study was to quantify cell production in human oral epithelium. While obtaining normal values, the range of physiological variation and reliability of quantitative assessment should be evaluated before such quantitations are employed in diagnostic fields. From the available data on animal studies it was apparent that one of the physiological factors which may considerably influence the estimated labelling index was that due to diurnal periodicity. As this was the first instance in which cell proliferation of human buccal mucosa had been systematically investigated using ^3H thymidine techniques, it was necessary to obtain the range in diurnal variation in cell kinetic parameters. By using a 24 hour sampling procedure, as described in Chapter 4, a diurnal variation in the labelling index was observed. The labelling index was found to be highest at 2200 hours, this value being significantly different from the rest of the times of the day when it was

measured. During daytime, when clinical estimations are most probably undertaken, the range of variation in labelling index was found to be within narrow limits. Consistency of results obtained for the labelling index estimation was evaluated by repeating the in vitro labelling experiment on a second occasion on an increased number of volunteer subjects. In young adult males the labelling index for normal buccal mucosa was found to range from 1·99 per cent to 3. 39 per cent by the total cell index in 30 biopsies taken at different times of the day/night cycle. Labelling index data derived in this study can be used to describe cell production activity in human buccal mucosa on a quantitative basis. These baseline values with the estimated range for the diurnal periodicity obtained in the present study can be compared with further studies of mucosal pathology.

In a cell renewal population which demonstrates circadian variation in the cell cycle parameters, the labelling index rhythm can be the result of proportional changes in the durati on of S phase, or be due to the rhythmic changes in the flow rate of cells into S phase. In the experimental study reported in Chapter 4, it was observed that T_s variations were not consistent and therefore could not account for the rise in labelling index at 2200 hours. It was observed that the fluctuations in the rates of cell entry into S phase could account for the variations seen in the labelling index at particular times of the day and therefore that the results were suggestive of a partial synchronisation of cell movement through the cell cycle in human buccal epithelium.

Using 24 hour mean values for the labelling index and T_s estimations over a 16 hour period, a turnover time of 10 days was obtained. From this information it could be derived that human buccal mucosa would renew faster than the epidermis but slower than gastrointestinal tract epithelium. A similar suggestion has been made by other

workers using results from studies on animal oral tissues.

Human buccal mucosa was found to have a multilayered progenitor cell compartment as labelled cells were commonly observed superficial to the basal cell layers in sections taken perpendicular to the epithelial surface. This finding is supportive of similar findings by Alvares et al (1972) and Kaidbey and Kurban (1971). Labelling index estimations which refer only to a basal labelling index may therefore be misleading. In this study the progenitor cells in the normal human buccal epithelium of young adult males were found to be restricted to the deepest three cell layers.

Criteria for histological identification of progenitor cells were laid down in Chapter 5. However, it was indirectly estimated that the growth fraction of the progenitor compartment may not be 100 per cent. Improved experimental techniques are required to delineate the exact limits of the progenitor compartment or to identify the presence of G_o cells or mature cells in the progenitor compartment.

Stereological point counting on projected images of human buccal mucosa provided a method for estimating the proportion of total epithelial thickness formed by the progenitor compartment. Diurnal variations were observed in the proportion of progenitor and maturation compartments in this epithelium.

The labelling index, rate of cell entry into S phase, and the proportional changes in the thickness of the two cell compartments were found to occur, with peaks at different times of the day. It was suggested that the variations in the thickness of the two cell compartments may control cell movement in the dividing cell cycle by an alteration in

the concentration of G_1 and G_2 inhibitors, one of which is produced by each compartment. Further experimental studies are required to analyse this possibility.

As a preliminary study, cell production in oral leukoplakia was assessed by the same techniques as were used to analyse cell kinetics in the normal buccal mucosa. A study was conducted on eight patients from whom a biopsy of leukoplakia and one of a clinically normal site were obtained. It was demonstrated that cell production is significantly increased in leukoplakia. The labelling index values by total cell index ranged from 1·88 per cent to 3.39 per cent in 10 normal young adult males, but in the eight patients investigated the labelling index of clinically normal buccal epithelium ranged from 1·7 per cent to 4·8 per cent, and in leukoplakia lesions the values ranged from 4·28 per cent to 7·58 per cent. Although a distinct cut-off point at which normality and disease in relation to cell production in buccal epithelium could not be established from this preliminary study, it is possible to state that a labelling index above 3·5 per cent by the total cell index appears to constitute increased cell production in human buccal epithelium The rise in the labelling index was thought to be due to increased numbers of cells in the progenitor cell compartment entering the dividing cell cycle, probably due to stimulation of G_o cells in leukoplakia resulting in an increased growth fraction operating in this disease process.

As the labelling index values were significantly higher for leukoplakia than that for normal epithelium, this by itself may be a useful diagnostic index for further studies to examine pathogenesis of leukoplakia and other proliferative disorders of human oral epithelium.

7.3 SOME PRACTICAL PROBLEMS AND METHODOLOGICAL ERRORS

7.3.1 Labelling Index Corrections

Some authors have suggested that the labelling index data should be corrected to account for errors arising from differences in the maximum distance traversed by tritium particles and the section thickness of histological material used for autoradiography. This is on the basis that in autoradiographic preparations of tissue sections labelled nuclei will produce latent images only if the penetrance of radioactive particles exceeds the section thickness. The average path length of tritium particles is between 1·5 - 2 μm with a maximum of 8 μm (Lajtha and Oliver, 1959). Iversen and Evensen (1962) using 5 - 7 μm thick histological sections of epidermis obtained from in vivo labelled mice, derived a correction to take account of this factor. They estimated that about 30 per cent of cell nuclei lay 1·5 μm away from autoradiographic film and therefore multiplied the labelled cell count by a factor of 1·4 to correct for the actual number of DNA synthesising nuclei in their material. Simnett (1968) using in vitro labelled mouse lung sectioned at 2, 3, 5, 10 and 15 μm for autoradiographic studies, indicated that a correction factor was not required up to 5 μm thick histological sections. Gelfant (1962) found no difference in the labelling index in 2 μm and 7 μm histological sections used for comparative study.

A correction factor to account for ^{3}H particle penetrance was not used in the present study as 3 μm sections were used. As this material was only half as thick as that used by Iversen and Evensen (1962) even if a correction is required this would be much smaller than the factor of 1·4 as derived by these authors.

However, it would be important to undertake this correction if comparison of data using material of varying thicknesses beyond 2 μm

is attempted. This would be significant if the labelling index estimations are expressed by the cell count methods. When using surface length or basement membrane length indices this factor would not influence the labelling index estimation as histological sections thicker than 2 μm will act as sections of "infinite thickness" and labelled nuclei beyond the maximum penetrance of ^{3}H particles will not be visualised. Therefore comparisons can be made between the labelling indices expressed per unit epithelial surface estimated from histological sections of varying thickness, provided these exceed the mean path length of the emitted particles.

7.3.2 Cell Count Corrections

The importance of the corrections required to correct for nuclear fragments which cause an overestimate of cell counts was described in the relevant parts of this thesis. Such a correction is important because of differences in the nuclear diameter in progenitor and mature cells which result in fragmented nuclei of varying proportions in the two cell compartments. The application of Abercrombie's correction to labelling index data in normal human buccal mucosa was found to increase the estimated mean value from 2·48 per cent to 2·56 per cent by the total cell index. In leukoplakic biopsies following this correction the estimated labelling index increased from 5·71 per cent to 6·01 per cent. The correction of cell counts altered the estimated values to an insignificant extent in the normal specimens because of the small difference in nuclear size between progenitor and mature cells. However, in the leukoplakic lesions the difference in nuclear size between compartments was greater, accounting for the 5 per cent increase in labelling index when corrected cell counts were used. Marrable (1962) came to a similar conclusion following investigations of the importance of correction factors for mitotic index in teleost blastula. He commented that the effect from nuclear fragments should be considered in counting of cells and only rejected when it has proved

insignificant. From the experience gained in this study it can be recommended that, to exclude the complex and tedious steps of correcting for nuclear fragments in cell kinetic studies, a pilot survey of the nuclear diameter measurements of labelled cells and unlabelled cells used for reference index calculation in histological sections or examinations of nuclear diameter in material from different contrasting groups under investigation should allow an investigator to decide whether correction factors would make an appreciable difference to the labelling index.

7.3.3 Some Remarks on the Use of ^{3}H Thymidine Labelling and Autoradiography for Estimation of Cell Production in Oral Epithelium

In this study small fragments of tissue varying in size from 1 mm^3 to a maximum size of 3 mm^3 were used for labelling in vitro. It was apparent that the use of larger pieces of tissue does not allow diffusion of the label adequately into the centre of the tissue block. The main advantage of tritium labelling as a marker of cell proliferation is its anatomical selectivity in that the label enables identification of cells in one phase of the cell cycle, and allows localisation of the labelled product at a histological level with good microscopic resolution.

In the use of DNA replication as a measure of cell production, the assumption is made that the labelled cells all proceed to mitosis. Although this is true for the normal tissues, when dealing with diseased tissues, especially with degeneration, it is necessary to examine whether all labelled cells appear as mitoses or may become non vital as has been observed in lichen planus where eosinophilic cells, probably degenerating keratinocytes, are commonly observed (see comment by El-Labban and Kramer, 1974).

Although counting cells in S phase from autoradiographs is

considered superior to counting mitoses in histological sections (Section 2.2.4), a large number of methodological errors may arise if autoradiographic procedures are carried out without proper control and standardisation. In the present study attention was paid to standardise all autoradiographic steps. Material which was dipped in emulsion in different batches was treated identically during later stages in order to obtain comparable results. The use of proper controls for the analysis of background and of positive and negative chemographic effects are of considerable importance in any study dealing with quantitative evaluation of labelled cells.

7.3.4 Reliability of Double Labelling Techniques

The double labelling technique primarily used to estimate the duration of the S phase introduced some methodological problems which deserve clarification at this stage. These difficulties were not obvious in the early parts of this study, but became apparent during later experiments.

The main difficulty was in the identification of the two cell populations in double labelled cells when the grain count over some nuclei was close to the dividing level used for the differentiation.

Ideally, to obtain proper identification of heavily and weakly labelled cells, what is required is to produce grain yields which do not overlap in cells labelled by the two pulses. As the grain yield in identically prepared autoradiographs is mainly dependent on the isotope dose used for labelling, this factor is to some extent under the control of the investigator. In selecting the two doses of isotope, in order to obtain optimum results, the main concern is to establish as many fold difference in the concentration of the two doses without hindering autoradiographic identification of weakly labelled cells and also without influencing the cell kinetics by using an excessive dose of tritium or

thymidine in the heavy dose. It was observed in the present study that the ideal grain distribution for identification of cells labelled by the weak dose is in the range of 8 - 15. This enables the investigator to carry out visual grain counting readily in order to identify the weakly labelled cell population. A heavy dose grain count above 40 ensures that clusters of silver grains will appear over the heavily labelled nuclei. It is pertinent to discuss the ^{3}H thymidine doses used in the present study for double labelling by contrasting with those used by other workers.

Galand's group, who introduced the double labelling technique with two doses of ^{3}H thymidine in vitro, published several reports between 1968 and 1971 on the application of this technique (Galand et al, 1968; Galand and Chretien, 1969; Bleiberg et al, 1970, and Heenen and Galand, 1971). For in vitro labelling they used ^{3}H thymidine in doses of 1 µCi/ml and 10 µCi/ml (specific activity 5 Ci/mM) or 0·5 µCi/ml and 20 µCi/ml of the same specific activity. Lachapelle and Gillman (1969) recommended that for optimum labelling of human epidermis in vitro, 2 µCi/ml of ^{3}H thymidine (specific activity 25 Ci/mM) was required. In the preliminary studies conducted during this investigation it was apparent that a dose of 0·5 µCi/ml of ^{3}H thymidine (specific activity 22 - 26 Ci/mM) was not sufficient to obtain consistent in vitro labelling and that 1 µCi/ml of ^{3}H thymidine (specific activity 22 - 26 Ci/mM) provided the optimum dose for hamster tongue epithelium. This results in only a minimum quantity of free thymidine; a concentration of 0·011 µg of thymidine per ml of medium. The dose of 20 µCi/ml of ^{3}H thymidine at a specific activity of 5 Ci/m Mole as used by Galand's group in some parts of their experiments (Heenen and Galand, 1971) as the heavy dose would involve the addition of 0·96 µg of thymidine per ml of incubation medium. It was considered that this could have reached a concentration of free thymidine at which alterations in cell kinetics might occur. The presence

of excessive thymidine is known to influence cell kinetics in vivo (Section 2. 3. 2). Therefore the lower dose of 10 μCi/ml as used by Galand's group in some of their experiments (Bleiberg et al, 1970) was considered appropriate and by using a compound with a high specific activity (22 - 26 Ci/mM) in the present study the amount of free thymidine in the incubation mixture was further reduced to 0·11 μg/ml.

Having decided to use these two doses for labelling the following difficulties were encountered during the analysis of autoradiographs:-

1. Labelled cells were found demonstrating around 25 silver grains, which was the line of demarcation for identification of heavily and weakly labelled cells.

2. In single pulse labelled tissues, a few cells labelled with the low isotope dose showed grain count yields in the heavy dose range and vice-versa.

   Small nuclear fragments which might be a part of a heavily labelled nucleus would be producing only a weak response on the emulsion due to the small amount of activity in the fragmented part and therefore these would possibly be identified as weakly labelled cells in double labelled tissues. It is probably advisable to eliminate such small nuclear fragments from the count and appropriate cytological criteria must be defined for this purpose.

3. Heavily labelled cells produce a cluster of silver grains over the nuclei and these occasionally overlap on to a neighbouring nucleus which is probably not in fact labelled but may be falsely identified as weakly labelled.

Careful control is required of the exposure time and the development schedule during the preparation of autoradiographs in order to obtain an optimum grain density of 8 - 15 silver grains in the majority of single weakly labelled cells. This facilitates visual grain counting of these nuclei for their differentiation and ensures that the great majority of single weakly labelled cells have grain counts of from 4 - 25.

Similar problems appear to have confronted other workers using the technique. Schmid et al (1974) by observing the minor errors which may result from the problem of misidentification of cells labelled by each isotope dose, suggested an arithmetic correction to re-allocate the small number of labelled nuclei giving grain yields apparently inappropriate to the isotope dosage. Such a method may prove useful in improving this main shortcoming of this method and a few suggestions on this are included in Section 7.4.

7.4 FURTHER WORK AND FUTURE APPLICATIONS

It would be possible to apply the quantitative techniques described in this thesis to a large number of the disorders of oral epithelium. This could allow quantitation of changes which at present are only assessed subjectively. A series of investigations is under progress to quantitate the changes in oral epithelium in iron deficiency and in experimental carcinogenesis.

It is hoped that labelling index and cell turnover data in conjunction with other recent advances in quantitative approaches to the study of tissue changes by objective criteria may profitably be used to change the present totally subjective and non-quantitative approach to diagnostic histopathology.

Further studies are being undertaken for investigating the effect of block size, time delay in transfer of tissues to the laboratory

and to assess accurately the effect of varying doses of ^{3}H thymidine in vitro on the estimated labelling index. More detailed investigations are required to improve the currently employed double labelling technique and it is intended to establish corrective methods to eliminate the dubiety in the identification of labelled cells corresponding to each dose. The most appropriate idea appears to be to work out an arithmetical correction by closely examining grain yields corresponding to each dose in order to correct the estimated proportions of heavily and weakly labelled cells. In the light of experience gained during the present study, the use of different doses of ^{3}H thymidine in vitro further to improve the dose selections for double labelling appears to be relevant.

As suggested in Chapter 5, the hypothesis of diurnal variation of thickness of epithelial compartments controlling movement of cells in the dividing cell cycle with the probable result of partial synchronisation of cells in S and M phases at different times of the day leading to the commonly observed circadian rhythms in labelling index and mitotic index in epithelial tissues, needs further investigation using more refined quantitative techniques. In addition to the study of probable physiological variations existing in sizes of cell compartments, the use of a suitable experimental model by application of friction or stripping surface layers in order to reduce the size of the maturation compartment and thus to study the effects upon the G_1 inhibitor and on entry of cells into the S phase may elucidate the control mechanism regulating movement of cells from G_1 to the S phase and also from G_2 to the M phase.

A further but a more remote application of the use of quantitative estimations of mitotic activity, labelling index and cell turnover is to monitor changes in cell production in epithelia during experimental studies which are designed to evaluate the therapeutic

benefit of chalones or other agents in cancer treatment in experimental animals. The in vitro technique using small biopsies at intervals to estimate the progress of lesions during long term experiments may be more beneficial than in vivo incorporation of isotopes in these situations. In vitro labelling for the quantitation of cell production may also be applicable for longitudinal studies on oral premalignancy to predict the progress in such lesions and to allow identification of lesions which otherwise might progress to squamous cell carcinomas. To be able to use this technique accurately in such estimations it will be necessary to examine closely the prognostic value of labelling index studies by further and more detailed investigations.

ABBREVIATIONS

A	Area of section
A_m	Area of maturation compartment in section
A_p	Area of progenitor compartment in section
B.M.L.	Basement membrane length
C.F.	Correction factor (Abercrombie's Correction)
$C.F._p$	Correction factor for progenitor cells
$C.F._m$	Correction factor for mature cells
$C_{L.C.}$	Corrected labelled cells
$C_{M.C.}$	Corrected mature cells
$C_{P.C.}$	Corrected progenitor cells
$C_{T.N.C.}$	Corrected total nucleated cells
$C_{L.C./T.N.C.\%}$	Corrected labelling index by total cell index
G_1	Post-mitotic pre-synthetic gap in cell cycle
G_2	Post-synthetic pre-mitotic gap in cell cycle
H.L.C.	Heavily labelled cell or cell count
I	Number of intersection points
I_{bm}	Intercept points along basement membrane of epithelium
I_s	Intercept points along epithelial surface
L	Length of structure
L.C.	Labelled cell or labelled cell count
L.I.	Labelling index
$L.I._1$	Labelling index by a single pulse
$L.I._2$	Labelling index by double labelling

M	Mitosis
M.C.	Mature cell or mature cell count
M.C. (size)	Mature cell size
$M.C._{ND}$	Nuclear diameter of mature cells
M.K.	Mixed keratinization
N.D.	Nuclear diameter
N.K.	Non-keratinization
O.K.	Orthokeratinization
P	Number of point counts
P_p	Point counts in progenitor compartment
P_m	Point counts in mature compartment
P_t	Point counts in total epithelium
P.C.	Progenitor cells or progenitor cell count
P.C. (size)	Progenitor cell size
$P.C._{ND}$	Progenitor cell nuclear diameter
P.K.	Parakeratinization
S (phase)	DNA synthesis phase
S.D.	Standard deviation
S.L.	Surface length
T_c	Total cell cycle time
T_{g1}	Duration of G_1 phase in the cell cycle
T_{g2}	Duration of G_2 phase in the cell cycle
T_m	Mitotic duration
T_p	Thickness of progenitor compartment
T_s	Duration of S phase
T_t	Total epithelial thickness
T.L.C.	Total labelled cells
T.N.C.	Total nucleated cells
W.L.C.	Weakly labelled cells
$\bar{x}$	Arithmetical mean

APPENDIX

APPENDIX 1 - FIXATIVE

Bouin's fluid

Picric Acid, saturated aqueous solution	70 ml
Formalin (40% filtered neutral formaldehyde)	30 ml

APPENDIX 2 - TISSUE PROCESSING CYCLE

Automatic tissue processor with following cycle:-

1.	70% methylated spirits	30 minutes
2.	80% methylated spirits	1 hour
3.	8% phenol in methylated spirits	2 hours
4.	8% phenol in methylated spirits	1 hour
5.	8% phenol in methylated spirits	2 hours
6.	Absolute alcohol	2 hours
7.	Absolute alcohol	3 hours
8.	50% absolute alcohol, 50% chloroform	30 minutes
9.	Chloroform	3 hours
10.	Chloroform	4 hours
11.	Paraffin wax	2 hours
12.	Paraffin wax	2 hours

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CELL PROLIFERATION IN HUMAN ORAL MUCOSA

VOLUME 2 OF TWO VOLUMES

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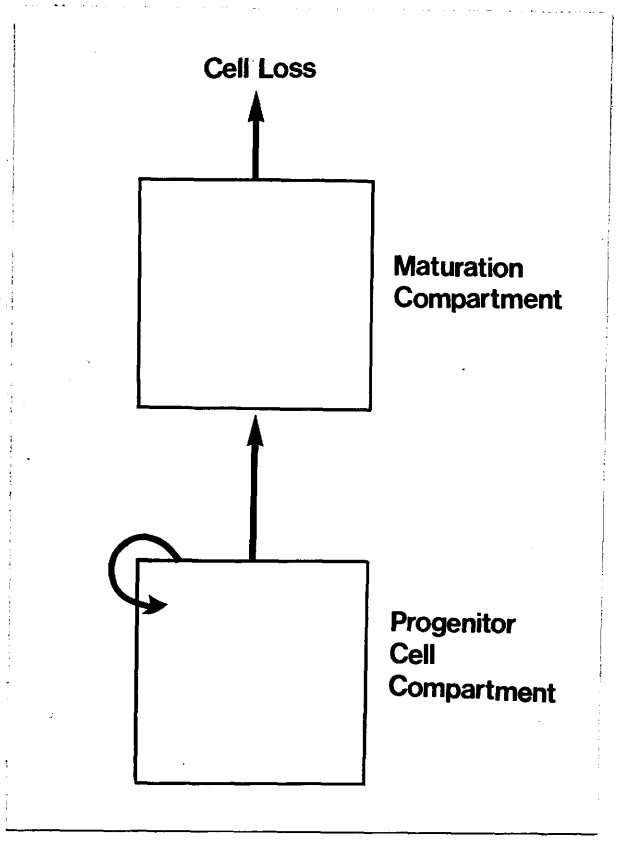


Fig. 1.1 Simple two compartmental cell renewal system with a progenitor and maturation compartment. Arrows indicate cell movement and cell loss.

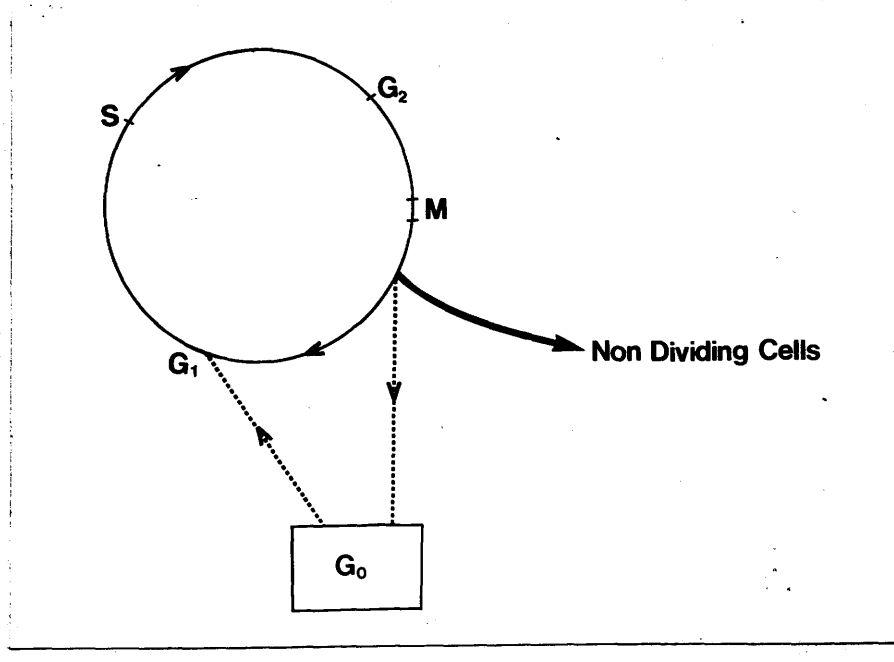


Fig. 1.2 Diagramatic illustration of cell cycle. M - mitosis; S - DNA synthesis phase; G_1 - postmitotic-presynthetic gap; G_2 - postsynthetic-premitotic gap. G_0 - non-proliferating cells, capable of dividing after appropriate stimulation.

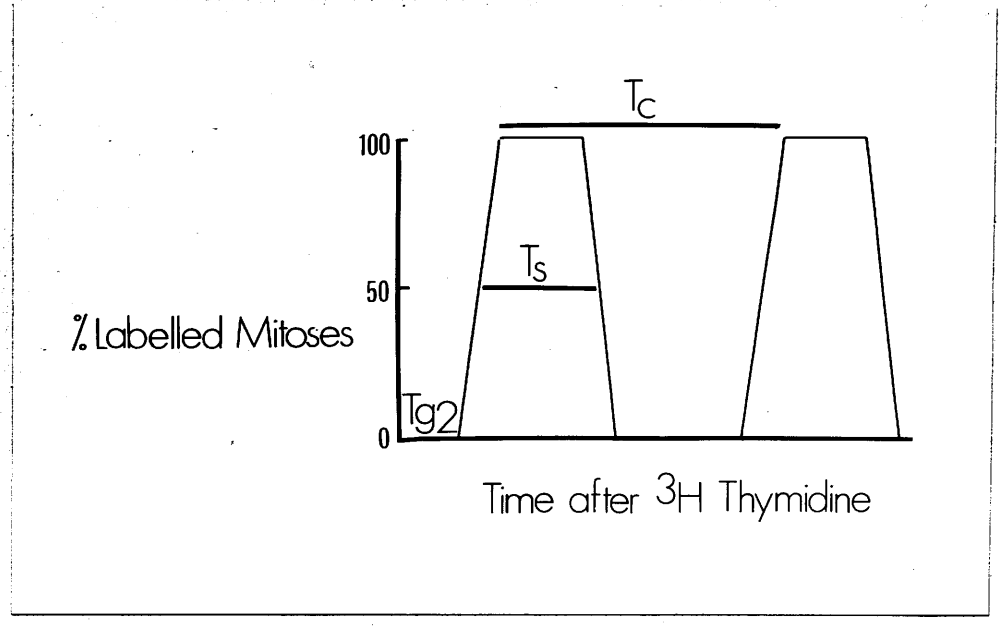
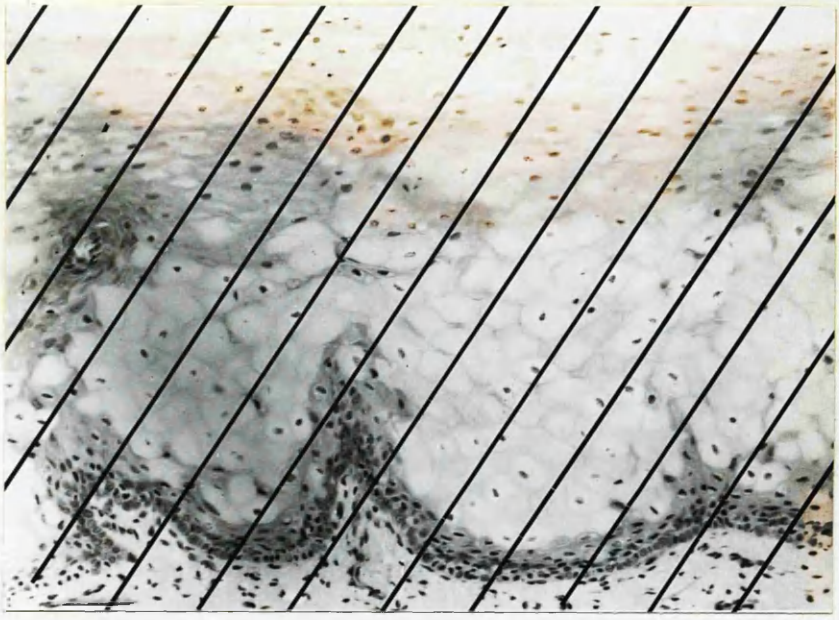


Fig. 2.1 Theoretical percentage labelled mitoses curve following administration of ^{3}H thymidine, assuming that all cells move through the dividing cycle at the same rate.
T_{g2} - duration of G_2; T_s - duration of S phase; T_c - duration of total dividing cell cycle.



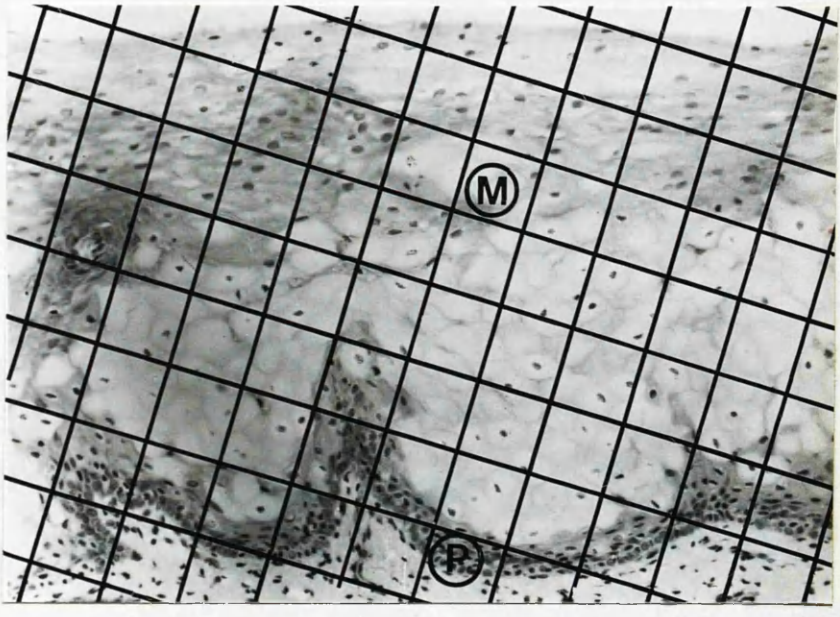


Fig. 2.2 Light microscopic view of human buccal mucosa.
Fig. 2.2.A with a superimposed grid for intercept point counting along the epithelial surface and the basement membrane x 200.
Fig. 2.2.B with a superimposed grid for stereologic point counting. M - maturation compartment; P - progenitor compartment x200.

Group No.	Hamster No.	Weight g	Time of Injection	Time of Sacrifice
I	1	93	0925	0955
	2	92	0925	1025
	3	90	0930	1130
	4	90	0930	1230
	5	87	0935	1335
	6	87	0935	1435
	7	90	0938	1538
	8	80	0938	1638
	9	98	0942	1742
	10	85	0942	1842
	11	98	0945	1945
	12	78	0945	2045
	13	83	0950	2150
II	19	90	1230	1330
	20	90	1232	1432
	21	87	1235	1535
III	22	90	1530	1730
	23	78	1532	1830

Table 3.1 Animal numbers, weights, times of injection of ^{3}H thymidine and times of sacrifice.

Fig. 3.1 Hamster in restraining device.

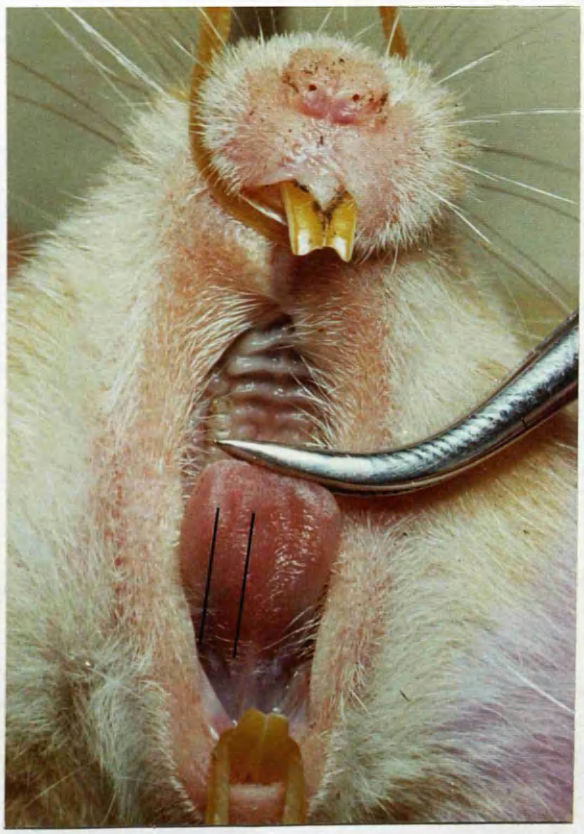


Fig. 3.2 Normal hamster ventral tongue. The initial vertical incisions are shown diagramatically.

Fig. 3.3 Specimen of ventral tongue mucosa before trimming.

Hamster No.	Weight g	Time of Biopsy
16	87	1000
14	85	1130
15+	85	1330
18	83	1435
17	85	1538
24*	100	1830

Table 3.2 Hamsters used for in vitro labelling (experiment 2) and as untreated controls for mitotic indices in experiment 3.
+ used for experiment 2 only.
* used for experiment 3 only.

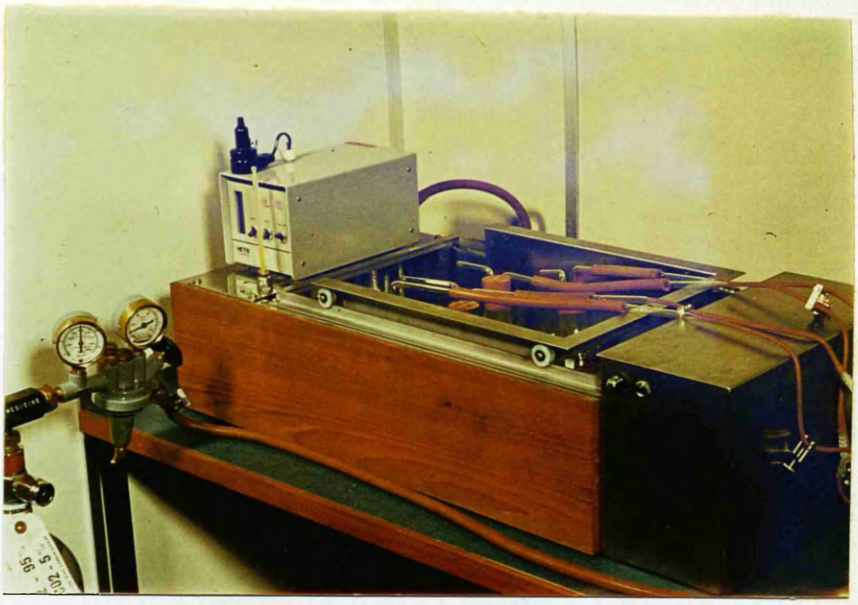


Fig. 3.4 Apparatus used for in vitro ^{3}H thymidine labelling.

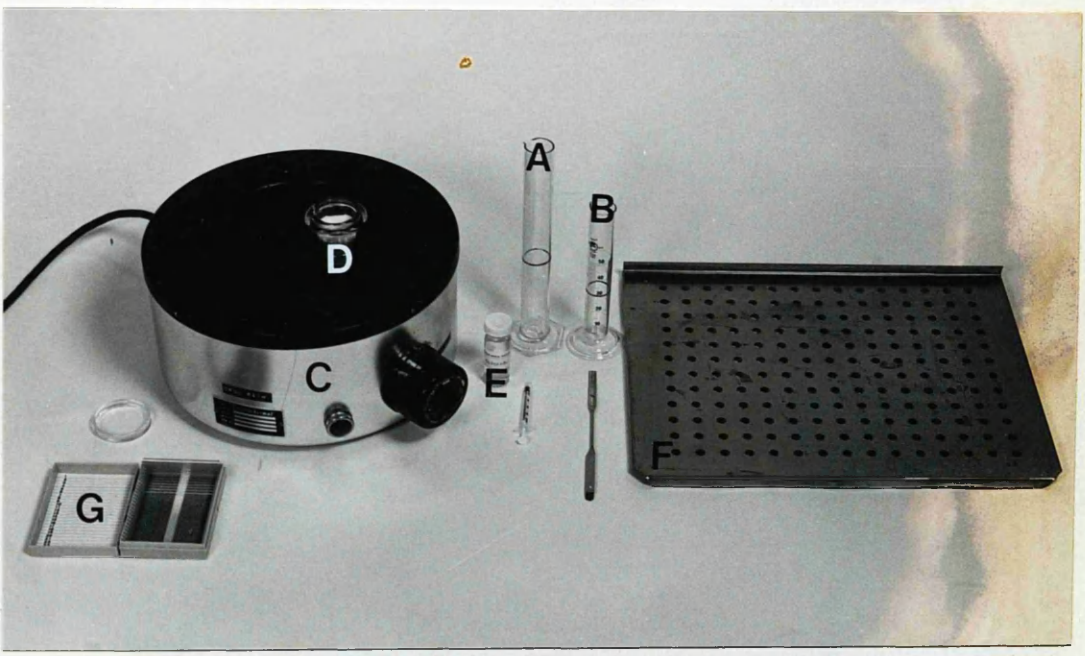


Fig. 3.5 Equipment used for autoradiography.
(A) and (B) measuring cylinders (see text)
(C) Water bath (D) Coplin jar with molten emulsion
(E) Glycerol (F) Plate for drying slides
(G) Light proof plastic box for exposure.

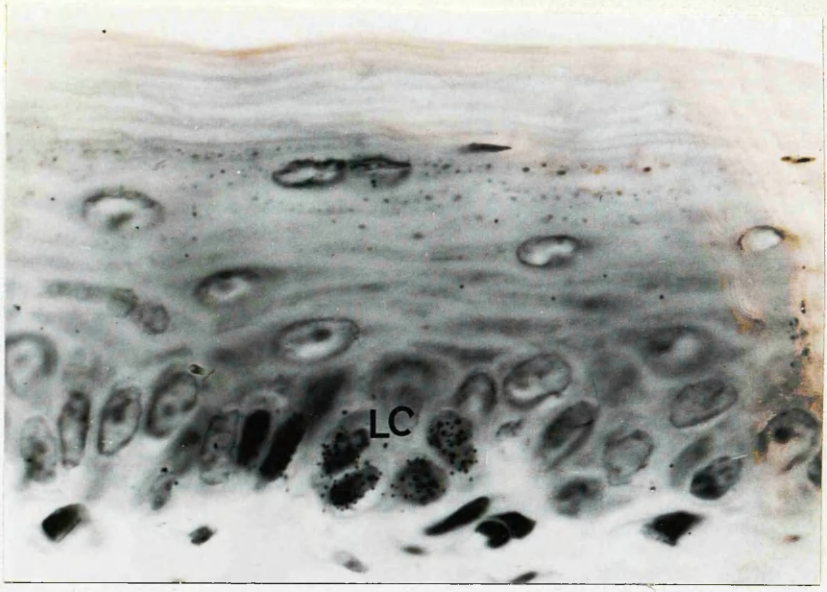


Fig. 3.6 Hamster ventral tongue following ^{3}H thymidine in vivo pulse labelling with 1 µCi/g body weight. Autoradiograph illuminated by transmitted light (x 1250). L.C. - labelled cells showing silver grains overlying the nuclei.

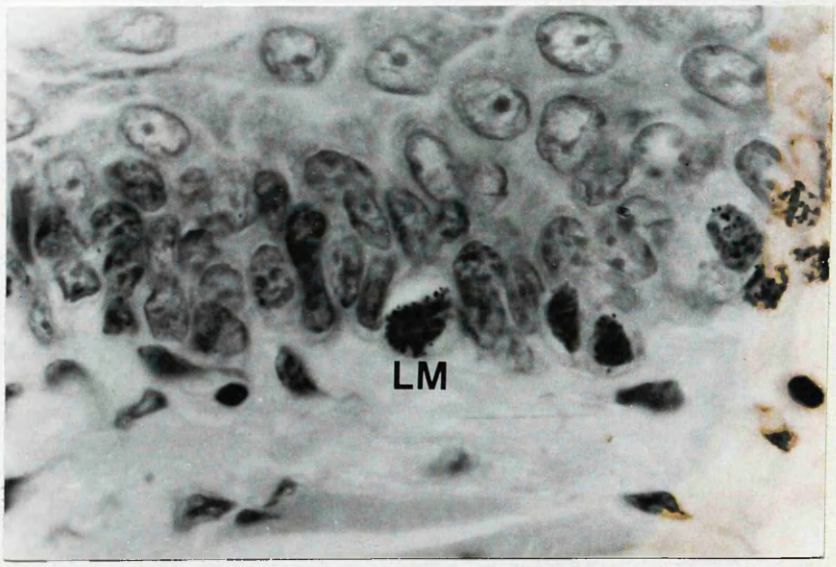


Fig. 3.7 Autoradiograph showing a labelled cell (LM) which has progressed to mitosis (prophase) in hamster number 8 (x 1250).

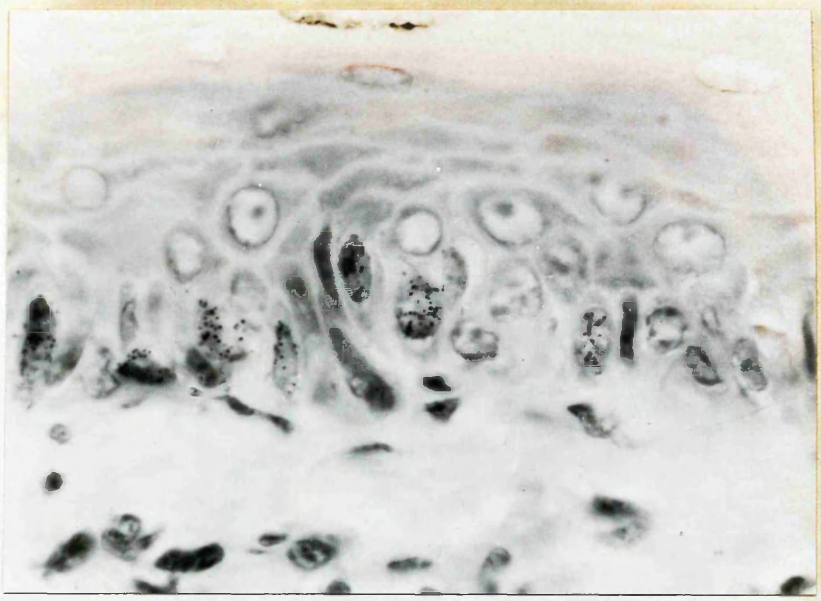


Fig. 3.8 Autoradiograph from in vitro labelled hamster tongue (single dose 1 µCi/ml) showing seven labelled cells. Individual silver grains are visible (x 1250).

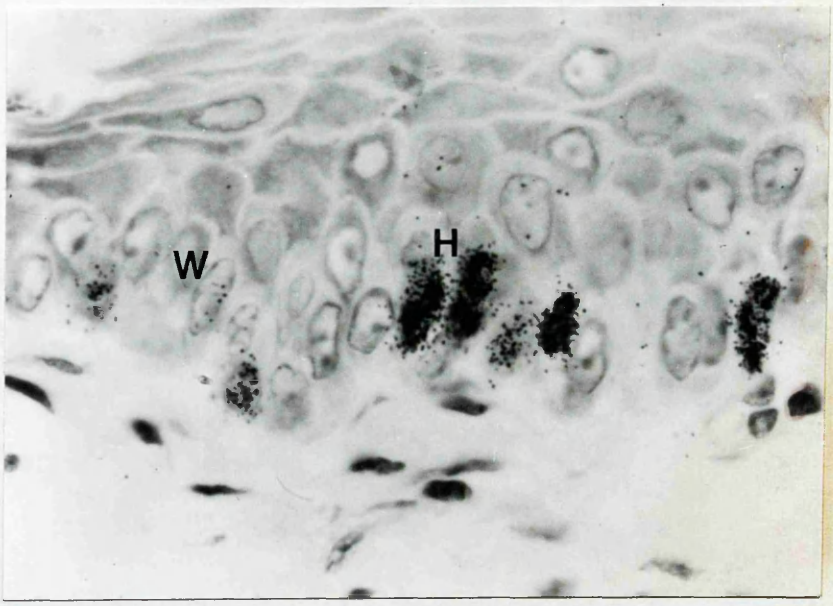


Fig. 3.9 Autoradiograph from in vitro labelled hamster tongue (double labelled with 1 µCi/ml and 10 uCi/ml). Heavily (H) and weakly (W) labelled cells are shown (x 1250).

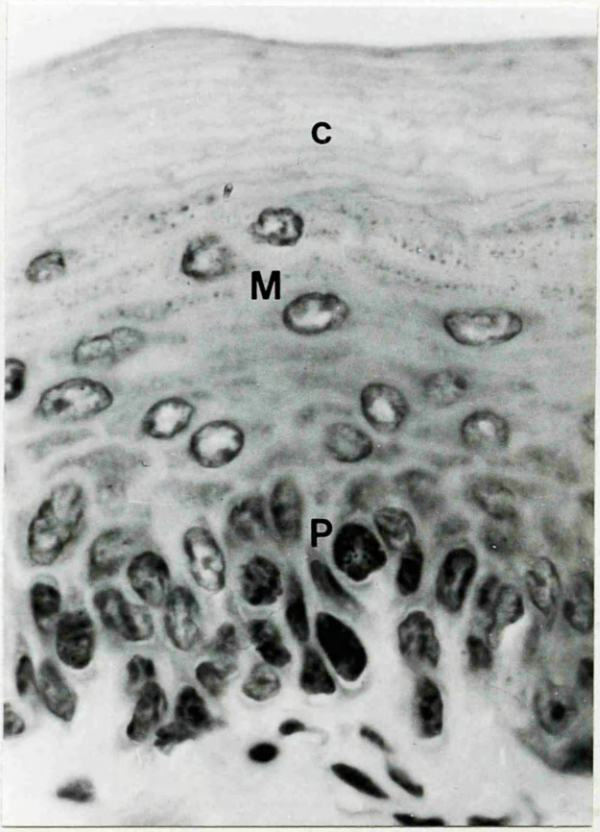


Fig. 3.10 Cell compartments of hamster ventral tongue epithelium. P - Progenitor Cell Compartment; M - Maturation Compartment; C - Cornified Compartment (x1250).

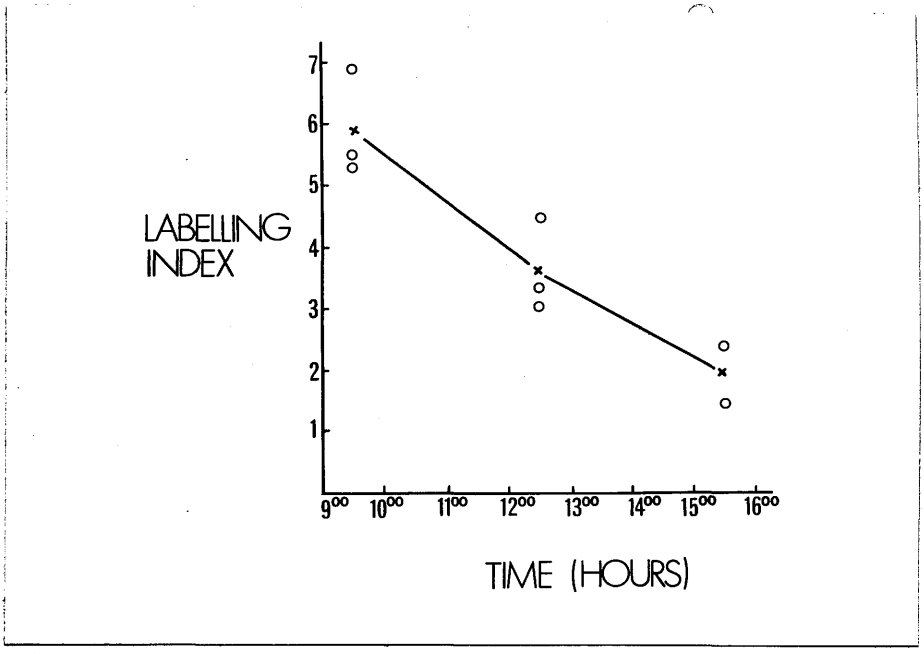


Fig. 3.11 Graph of labelling indices per 100 total nucleated cells estimated in vivo (detailed in Table 3.3).

Group No.	Hamster No.	Total Cells	Labelled Cells	Labelling Index	Labelling Index per Group
I	2	1739	93	5.35	5.92
	3	2014	112	5.50	
	4	2563	178	6.91	
II	19	1818	55	3.03	3.61
	20	1937	87	4.49	
	21	1942	64	3.30	
III	22	1921	28	1.45	1.93
	23	1774	43	2.41	

Table 3.3 Labelling indices of hamster ventral tongue epithelium by in vivo labelling.
Injection times:- Group I - 0925-0930; Group II - 1230-1235; Group III - 1530-1532.

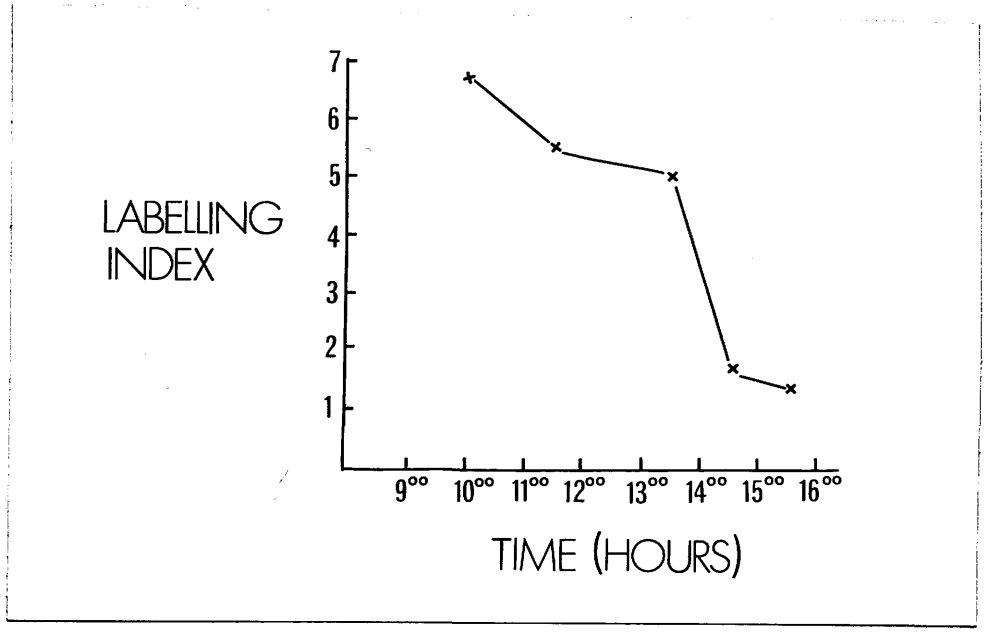


Fig. 3.12 Graph of labelling indices per 100 total nucleated cells estimated in vitro by single pulse labelling (detailed in Table 3.4).

Hamster No.	Time	Total Cells	Labelled Cells	Labelling Index
16	1000	1689	114	6.75
14	1130	811	45	5.55
15	1330	526	27	5.13
18	1435	1841	32	1.74
17	1538	1028	15	1.46

Table 3.4 Labelling indices by in vitro labelling (single pulse of 1 μCi/ml). The time shown is the time of biopsy.

Hamster No.	Time	Total Cells	Heavily Labelled Cells	Weakly Labelled Cells	Labelling Index	Ts (Hours)
16	1000	1830	112	16	6.12	7.00
14	1130	3696	206	28	5.57	7.36
15	1330	5190	223	30	4.30	7.43
18	1435	4624	66	10	1.42	6.60
17	1538	3268	44	8	1.35	5.50

Table 3.5 Proportions of heavily to weakly labelled cells and estimated T_s in autoradiographs from in vitro double labelled hamster ventral tongue. The labelling indices using counts of heavily labelled cells are also shown.

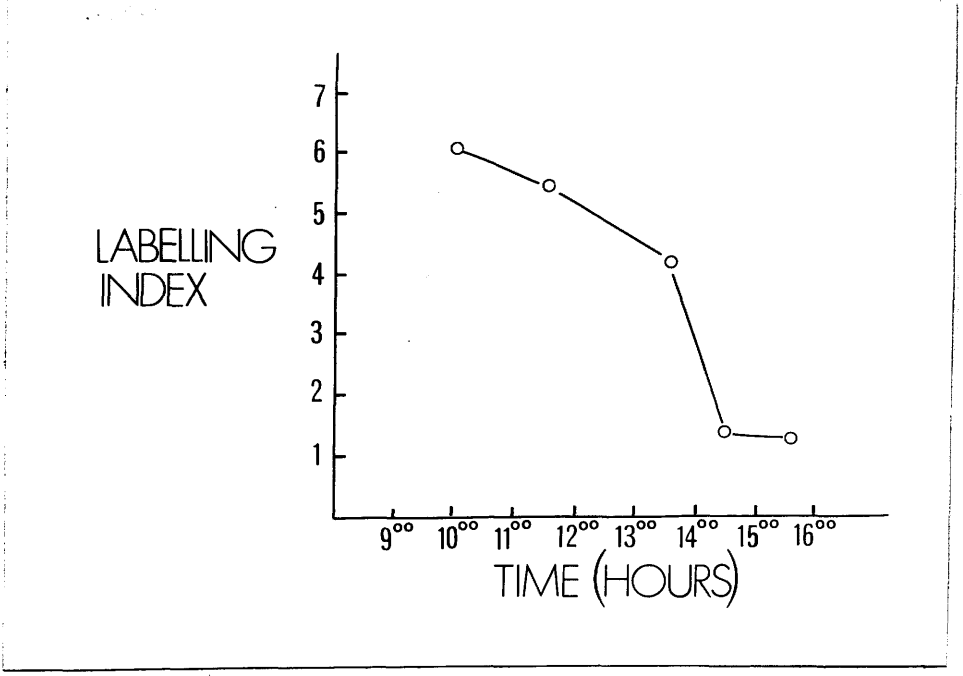


Fig. 3.13 Graph of labelling indices following in vitro labelling by counting cells labelled by the heavy pulse of 10 µCi/ml in double labelled material (detailed in Table 3.5).

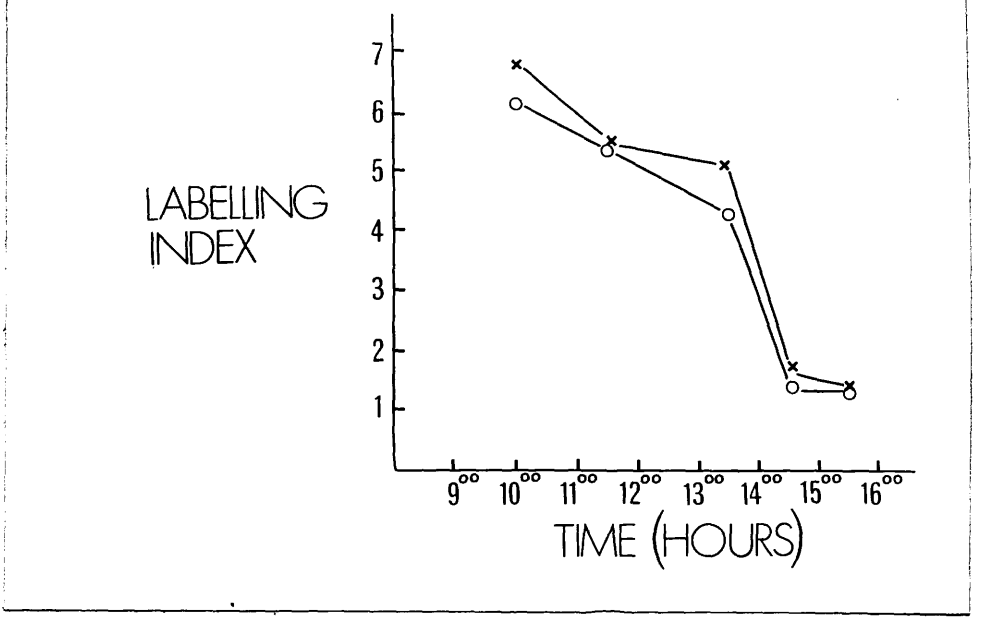


Fig. 3.14 Comparison of labelling indices estimated by in vitro labelling, using a single pulse and using counts of heavily labelled cells only in double labelled material.
x - x in vitro single; o - o HLC index in double.

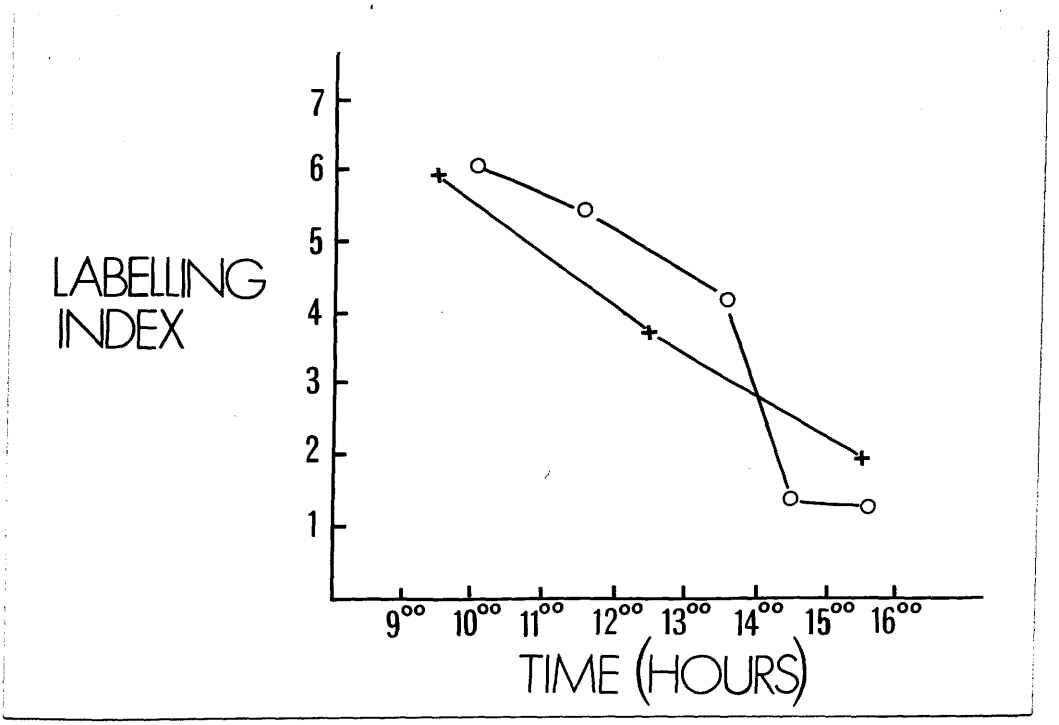


Fig. 3.15 Comparison of labelling indices obtained by in vivo and in vitro pulse labelling.
o - o in vitro.
x - x in vivo.

Hamster No.	Time between Injection and Sacrifice (Hours)	Labelled Mitoses	Unlabelled Mitoses	Total Mitoses	Percentage Labelled Mitoses
1	$\frac{1}{2}$	O	19	19	O
2	1	3	55	58	5
3	2	60	117	177	34
4	3	87	7	94	92
5	4	59	1	60	98
6	5	51	O	51	100
7	6	50	O	50	100
8	7	81	O	81	100
9	8	79	5	84	94
10	9	75	8	83	90
11	10	42	12	54	78
12	11	28	24	52	53
13	12	11	15	26	42

Table 3.6 Labelled and unlabelled mitoses counted in autoradiographs from hamsters number 1-13 (Group I) with estimates of the percentages of labelled mitoses at sequential sacrifice.

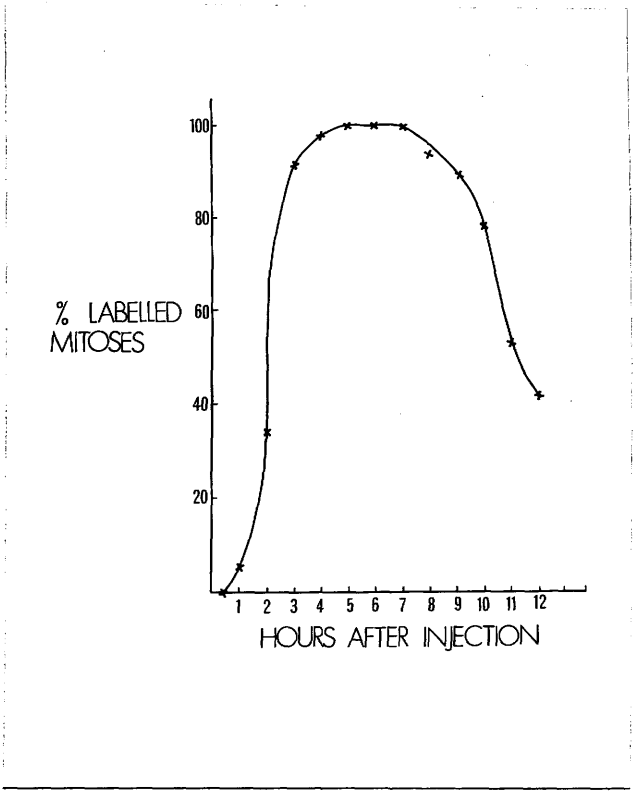


Fig. 3.16 Percentage labelled mitoses in hamster ventral tongue plotted against time after injection of ^{3}H thymidine - from data in Table 3.6.

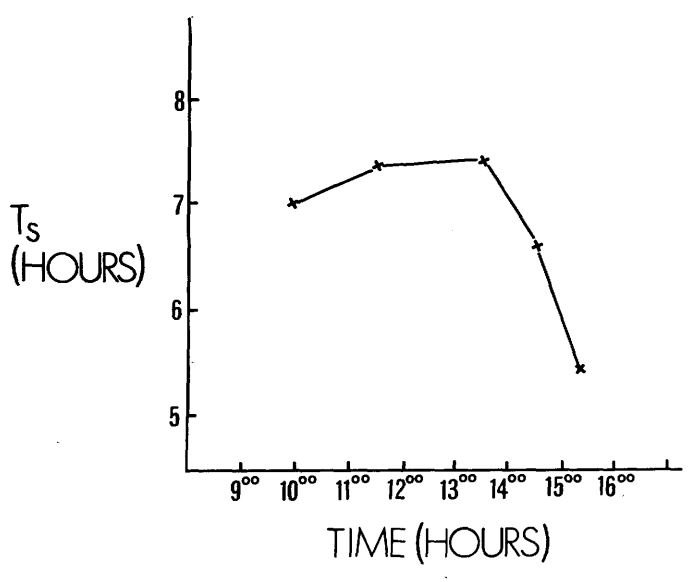


Fig. 3.17 T_s estimations by double labelling method (data in Table 3.5).

Hamster No.	Time	Total Cells	Surface Length	Mitoses	Mitoses per 1000 cells	Mitoses per mm.
1	0955	3475	8.160	19	5.522	2.350
3	1130	4417	11.645	47	10.513	4.031
5	1335	4713	11.390	51	10.840	4.485
7	1538	4576	10.455	50	10.928	4.785
10	1842	4452	11.220	40	8.986	3.572

Table 3.7 Counts of mitoses, total cells and estimated surface length, for ^{3}H thymidine injected hamsters. Mitotic indices by total cell index and per millimetre epithelial surface.

Hamster No.	Time	Total Cells	Surface Length	Mitoses	Mitoses per 1000 cells	Mitoses per 1 mm.
16	1000	1502	3.455	14	9.32	4.017
14	1130	3063	8.330	33	10.77	3.962
18	1435	2698	6.800	28	10.38	4.118
17	1538	12702	27.625	144	11.45	5.124
24	1830	4497	12.580	45	10.00	3.734

Table 3.8 Counts of mitoses, total cells and estimated surface length for control animals. Mitotic indices by total cell index and per millimetre epithelial surface.

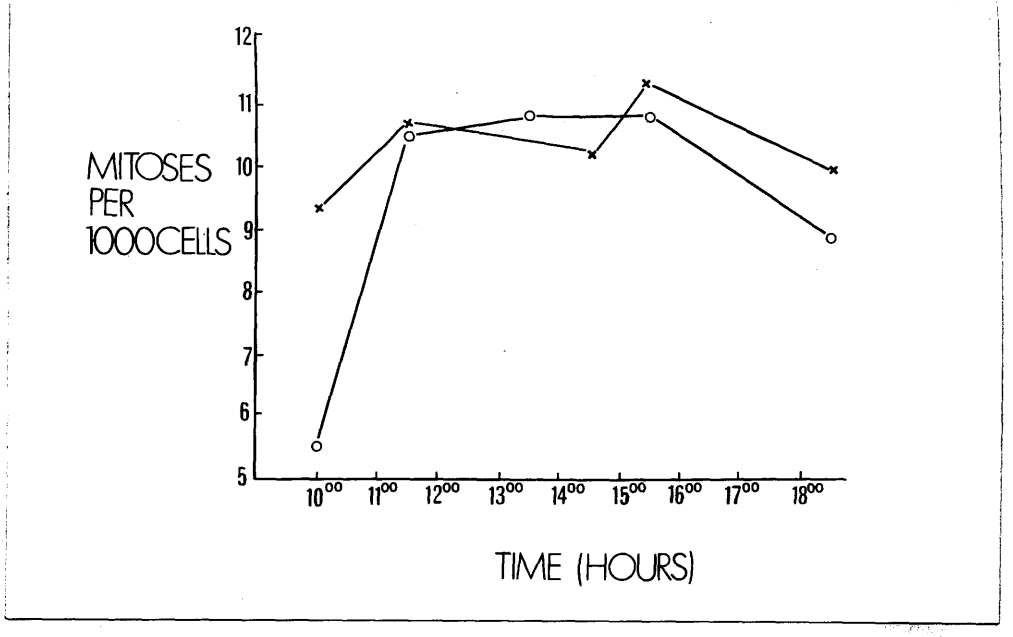


Fig. 3.18 Mitotic index by cell count method for ventral tongue epithelium in ^{3}H thymidine injected and control hamsters.
x - x control; o - o injected group.
(Values are shown against the time of day at sacrifice).

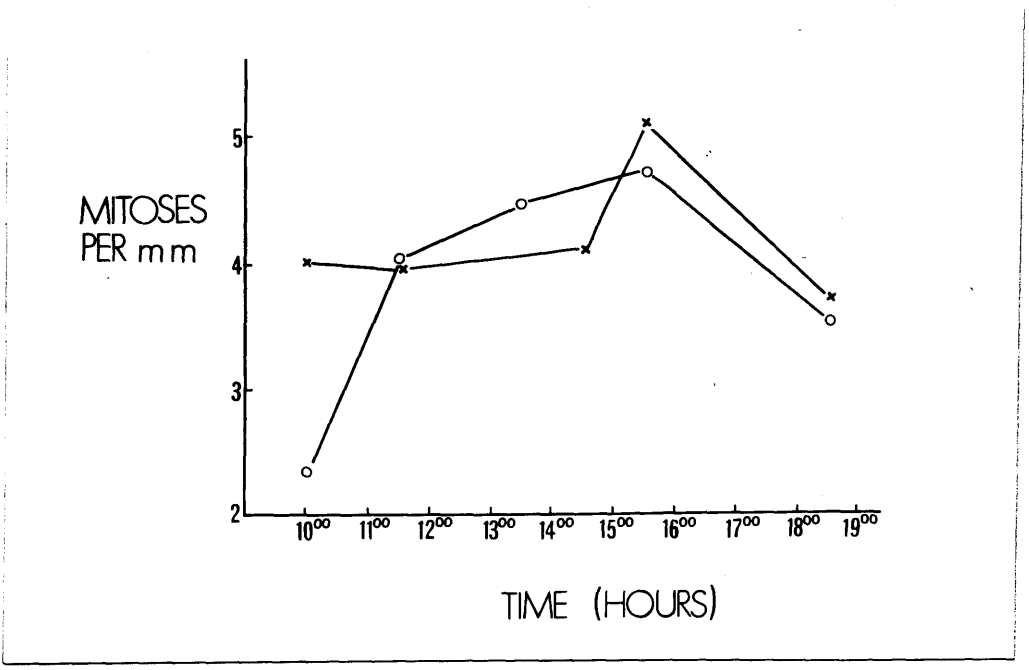


Fig. 3.19 Mitoses per millimetre surface length for ventral tongue epithelium of ^{3}H thymidine injected and the control group of hamsters.
x - x control; o - o injected.
(values are shown against the time of day at sacrifice).

	Time	Labelling Index	Ts (Hours)	Turnover Time (Days)
In Vitro	1000	6.12	7.00	4.76
	1130	5.57	7.36	5.51
	1330	4.30	7.43	7.20
	1435	1.42	6.60	19.37
	1538	1.35	5.50	16.97
				$\bar{x}$ 10.76
	Time	Mean Labelling Index	Mean Ts (Hours)	Turnover Time (Days)
In Vivo	0930	5.9	9.0	6.36
	1230	3.6	9.0	10.41
	1530	1.9	9.0	19.74
				$\bar{x}$ 12.17

Table 3.9 The table shows the variations in estimated turnover time, by using labelling index and T_s data obtained at different time periods.

Fig. 4.1 Normal buccal mucosa.

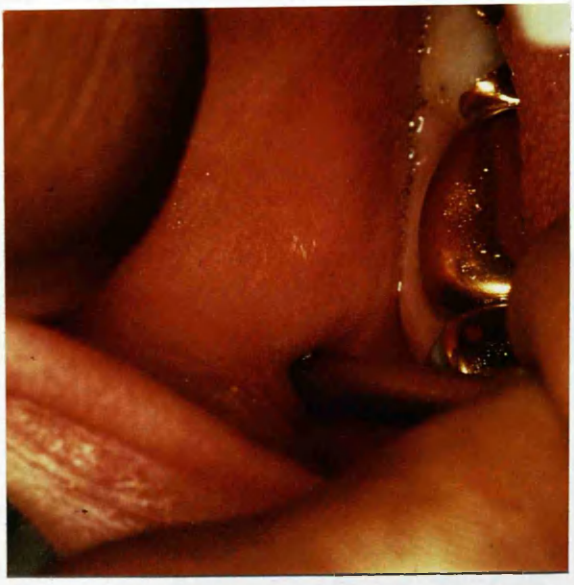


Fig. 4.2 Using a trephine type punch of 3 millimetre diameter to make a circular incision.

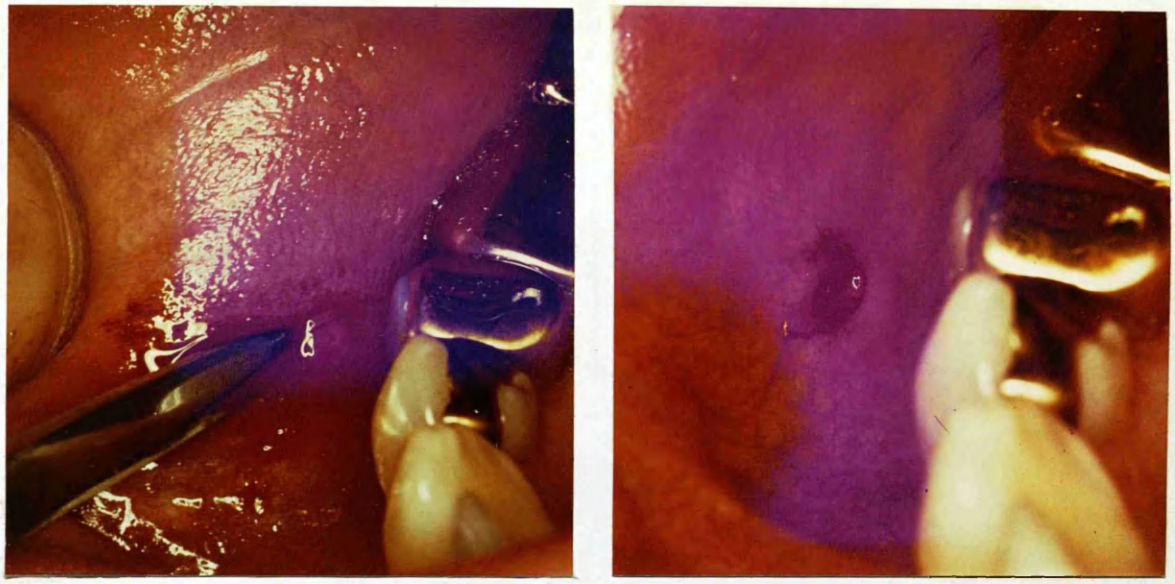


Fig. 4.3 The base of the punched mucosa is cut with a pair of fine scissors leaving a small wound which does not require suturing.

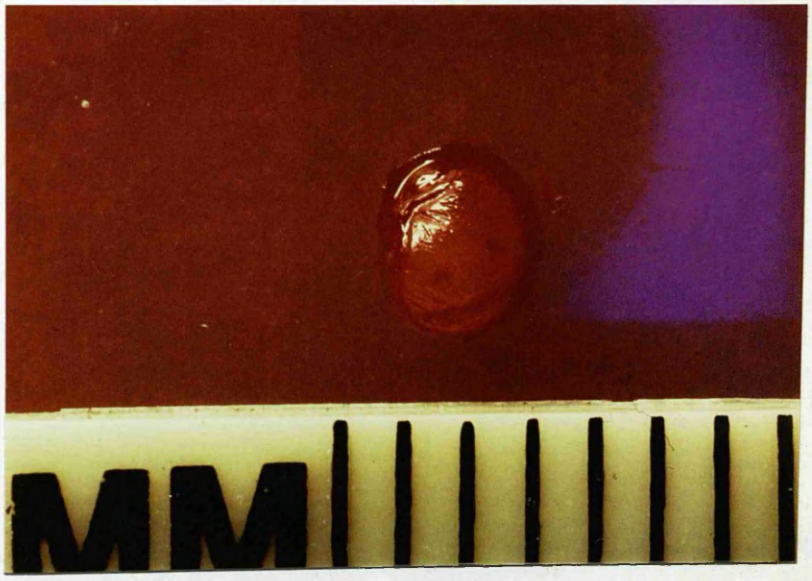


Fig. 4.4 "Punch Biopsy".

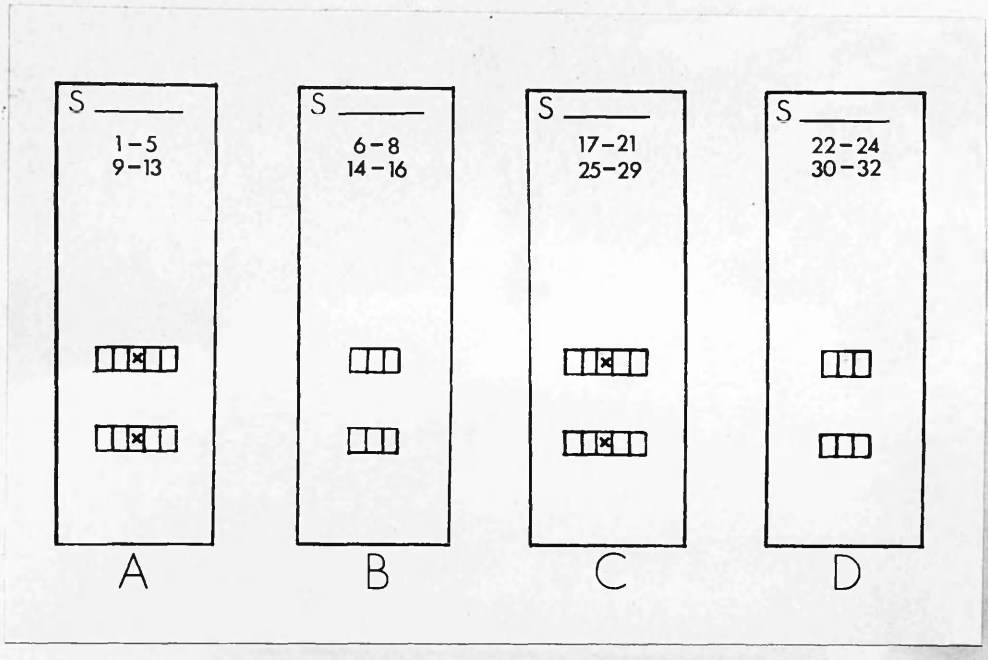


Fig. 4.5 Arrangement of serial sections on subbed slides for autoradiography. Slides A and C are used and B and D kept as spares. X marks every eighth section used for counting.

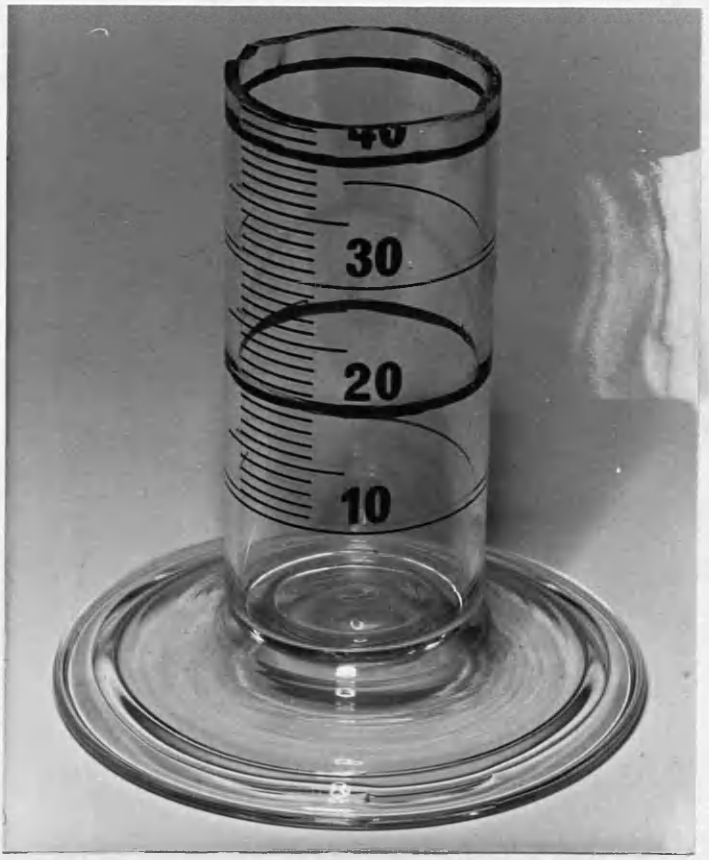


Fig. 4.6 Dipping jar for autoradiography made by cutting a 100 ml measuring cylinder.

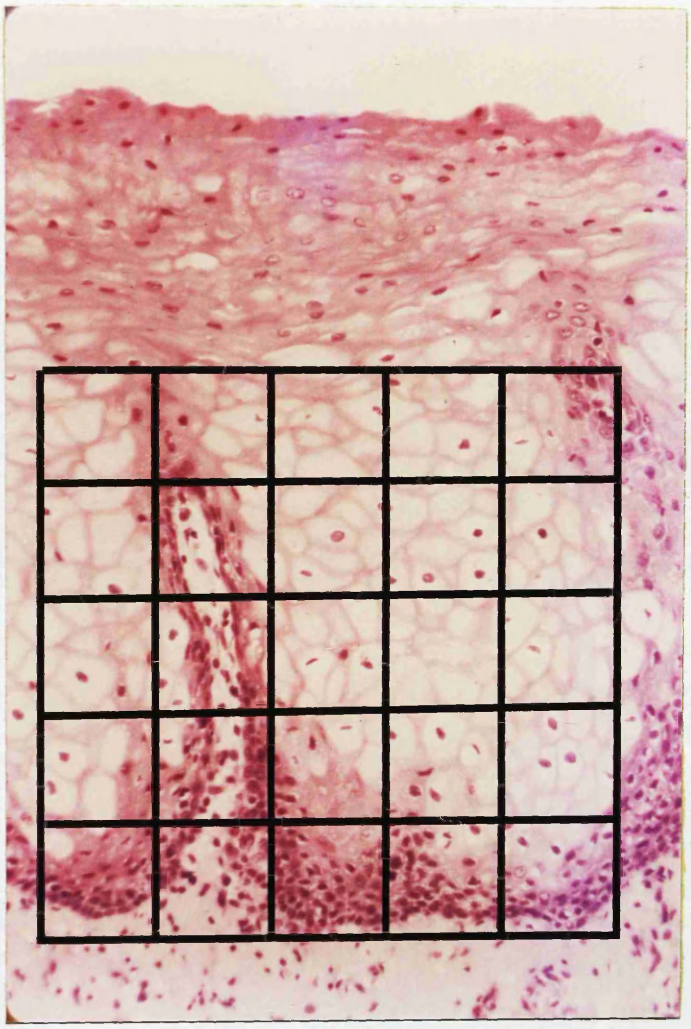


Fig. 4.7 Normal buccal epithelium illustrating the use of a microscopic eyepiece graticule with 25 squares, to delineate small units for cell counting. x160

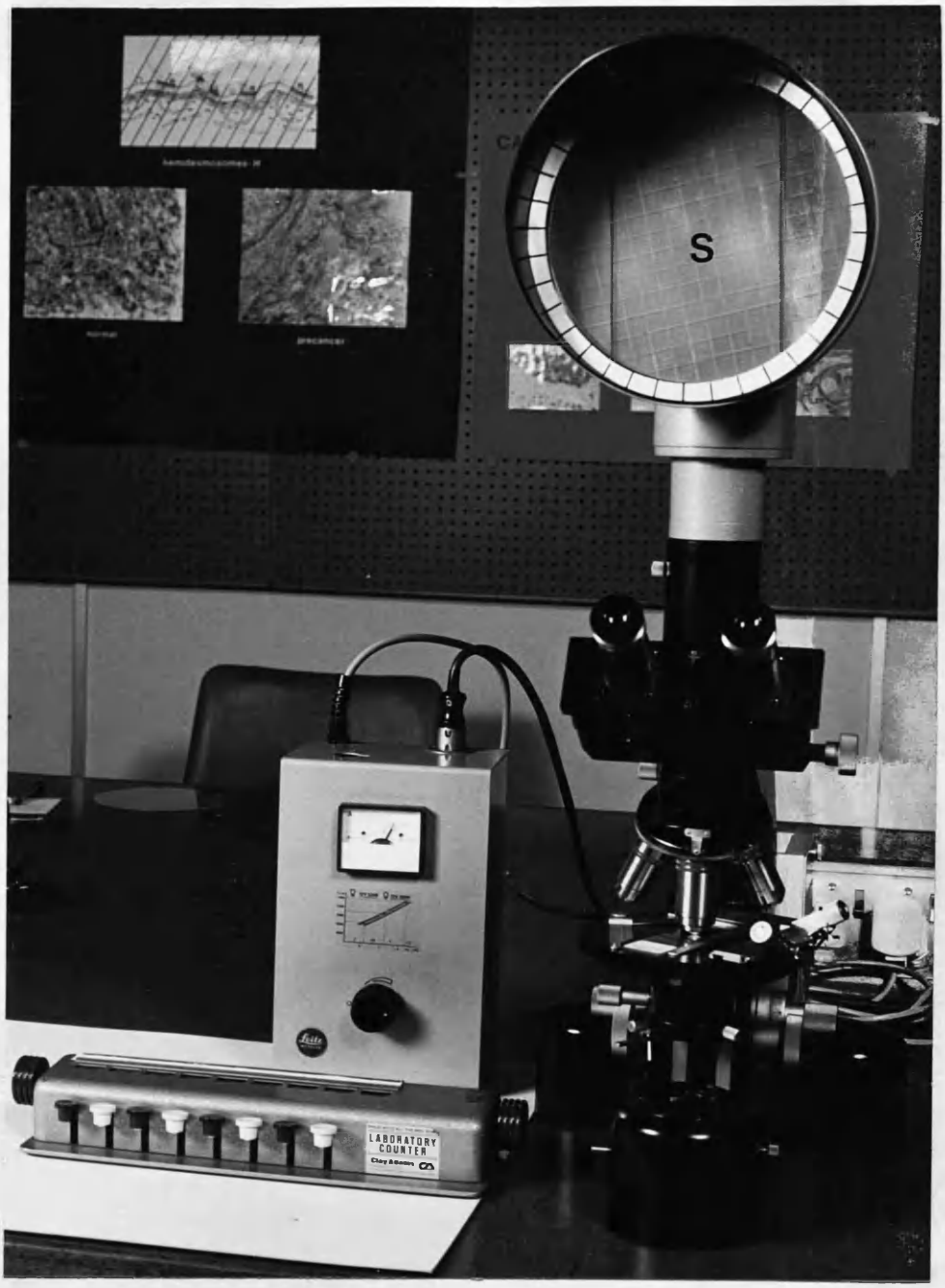
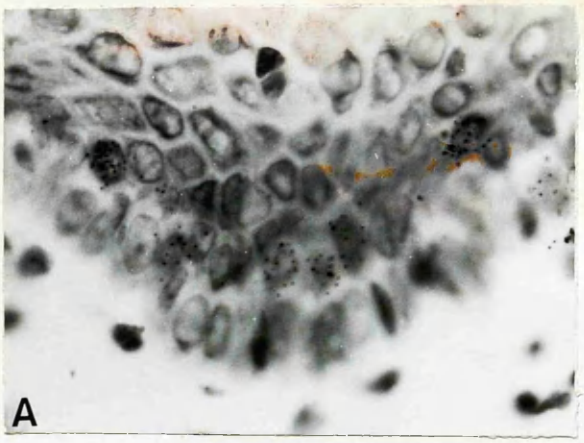
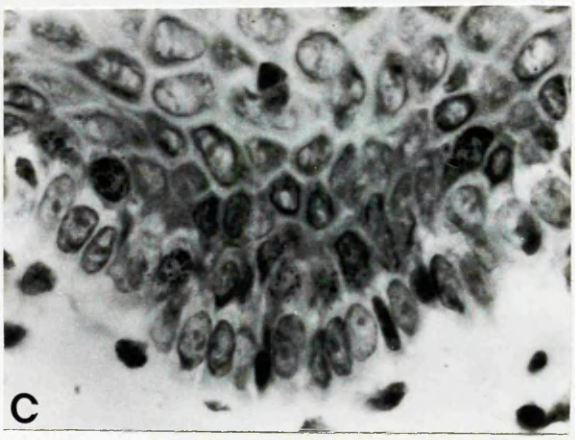
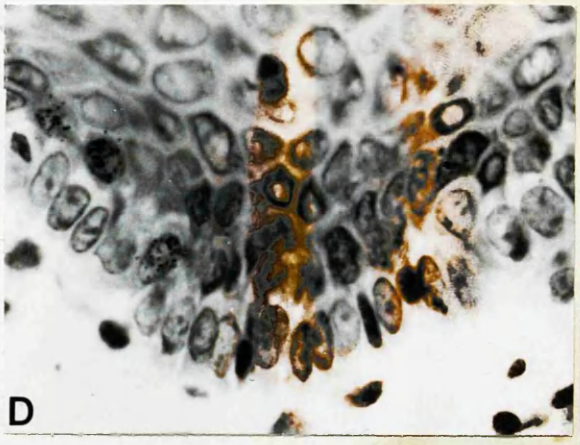


Fig. 4.8 Leitz Ortholux microscope with projection screen (s) upon which a circular transparent grid has been placed.







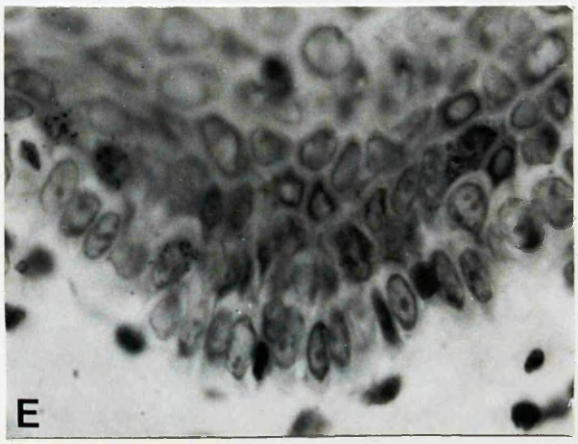


Fig. 4.9 A through focus series of photomicrographs taken 1 um apart. Fig. A shows the focal plane 1 um above the cellular layer at which most of the silver grains of the autoradiographs are in focus.

Subject Number	Age (Years)	Time of Biopsy (Hours)					
		1000	1400	1800	2200	0200	0600
1	32	x	x	x	x	x	x
2	26	x	x	x	x	x	x
3	28	x	x	x	x	x	x
4	32	x	x				
5	21	x	x				
6	27	x	x				
7	21	x	x				
8	26			x	x		
9	25			x	x		
10	24			x	x		
11	28			x	x		

Table 4.1 Ages of subjects and times at which biopsies were taken (Experiment 4A and 4B).

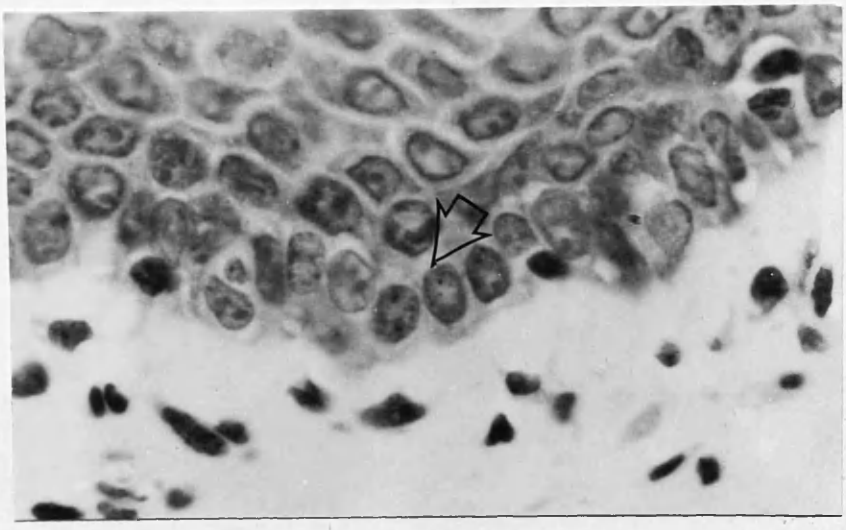


Fig. 4.10 Autoradiograph showing cells labelled in human buccal epithelium, with a weak dose of ^{3}H thymidine, transmitted light, x1250.

Time	No.	P.C.	M.C.	TNC	I_s	I_{bm}	S.L.	B.M.L.	L.C.	LC/TNC %	LC/PC%	LC/SL	LC/BML
1000	1	1118	1210	2328	37.2	44.9	2.92	3.53	45	1.93	4.03	15.40	12.75
	2	1518	1610	3128	58.3	62.6	4.58	4.92	85	2.72	5.60	18.55	17.28
	3	2165	2483	4648	91.6	102.3	7.20	8.04	93	2.00	4.30	12.92	11.57
1400	1	1188	982	2170	30.3	42.3	2.38	3.32	45	2.07	3.79	18.90	13.55
	2	836	759	1595	30.9	40.2	2.43	3.16	45	2.82	5.38	18.51	14.24
	3	1112	1034	2146	45.6	54.9	3.58	4.32	57	2.66	5.13	15.91	13.19
1800	1	1308	920	2228	45.5	57.6	3.58	4.52	64	2.87	4.89	17.90	14.16
	2	1161	1196	2357	34.6	43.8	2.72	3.44	64	2.72	5.51	23.53	18.60
	3	1666	2342	4008	75.8	107.1	5.96	8.41	104	2.59	6.24	17.46	12.36
2200	1	1528	1420	2948	34.1	41.1	2.68	3.23	100	3.39	6.54	37.40	30.96
	2	1365	1309	2674	30.3	47.2	2.38	3.71	81	3.03	5.93	34.02	25.55
	3	1673	1386	3059	48.8	55.2	3.84	4.33	97	3.17	5.80	25.29	22.40
0200	1	2342	1722	4064	79.3	95.0	6.23	7.46	87	2.14	3.71	13.96	11.66
	2	2826	2085	4911	88.6	114.3	6.96	8.98	116	2.36	4.10	16.65	12.92
	3	1192	868	2060	52.3	56.6	4.11	4.45	44	2.14	3.69	10.70	9.89
0600	1	2093	1817	3910	51.3	66.9	4.03	5.26	84	2.15	4.01	20.83	15.97
	2	1247	1006	2253	31.6	34.3	2.48	2.69	41	1.82	3.29	16.51	15.24
	3	1221	731	1952	43.6	51.6	3.43	4.05	40	2.05	3.28	11.67	9.88

Table 4.2 Estimation of labelling index of human buccal epithelium -Subjects 1-3, Experiment 4A (see Appendix A for definition of table abbreviations).

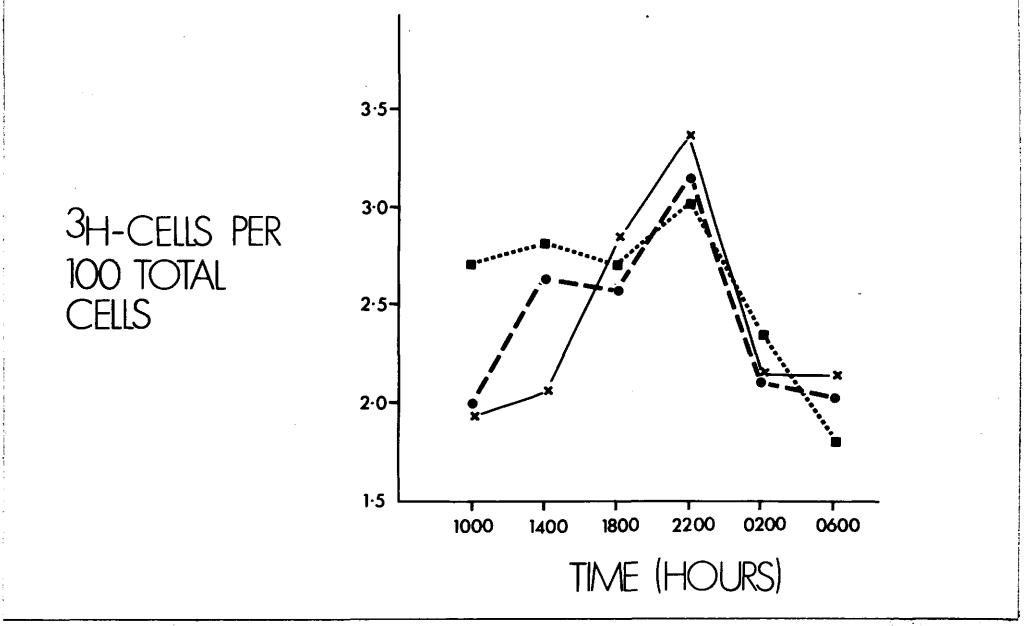


Fig. 4.11 Labelling index of buccal epithelium in the three subjects expressed as labelled cells per 100 total nucleated cells at six time periods. (In all graphs relating to Experiment 4A each of the three subjects is individually designated by
1 ×—× 2 ▪·····▪ 3 ●- - -●

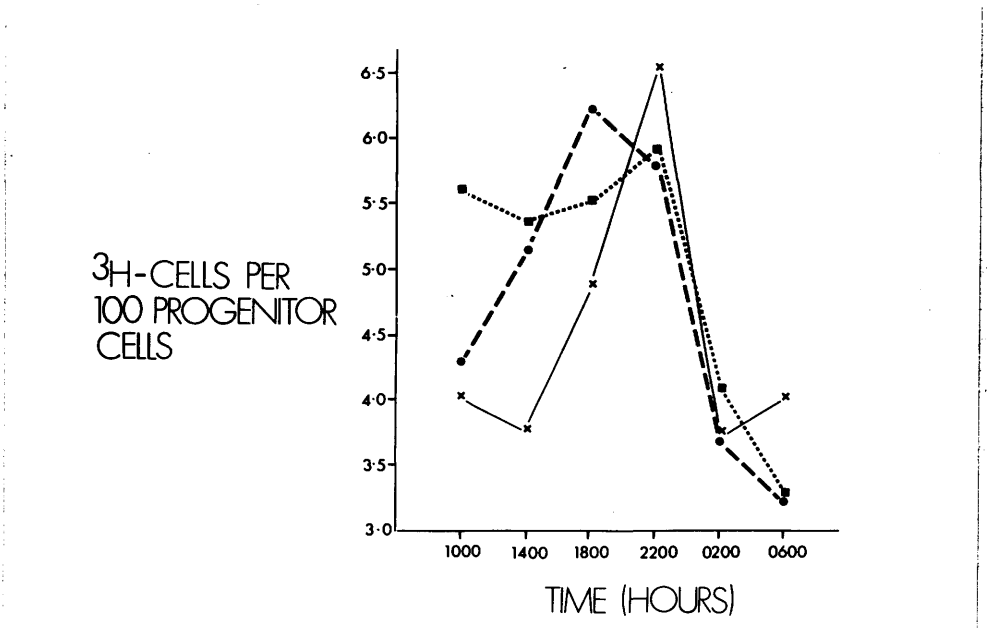


Fig. 4.12 Labelling index of buccal epithelium in the three subjects expressed as labelled cells per 100 progenitor cells at six time periods.

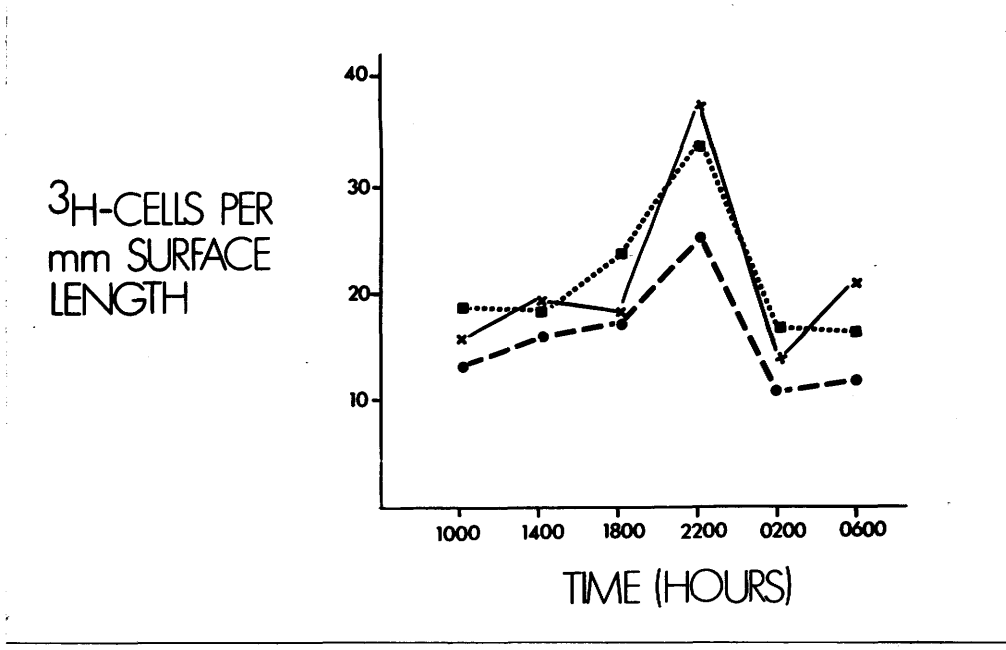


Fig. 4.13 Labelling index of human buccal epithelium expressed as labelled cells per millimetre of epithelial surface.

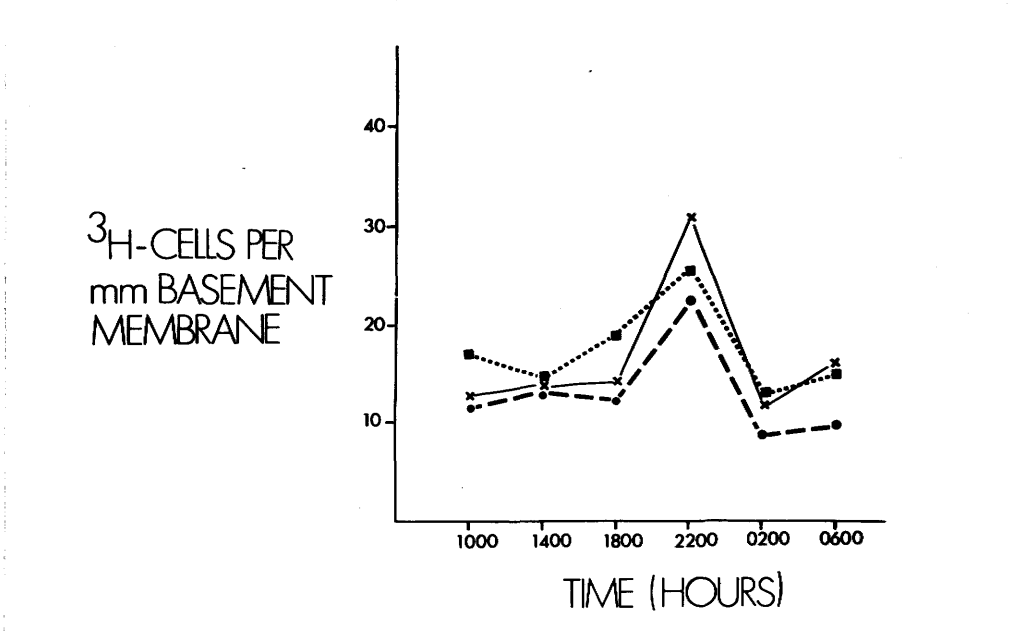


Fig. 4.14 Labelling index of human buccal epithelium expressed as labelled cells per millimetre of basement membrane.

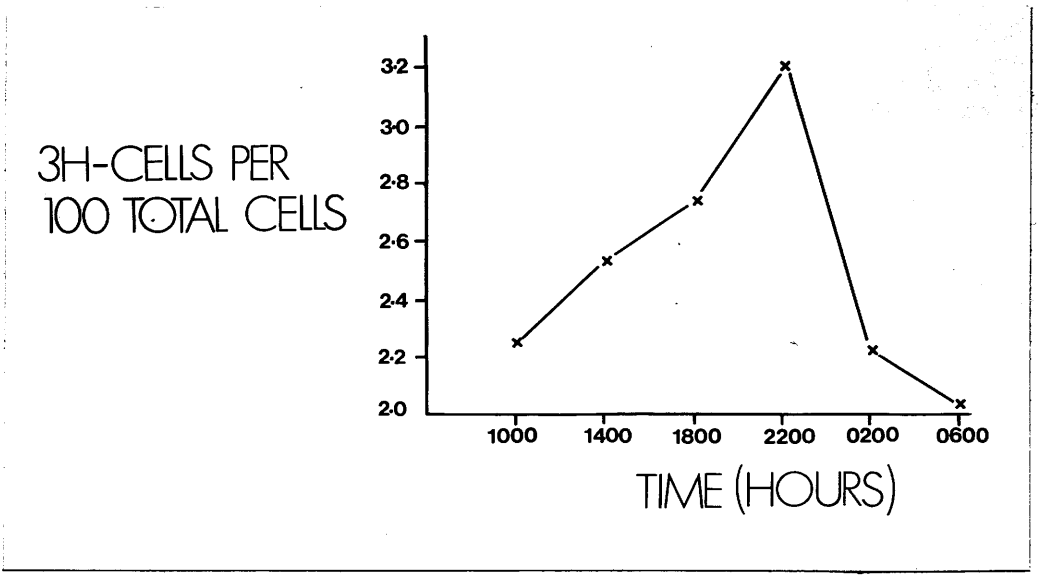


Fig. 4.15 Mean labelling indices for the three subjects (Experiment 4A) at six time periods of the day.

SUBJECT 1		SUBJECT 2		SUBJECT 3	
LI	(Rank)	LI	(Rank)	LI	(Rank)
1•93	(2)	2•72	(12•5)	2•00	(3)
2•07	(5)	2•82	(14)	2•66	(11)
2•87	(15)	2•72	(12•5)	2•59	(10)
3•39	(18)	3•03	(16)	3•17	(17)
2•14	(6•5)	2•36	(9)	2•14	(6•5)
2•15	(8)	1•82	(1)	2•05	(4)
R_1 = 54•5		R_2 = 65•0		R_3 = 51•5	

$$H = \frac{12}{N\,(N+1)} \sum_{j=1}^{k} \frac{R_j^2}{n_j} - 3\,(N+1)$$

$$= \frac{12}{18\,(19)} \left[\frac{(54{\cdot}5)^2}{6} \quad \frac{(65)^2}{6} \quad \frac{(51{\cdot}5)^2}{6} \right] - 3\,(19)$$

$$= 4{\cdot}41$$

$$P > 0{\cdot}10$$

Table 4.3 The Kruskall-Wallis one-way analysis of variance by ranks for comparison of labelling index data for the three subjects. Siegel (1956).

1000		1400		1800		2200		0200		0600	
LI	Rank	LI	Rank	LI	Rank	LI	Rank	LI	Rank	LI	Rank
1•93	2	2•07	5	2•87	15	3•39	18	2•14	6•5	2•15	8
2•72	12•5	2•82	14	2•72	12•5	3•03	16	2•36	9	1•82	1
2•00	3	2•66	11	2•59	10	3•17	17	2•14	6•5	2•05	4
R_1=17•5		R_2=30		R_3=37•5		R_4=51		R_5=22		R_6=13	

$$H = \frac{12}{N(N+1)} \sum_{j=1}^{k} \frac{R_j^2}{n_j} \quad 3(N+1)$$

$$= \frac{12}{18(19)} \left[\frac{(17•5)^2}{3} + \frac{(30)^2}{3} + \frac{(37•5)^2}{3} + \frac{(51)^2}{3} + \frac{(22)^2}{3} + \frac{(13)^2}{3} \right] - 3(19)$$

$$= 11•61$$

$P < 0•05$

Table 4.4 The Kruskall-Wallis one-way analysis of variance by ranks for comparison of labelling index data at six times of the day.

Siegel (1956).

LI at 2200	Rank	LI at other times	Rank
37•40	18	15•40	5
34•02	17	18•55	12
25•29	16	12•92	3
		18•90	13
		18•51	11
		15•91	6
		17•90	10
		23•53	15
		17•46	9
		13•96	4
		16•65	8
		10•70	1
		20•83	14
		16•51	7
		11•67	2
	$R_1 = 51$		$R_2 = 120$

$$U = n_1 n_2 + n_1 \frac{(n_1 + 1)}{2} - R_1$$

$$= 3 (15) + 3 \frac{(3 + 1)}{2} - 51$$

$$= 0$$

$$P = 0{\cdot}002$$

Table 4.5 The Mann-Whitney U Test applied to Labelling index data by surface length index to compare values at 2200 hours with those at other times of the day.

Siegel (1956).

Time	No.	$P.C._{ND}$	$M.C._{ND}$	$C.F._{p}$	$C.F._{m}$	UNCORRECTED P.C.	UNCORRECTED M.C.	UNCORRECTED L.C.	CORRECTED $C_{P.C.}$	CORRECTED $C_{M.C.}$	CORRECTED $C_{T.N.C.}$	CORRECTED $C_{L.C.}$	$LC/_{TNC}\%$	$LC/_{PC}\%$
1000	1	5.05	6.43	.409	.352	1118	1210	45	457	426	883	18.4	2.08	4.03
	2	5.37	5.60	.395	.385	1518	1610	85	600	620	1220	33.6	2.75	5.60
	3	6.36	6.53	.355	.349	2165	2483	93	767	867	1636	33.0	2.02	4.29
1400	1	5.93	7.06	.371	.331	1188	982	45	441	325	766	16.7	2.18	3.79
	2	6.08	6.32	.365	.356	836	759	45	305	283	588	16.4	2.79	5.38
	3	6.51	6.86	.350	.338	1112	1034	57	389	349	738	19.9	2.70	5.11
1800	1	5.73	6.31	.379	.357	1308	920	64	496	328	824	24.3	2.95	4.90
	2	6.43	7.43	.353	.320	1161	1196	64	410	383	793	22.6	2.85	5.51
	3	5.88	6.46	.373	.351	1666	2342	104	621	822	1443	38.8	2.69	6.25
2200	1	4.73	6.28	.401	.358	1528	1420	100	613	508	1121	40.1	3.58	6.54
	2	6.08	6.72	.365	.342	1365	1309	81	498	448	946	29.6	3.13	5.94
	3	5.02	5.93	.411	.371	1673	1386	97	688	514	1202	39.9	3.32	5.80
0200	1	5.37	6.55	.404	.348	2342	1722	87	946	599	1545	35.1	2.27	3.71
	2	5.90	6.29	.372	.358	2826	2085	116	1051	746	1797	43.2	2.41	4.11
	3	6.23	6.66	.360	.344	1192	868	44	429	299	728	15.8	2.17	3.68
0600	1	5.61	7.01	.384	.333	2093	1817	84	804	605	1409	32.3	2.29	4.02
	2	6.18	7.47	.362	.319	1247	1006	41	451	321	772	14.8	1.92	3.28
	3	5.75	6.79	.378	.340	1221	731	40	462	249	711	15.1	2.12	3.27

Table 4.6 Application of Abercrombie's correction to cell counts.

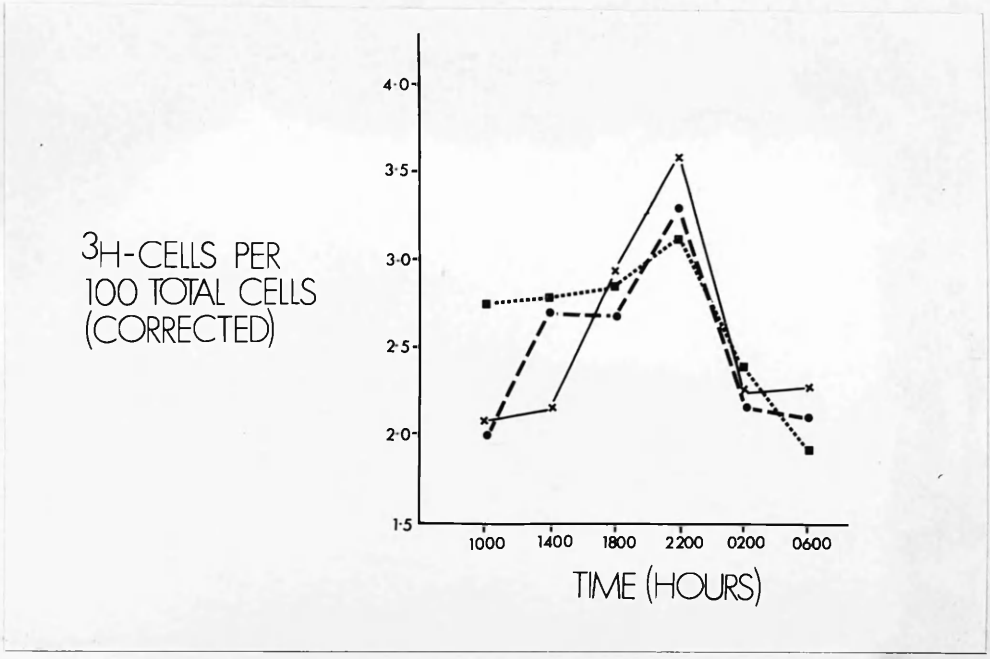


Fig. 4.16 Labelling indices expressed by the corrected total cell index using Abercrombie's correction.

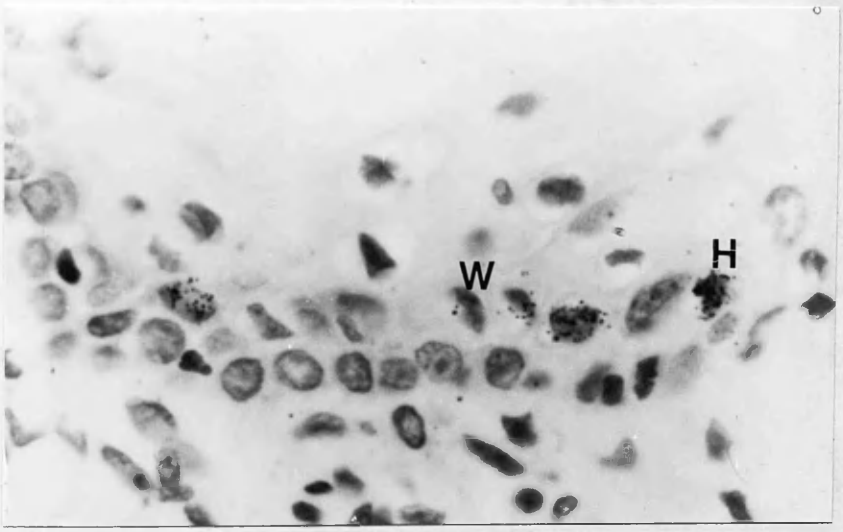


Fig. 4.17 An autoradiograph showing double labelling with two doses of ^{3}H thymidine (H) Heavily Labelled Cells; (W) Weakly Labelled Cells.

Time	No.	T.N.C.	H.L.C.	W.L.C.	T_s
1000	1	4625	125	23	5.4
	2	2758	76	15	5.1
	3	3250	78	14	5.6
1400	1	2471	77	13	5.9
	2	3351	92	15	6.1
	3	3087	85	13	6.5
1800	1	1822	60	9	6.7
	2	2483	78	13	6.0
	3	2319	81	13	6.2
2200	1	1835	62	9	6.9
	2	2584	89	15	5.9
	3	1688	55	9	6.1
				T.L.C.	
0200	1	3421		86	
	2	2295		53	
	3	2169		46	
0600	1	2089		63	
	2	2355		66	
	3	2328		64	

Table 4.7 T_s estimation using counts of heavily (HLC) and weakly (WLC) labelled cells. For 0200 and 0600 hours total labelled cells (HLC + WLC) are shown (see text).

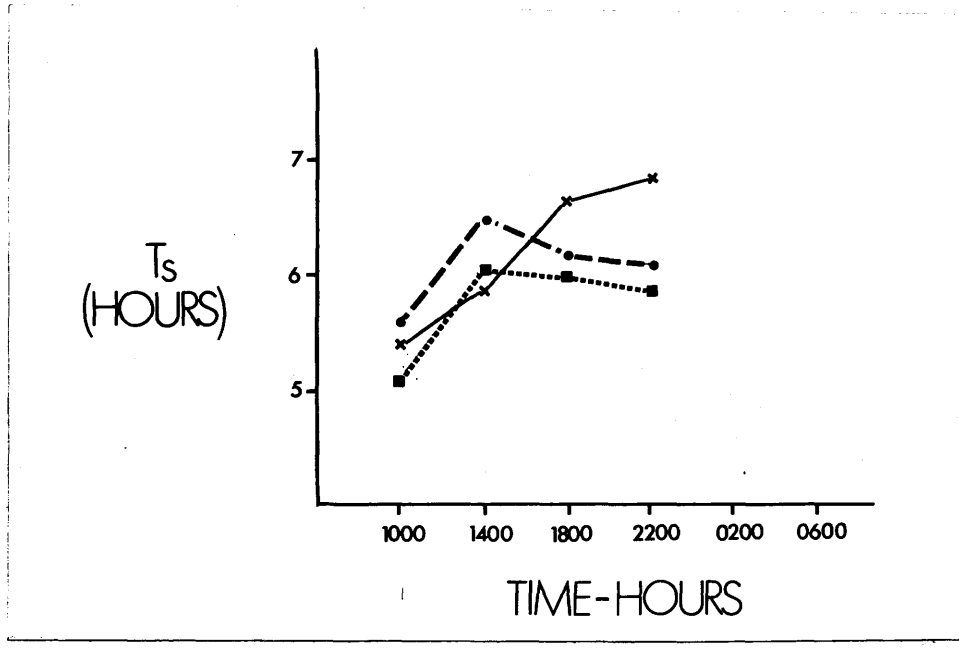


Fig. 4.18 Estimated T_s for human buccal epithelium at four time periods.

Time	No.	Single Labelled			Double Labelled				Cell Entry per Hour per 100 Cells
		T.N.C.	L.C.	$L.I._1$	T.N.C.	T.L.C.	$L.I_2$	$L.I._2 - L.I._1$	
1000	1	2328	45	1.93	4625	148	3.20	1.27	1.02
	2	3128	85	2.72	2758	91	3.30	0.58	0.46
	3	4648	93	2.00	3250	92	2.83	0.83	0.66
1400	1	2170	45	2.07	2471	90	3.64	1.57	1.26
	2	1595	45	2.82	3351	107	3.19	0.37	0.29
	3	2146	57	2.66	3087	98	3.17	0.51	0.41
1800	1	2228	64	2.87	1822	69	3.79	0.92	0.74
	2	2357	64	2.72	2483	91	3.66	0.94	0.75
	3	4008	104	2.59	2319	94	4.05	1.46	1.17
2200	1	2948	100	3.39	1835	71	3.87	0.48	0.38
	2	2674	81	3.03	2584	104	4.03	1.00	0.80
	3	3059	97	3.17	1688	64	3.79	0.62	0.50
0200	1	4064	87	2.14	3421	86	2.51	0.38	0.30
	2	4911	116	2.36	2295	53	2.31	- 0.05	0
	3	2060	44	2.14	2169	46	2.12	- 0.02	0
0600	1	3910	84	2.15	2089	63	3.01	0.86	0.69
	2	2253	41	1.82	2355	66	2.80	0.98	0.78
	3	1952	40	2.05	2328	64	2.75	0.70	0.56

Table 4.8 Rate of cell entry into S phase per 100 cells per hour estimated by the difference in labelling index by single pulse label and by two labels within 75 minutes.

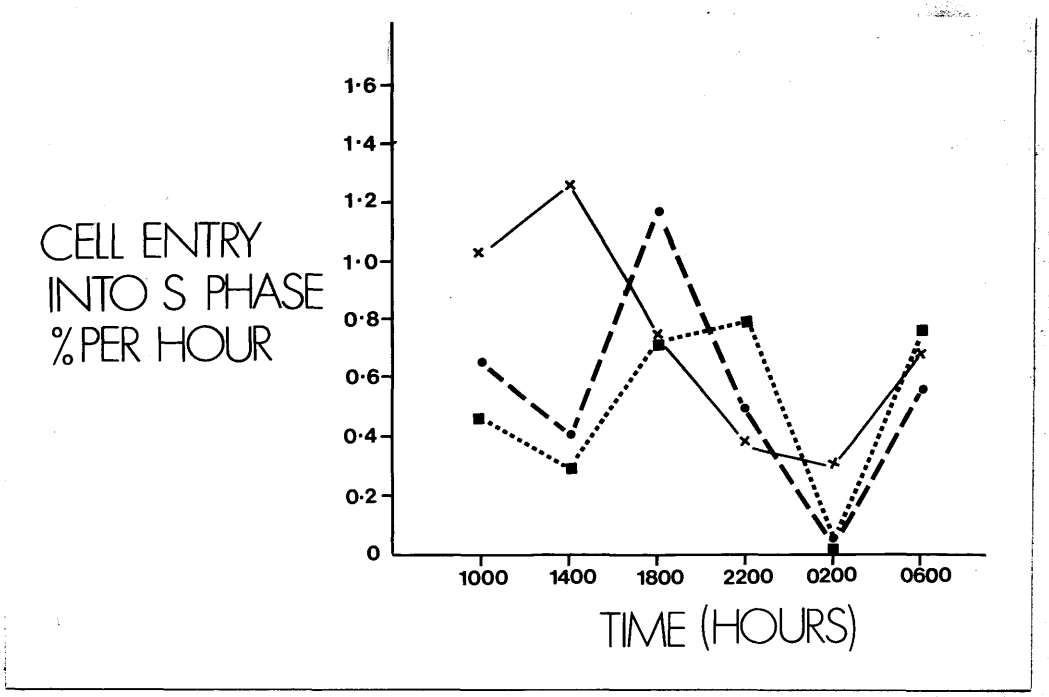


Fig. 4.19 The rates of cell entry into S phase per 100 cells per hour for the three subjects in Experiment 4A.

Time	No.	P.C.	M.C.	T.N.C.	I_s	I_{bm}	S.L. (mm)	B.M.L. (mm)	L.C.	$LC/_{TNC}$%	$LC/_{SL}$
1000	4	1536	1744	3280	55.8	77.0	4.38	6.04	126	3.84	28.75
	5	991	1066	2057	27.5	31.8	2.16	2.50	45	2.19	20.83
	6	1111	1002	2113	38.2	49.3	3.00	3.87	47	2.22	15.67
	7	1613	1189	2802	59.0	88.0	4.63	6.91	62	2.21	13.39
1400	4	1081	1073	2154	40.3	55.0	3.17	4.32	73	3.39	23.03
	5	754	1171	1925	26.8	35.8	2.11	2.81	43	2.23	20.38
	6										
	7	1835	1565	3400	76.7	97.3	6.02	7.64	80	2.35	13.29
1800	8	632	981	1613	26.3	31.0	2.07	2.43	53	3.29	25.60
	9	1221	1329	2550	40.5	53.5	3.18	4.20	48	1.88	15.09
	10	1246	1680	2926	51.1	69.2	4.02	5.43	64	2.19	15.92
	11	1886	1956	3842	51.8	67.5	4.07	5.30	127	3.31	31.20
2200	8	1251	1845	3096	52.8	59.7	4.15	4.68	85	2.75	20.48
	9	1167	1139	2306	38.7	56.0	3.04	4.39	66	2.86	21.71
	10	1733	1749	3482	52.2	89.8	4.10	7.05	119	3.42	29.02
	11	1643	1543	3186	52.2	90.0	4.10	7.07	102	3.20	24.88

Table 4.9 Labelling indices of subjects in Experiment 4B.

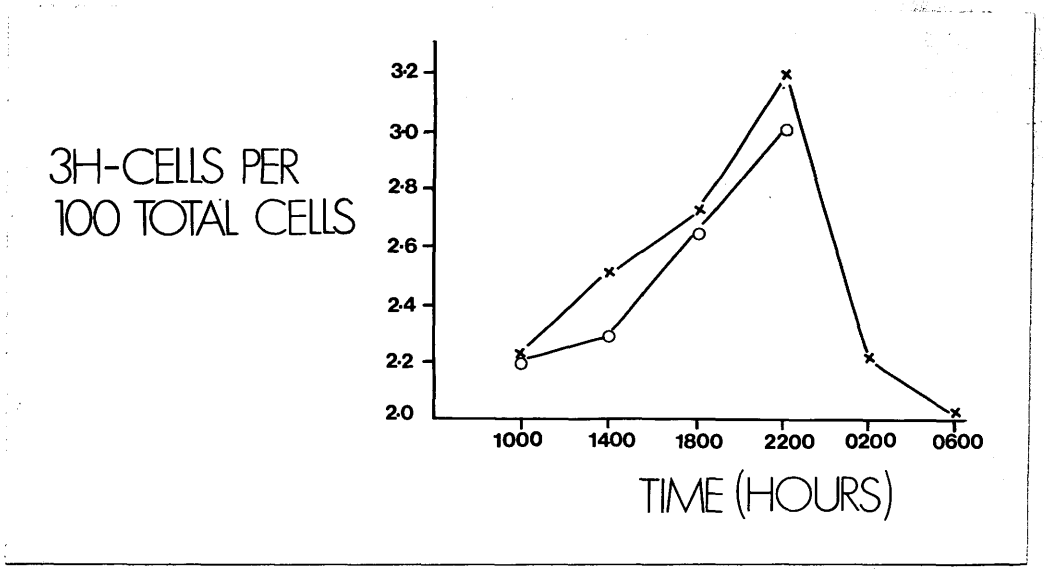


Fig. 4.20 Comparison of mean labelling indices by total cell index for the three subjects in Experiment 4A × —— × and seven subjects in Experiment 4B o —— o.

Time (Hours)	Subject No.	T.N.C.	H.L.C.	W.L.C.	T.L.C.	T_s	$L.I_2$	$L.I_2 - L.I_1$	Rate of Cell Entry % per Hour
1000	4	3036	94	19	113	4.9	3.72	-.12	-.10
	5	860	20	4	24	5.0	2.71	.53	.42
	6	3526	77	17	94	4.5	2.60	.38	.30
	7	3292	72	14	86	5.1	2.61	.40	.32
1400	4	1240	46	12	58	3.8	4.68	1.29	1.03
	5	1277	45	10	55	4.5	4.31	2.08	1.66
	6								
	7	1228	34	8	42	4.3	3.40	1.05	.84
1800	8	2833	111	20	131	5.5	4.94	1.65	1.32
	9	3357	79	11	90	7.2	2.68	.79	.63
	10								
	11	1817	58	10	68	5.8	3.74	.44	.35
2200	8	3228	84	17	101	4.9	3.13	.39	.31
	9	2057	60	13	73	4.6	3.55	.69	.55
	10								
	11	3191	96	18	114	5.3	3.57	.37	.30

Table 4.10 T_s and rate of cell entry per cent per hour for biopsies from subjects in Experiment 4B.

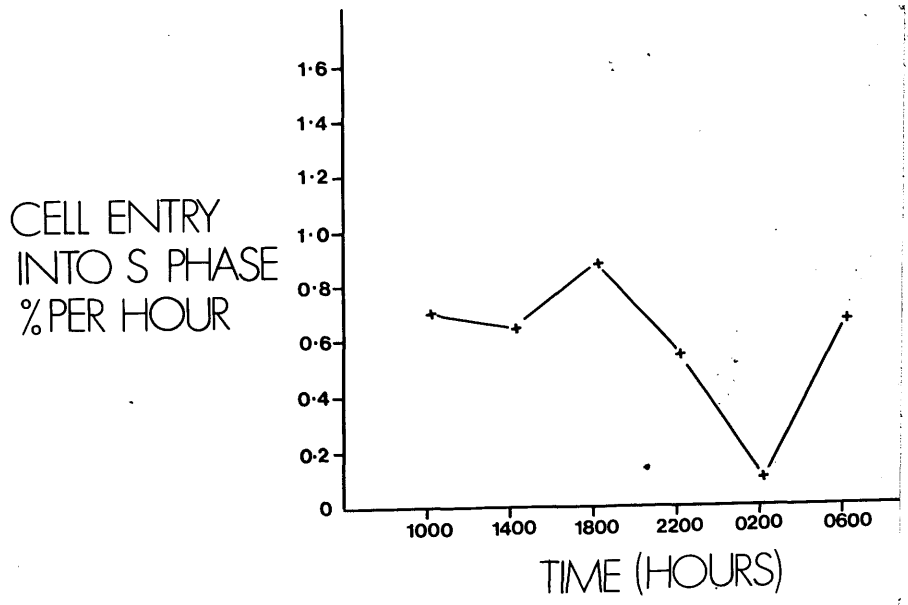


Fig. 4.21 The mean rate of cell entry into S phase per 100 cells per hour for the three subjects (Experiment 4A).

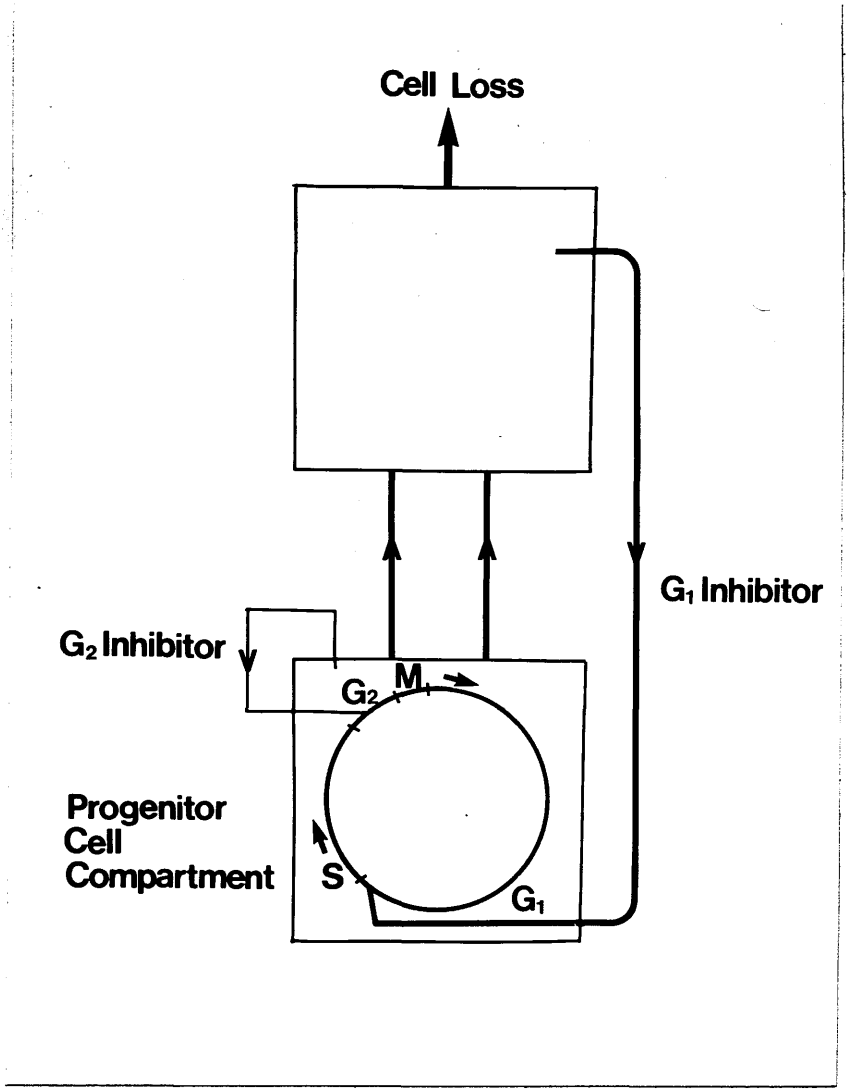
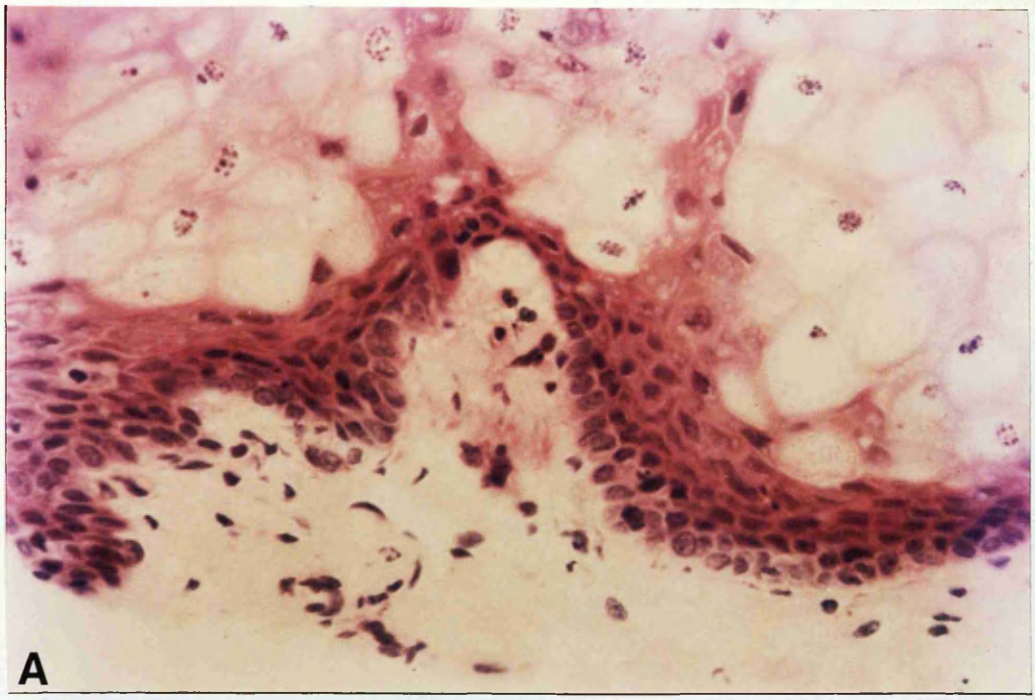


Fig. 5.1 Suggested mechanisms of control of the cell cycle in the two compartment system of normal buccal epithelium. A G_1 inhibitor is produced by mature cells and controls the entry of cells into the S phase. A G_2 inhibitor is produced by progenitor cells and acts by inhibiting the entry of cells into mitosis (modified from Elgjo, Laerum and Edgehill 1971; 1972).



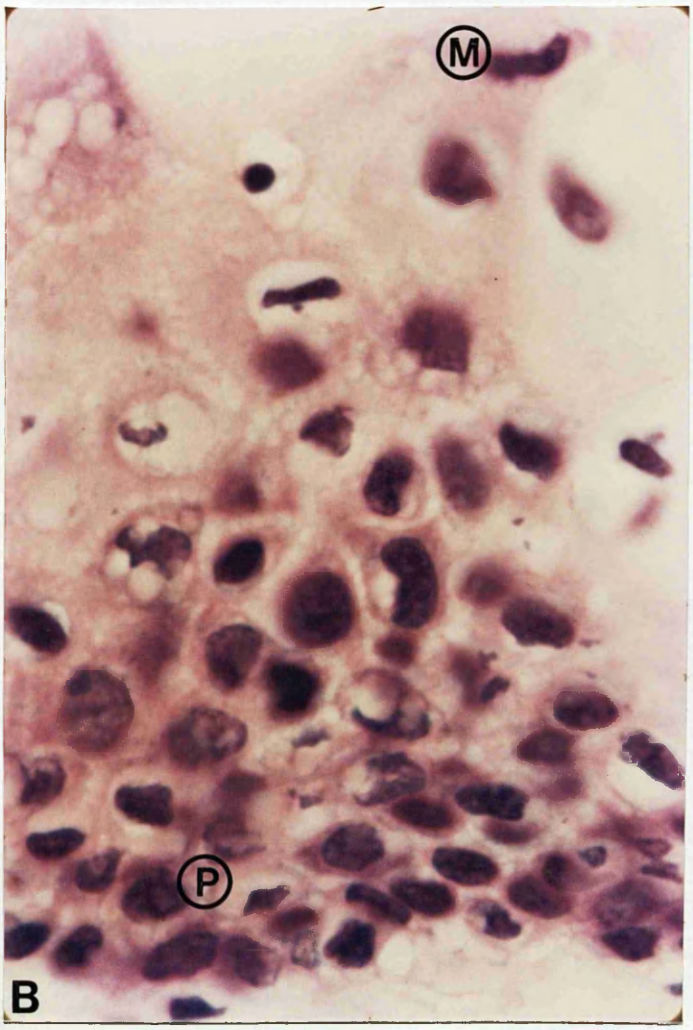


Fig. 5.2 Cell compartments in normal buccal epithelium.

A Low power photomicrograph showing the distribution of the two cell compartments.
H. & E. x 400.

B High power view of deeper cell layers.
H. & E. x 1000.
(P) Progenitor cells.
(M) Mature cells.

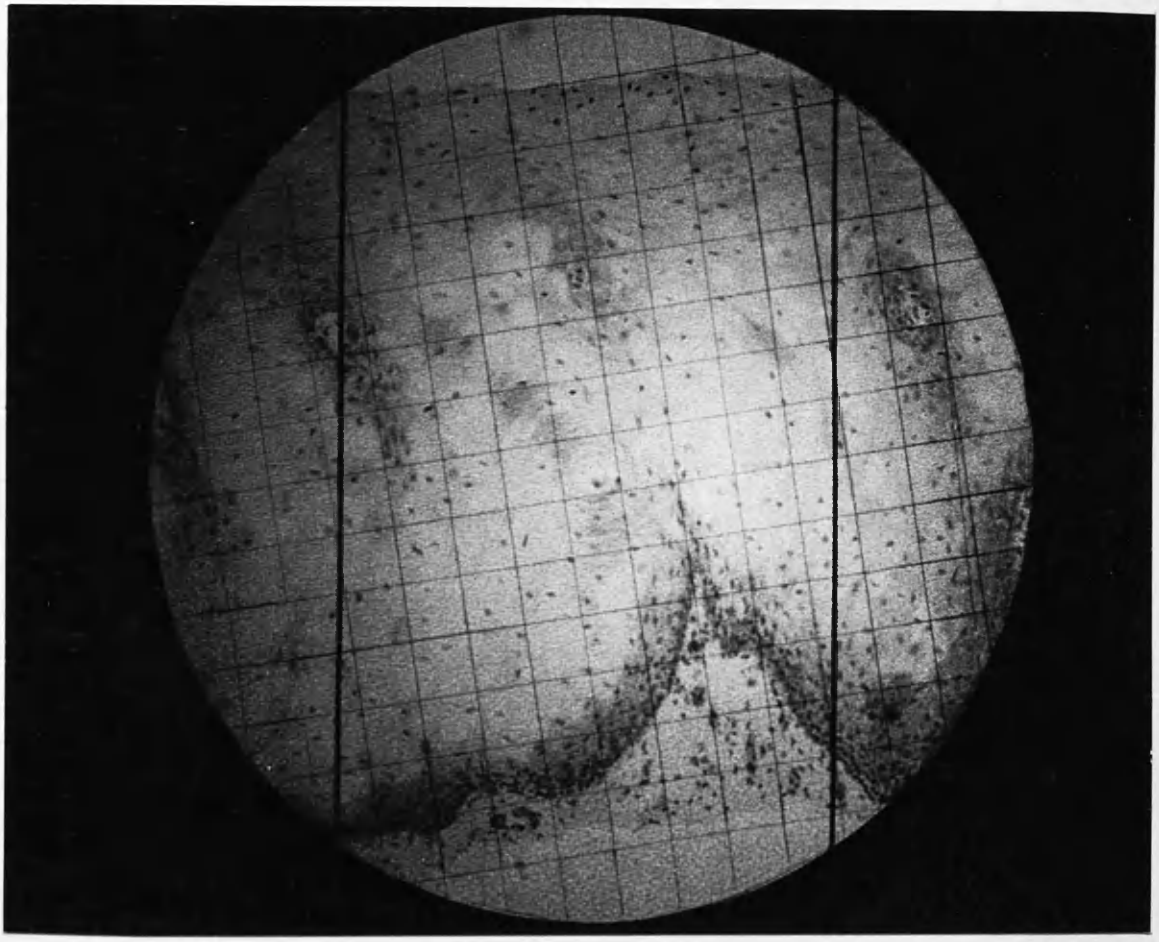


Fig. 5.3 A view of the projection head of the Leitz Ortholux microscope showing a superimposed grid for point counting. The two vertical lines define the column of epithelium used for counting.

Time	No.	P_p	P_m	P_t	P_p/P_t %
1000	1	93	431	524	17.75
	2	53	655	708	7.49
	3	85	757	842	10.10
1400	1	134	878	1012	13.24
	2	105	761	866	12.12
	3	115	674	789	14.58
1800	1	66	285	351	18.80
	2	126	1243	1369	9.20
	3	86	414	500	17.20
2200	1	77	735	812	9.48
	2	131	1340	1471	8.91
	3	74	493	567	13.05
0200	1	70	665	735	9.52
	2	76	806	882	8.61
	3	43	467	510	8.43
0600	1	113	656	769	14.69
	2	129	680	809	15.94
	3	124	436	560	22.14

Table 5. 1 Stereologic point counts in each cell compartment and the proportion of points on progenitor compartment expressed as a percentage of total points.

Time	No.	P_p	$P_p/12$	A_p mm^2 (.42mm Col)	Thickness (μm)	PC/mm	PC Size um^2
1000	1	93	7.750	.0194	46	382.8	120.7
	2	53	4.417	.0110	26	331.5	79.0
	3	85	7.083	.0177	42	300.9	140.1
1400	1	134	11.167	.0279	66	498.5	133.3
	2	105	8.750	.0219	52	344.1	151.5
	3	115	9.583	.0240	57	310.7	183.9
1800	1	66	5.50	.0138	32	365.3	89.9
	2	126	10.50	.0263	62	426.5	146.8
	3	86	7.167	.0179	42	278.8	152.9
2200	1	77	6.417	.0160	38	567.0	67.2
	2	131	10.917	.0273	65	573.6	113.3
	3	74	6.167	.0154	36	434.0	84.5
0200	1	70	5.833	.0146	34	376.1	92.4
	2	76	6.333	.0158	37	406.1	92.6
	3	43	3.583	.0090	21	290.2	73.8
0600	1	113	9.417	.0235	56	519.5	107.7
	2	129	10.750	.0269	64	501.6	127.7
	3	124	10.333	.0258	61	356.3	172.4

Table 5.2 Quantitative features on progenitor compartment thickness and cell size in normal human buccal epithelium.

Time	No.	P_m	$P_m/12$	Am mm^2 (.42 mm Column)	Thickness um	MC/mm	MC Size μm^2
1000	1	431	35.917	.0898	214	414.4	515.9
	2	655	54.583	.1365	325	351.5	924.6
	3	757	63.083	.1577	376	344.9	1088.7
1400	1	878	73.167	.1829	436	412.6	1055.5
	2	761	63.417	.1585	377	312.3	1208.4
	3	674	56.167	.1404	334	288.8	1157.5
1800	1	285	23.750	.0594	141	257.0	550.1
	2	1243	103.583	.2590	618	439.7	1402.5
	3	414	34.500	.0863	205	393.0	522.6
2200	1	735	61.250	.1531	365	530.0	687.7
	2	1340	111.667	.2792	665	550.0	1208.7
	3	493	41.083	.1027	245	360.9	677.4
0200	1	665	55.417	.1385	330	276.4	1193.1
	2	806	67.167	.1679	400	300.0	1332.6
	3	467	38.917	.0973	232	211.2	1097.0
0600	1	656	54.500	.1363	325	450.9	719.6
	2	680	56.667	.1417	337	405.6	831.8
	3	436	36.333	.0908	216	213.1	1014.5

Table 5.3 Quantitative features of maturation compartment thickness and cell size.

Time	No.	P_t	Thickness (μm)
1000	1	524	260
	2	708	351
	3	842	418
1400	1	1012	502
	2	866	429
	3	789	391
1800	1	351	174
	2	1369	679
	3	500	248
2200	1	812	403
	2	1471	730
	3	567	281
0200	1	735	365
	2	882	438
	3	510	253
0600	1	769	386
	2	809	448
	3	560	278

Table 5.4 Mean thickness of human buccal epithelium in 18 biopsies taken from three subjects at six times of the day.

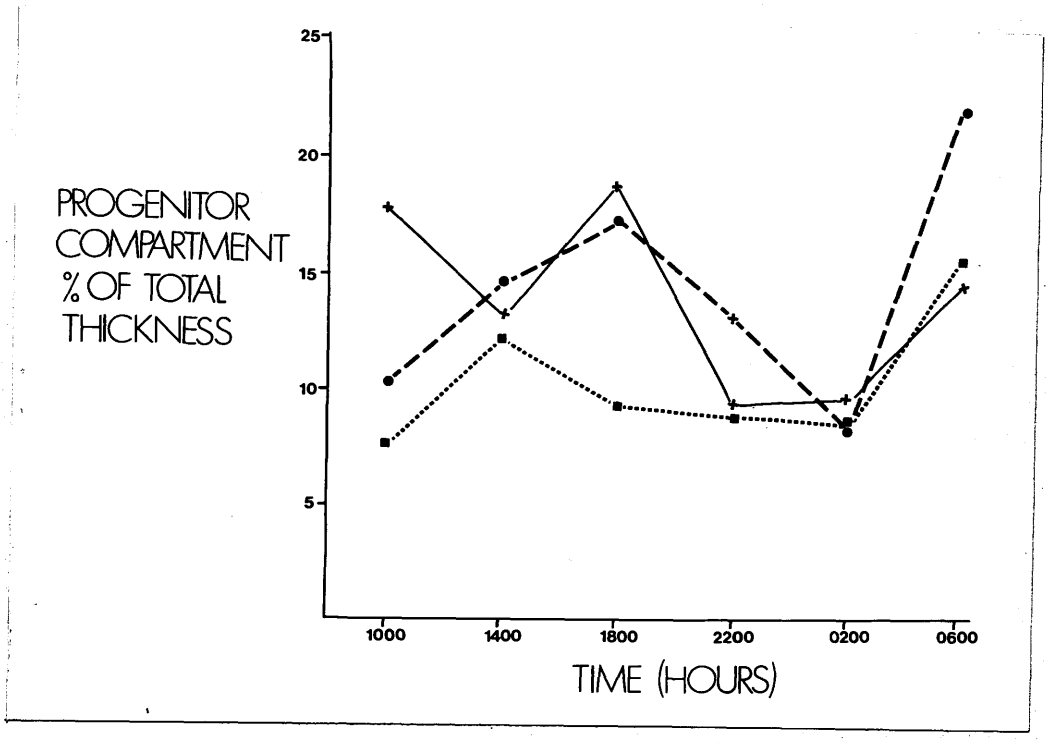


Fig. 5.4 Thickness of progenitor compartment at six time periods.

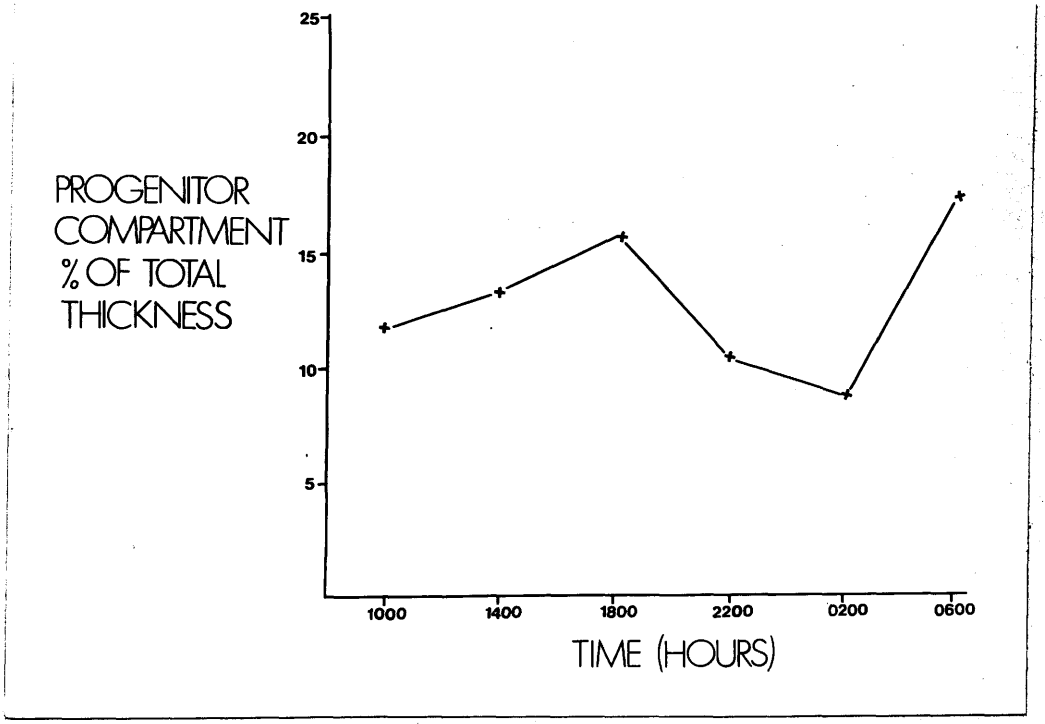


Fig. 5.5 Progenitor compartment - mean values for the three subjects of the proportion of total thickness of epithelium formed by the progenitor compartment.

	CONDITIONS					
	Morning		Afternoon		Night	
		Rank		Rank		Rank
	14•69	3	13•24	2	9•48	1
	15•94	3	12•12	2	8•91	1
	22•14	3	14•58	2	13•05	1
	17•75	2	18•80	3	9•52	1
	7•49	1	9•20	3	8•61	2
	10•10	2	17•20	3	8•43	1
R_j =		14		15		7

$$X_r^2 = \frac{12}{Nk\,(k+1)} \sum_{j=1}^{k} (R_j)^2 - 3N\,(k+1)$$

$$= \frac{12}{(6)\,(3)\,(4)} \left[(14)^2 + (15)^2 + (7)^2\right] - (3)(6)(4)$$

$$= 6{\cdot}3$$

$P < 0{\cdot}05$

Table 5.5 Friedman two way analysis of variance for the proportions of epithelium formed by progenitor compartment.
Siegel (1956).

Time	No.	$C_{P.C.}$	$C_{M.C.}$	$C_{T.N.C.}$	$C_{P.C.}/C_{T.N.C.}$ %
1000	1	457	426	883	51.76
	2	600	620	1220	49.18
	3	767	867	1634	46.94
1400	1	441	325	766	57.57
	2	305	283	588	51.87
	3	389	349	738	52.71
1800	1	496	328	824	60.19
	2	410	383	793	51.70
	3	621	822	1443	43.04
2200	1	613	508	1121	54.68
	2	498	448	946	52.64
	3	688	514	1202	57.24
0200	1	946	599	1545	61.23
	2	1051	746	1797	58.49
	3	429	299	728	58.93
0600	1	804	605	1409	57.06
	2	451	321	772	58.42
	3	462	249	711	64.98

Table 5.6 Proportions of cell compartments by corrected cell numbers.

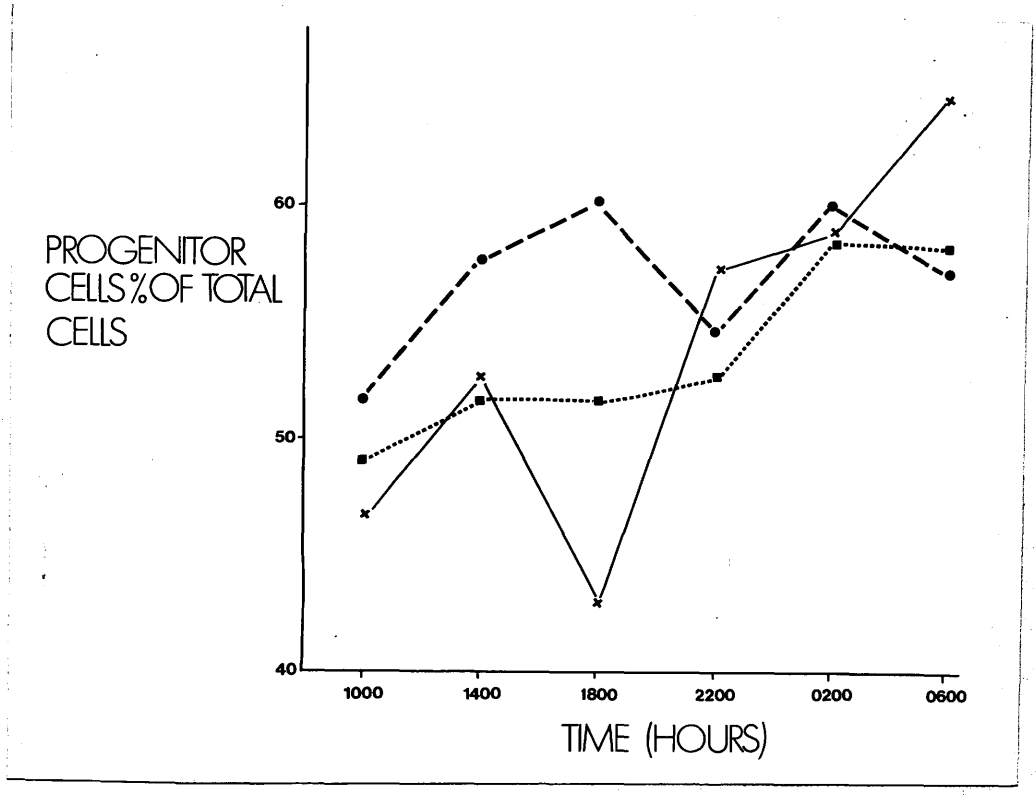


Fig. 5.6 Cell numbers in progenitor compartment at six times of the day expressed as a percentage of total cell numbers.

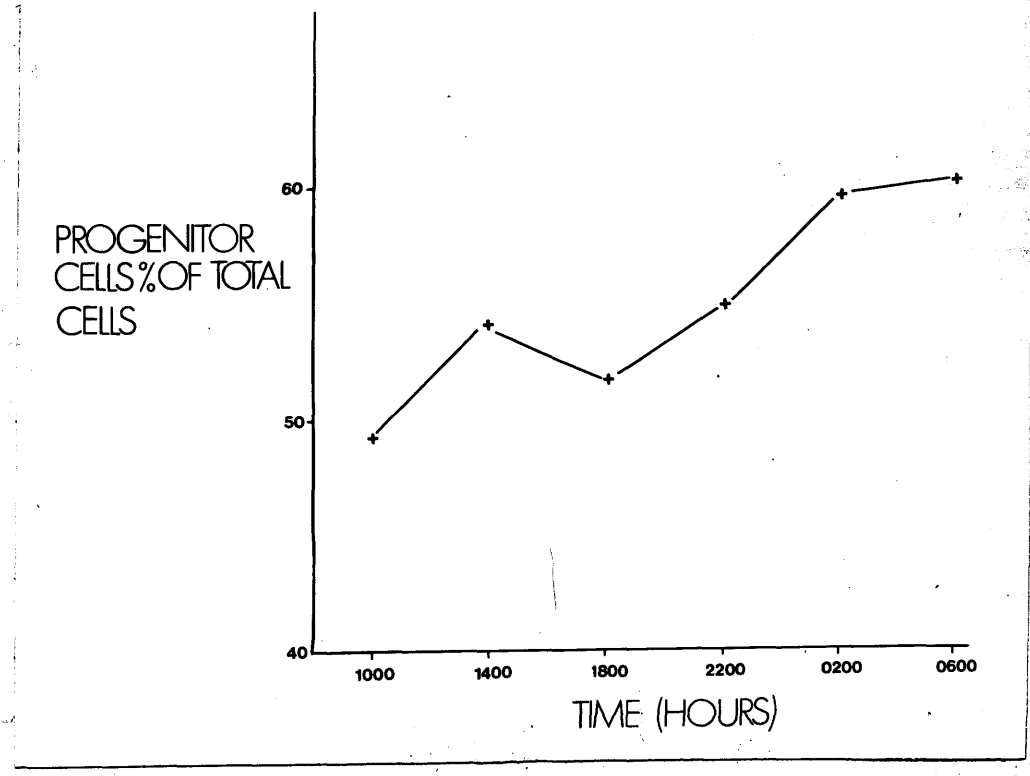


Fig. 5.7 Mean values of progenitor cell numbers for the three subjects.

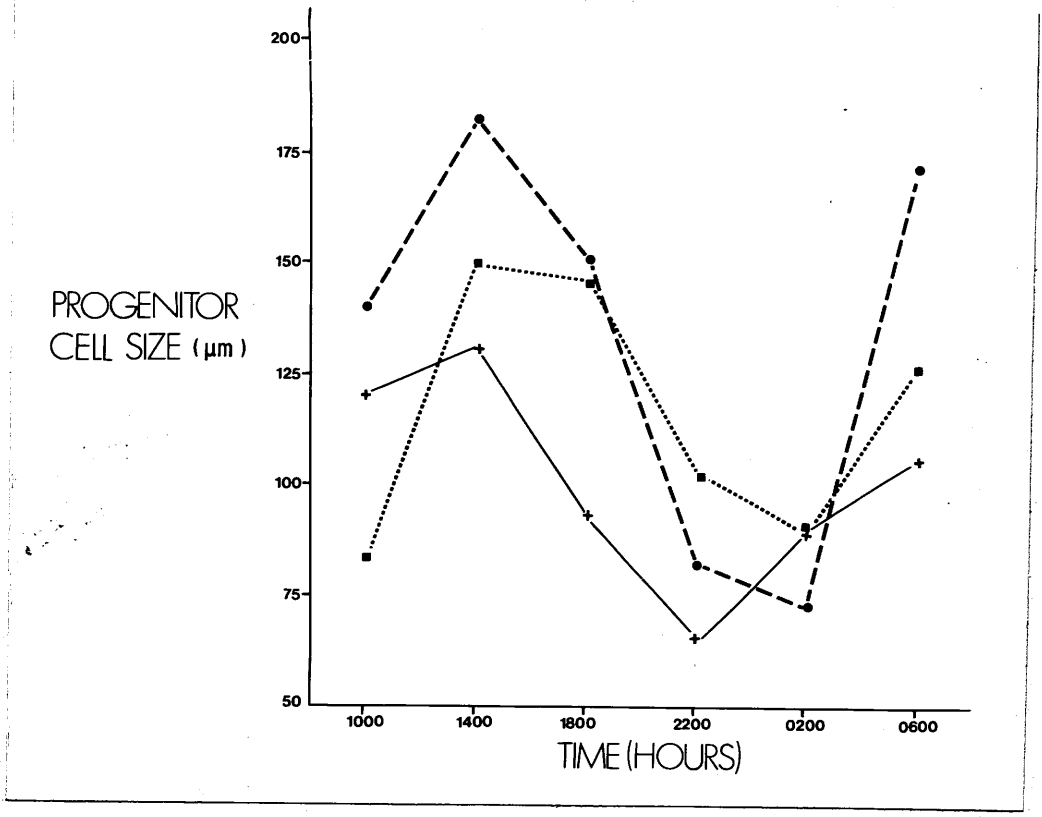


Fig. 5.8 Mean section area of progenitor cells (cell size) of each subject at different times (data in Table 5.2).

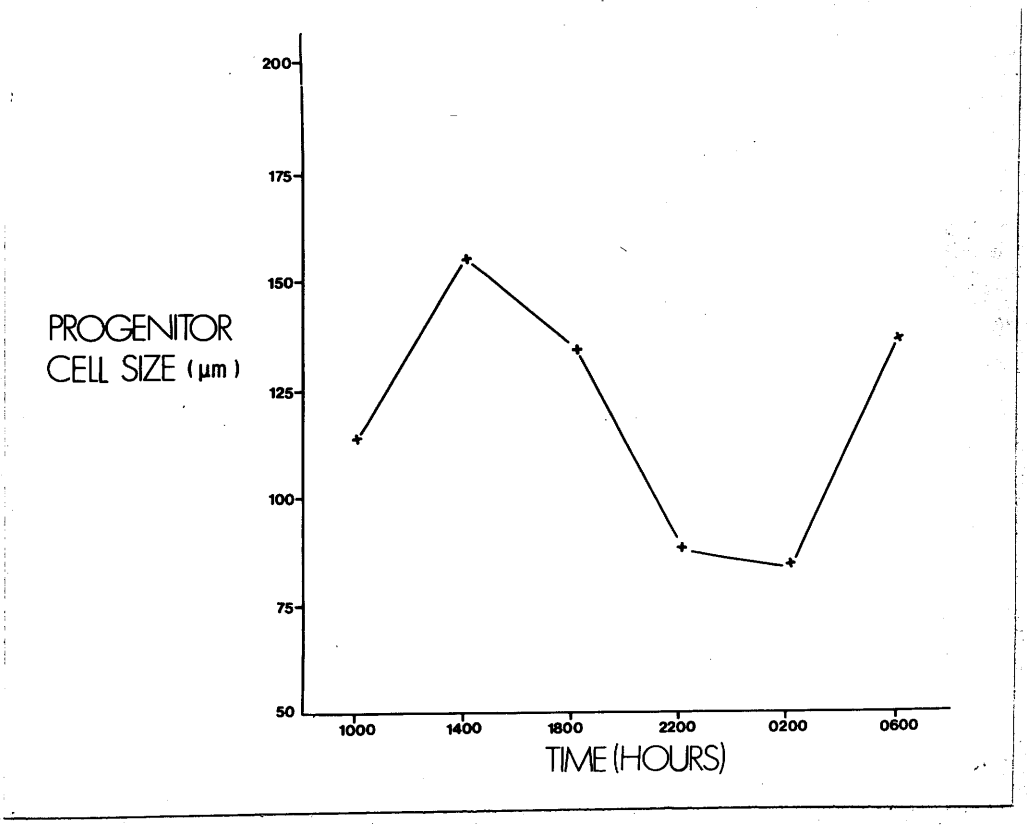


Fig. 5.9 Mean progenitor cell size values for three subjects shown individually in Fig. 5.8.

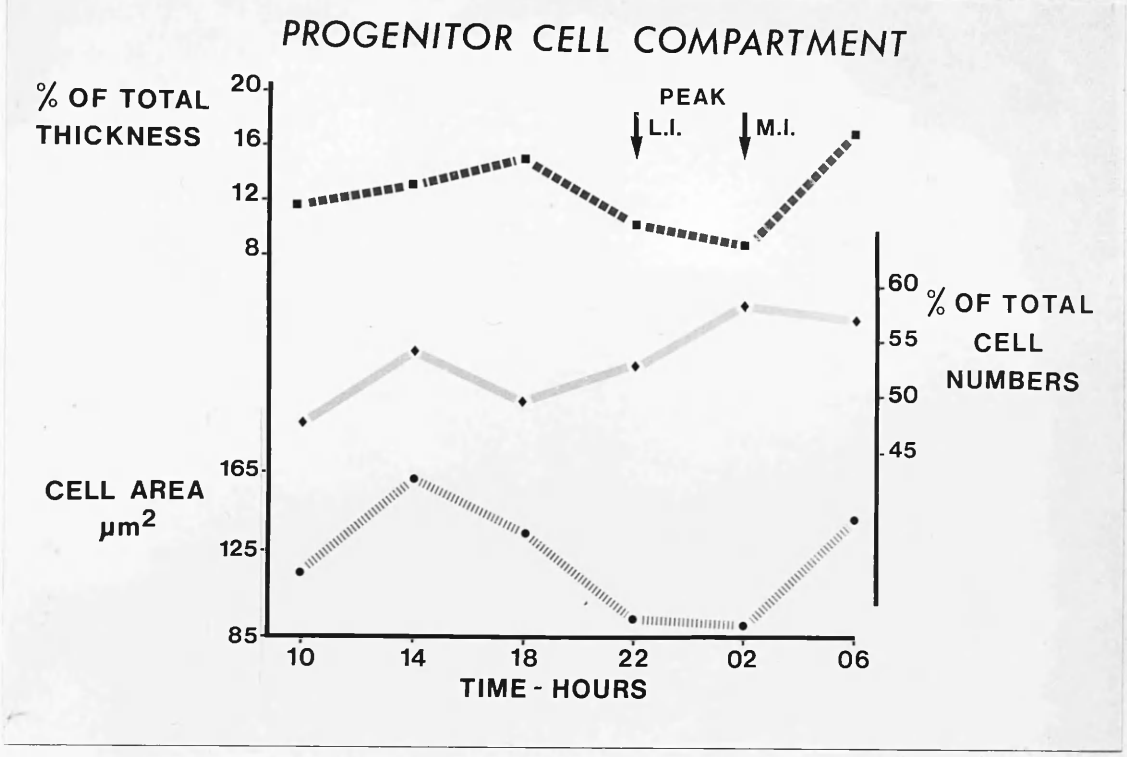


Fig. 5.10 Comparison of means of percentage total thickness, percentage of cell numbers and section area for progenitor cells at six time periods (data from Table 5.7).

Time	L1/SL	P_p/P_t %	$P/_T$ Cell Nos. %	P.C. Size (um^2)	M.C. Size (um^2)
1000	15.62	11.78	47.71	113.3	843.1
1400	17.77	13.31	53.99	156.2	1140.5
1800	19.63	15.07	49.84	129.9	825.1
2200	32.24	10.48	52.52	88.3	857.9
0200	13.77	8.85	57.68	86.3	1207.6
0600	16.34	17.59	57.14	135.9	855.3

Table 5.7 Mean values from Experiment 4A and cell compartment study (Experiment 5).

Patient No.	Sex	Age (years)	Time of Biopsy
P1	M	56	1500
P2	F	70	1620
P3	F	54	1130
P4	M	61	1115
P5	F	41	0950
P6	F	57	1445
P7	F	77	1030
P8	M	62	1620

Table 6.1 Age, sex and times of biopsy of patients included in Experiment 6.

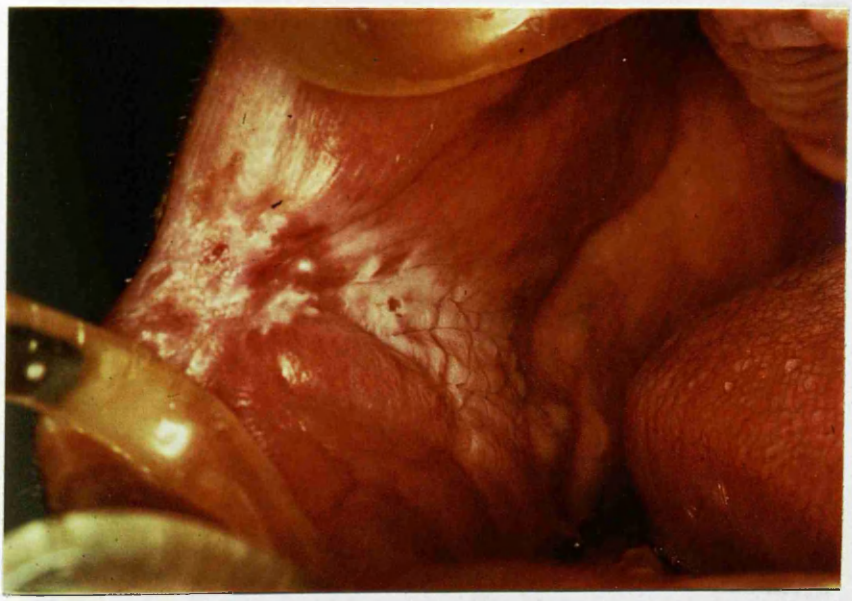


Fig. 6.1 Leukoplakia lesion in right buccal mucosa in patient (P1) - Clinically diagnosed as 'speckled leukoplakia'.

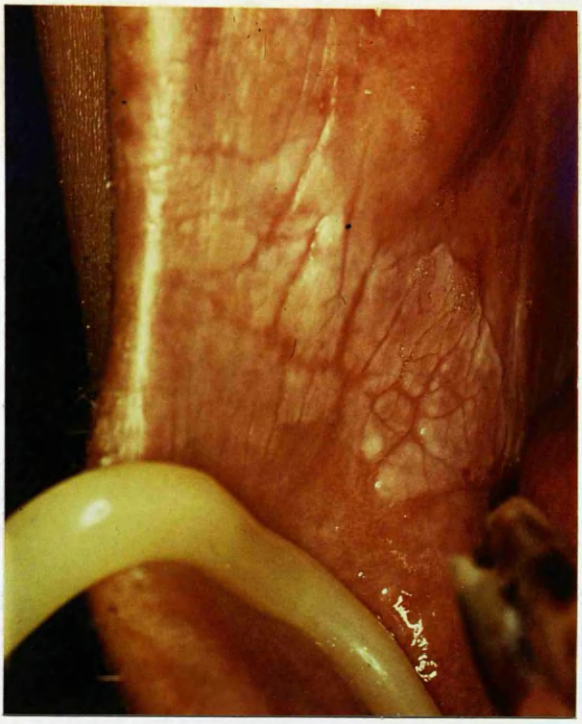


Fig. 6.2 'Homogenous leukoplakia' in patient (P8).

Fig. 6.3 A punch biopsy taken from leukoplakia lesions in patient (P3).

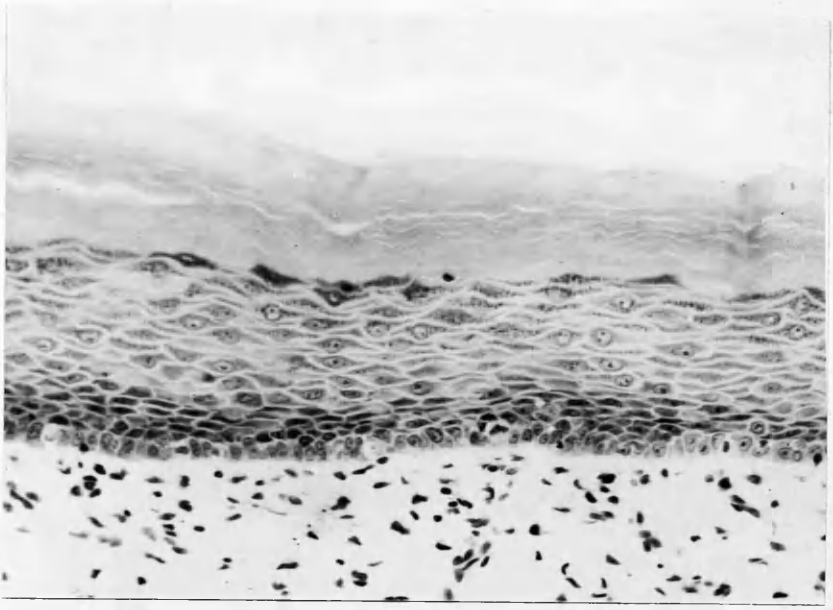


Fig. 6.4 Photomicrograph of human buccal mucosa from a leukoplakia lesion (P2) showing extensive keratinization and atrophy of the epithelium. H. and E., x 312·5.

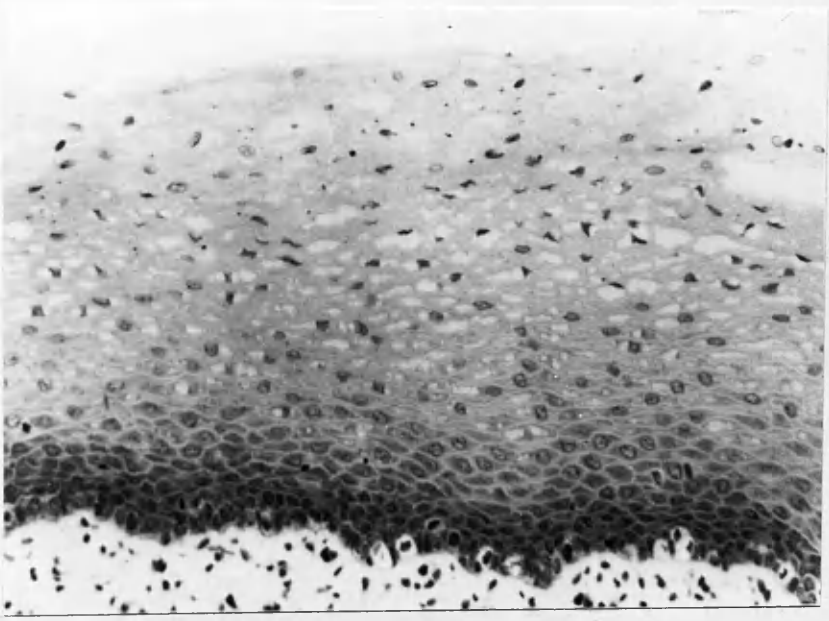


Fig. 6.5 Non-keratinized human buccal epithelium from the control site in patient (P2) photographed at the same magnification as Fig. 6.4. H. and E., x 312·5.

Patient No.	Keratinization		Candida		Atypia Score	
	Control	Leuko	Control	Leuko	Control	Leuko
P1	NK	OK	-	-	13	30
P2	NK	OK	-	-	10	9
P3	NK	OK	-	-	6	18
P4	NK	MK	-	-	9	17
P5	NK	PK	-	-	8	18
P6	NK	OK	-	-	2	21
P7	NK	OK	-	+	8	28
P8	NK	OK	-	-	16	32

Table 6.2 Histological findings of keratinization and candidal invasion in the epithelium of control and leukoplakia biopsies. The atypia score was obtained by the Smith and Pindborg technique and is out of a maximum value of 75.

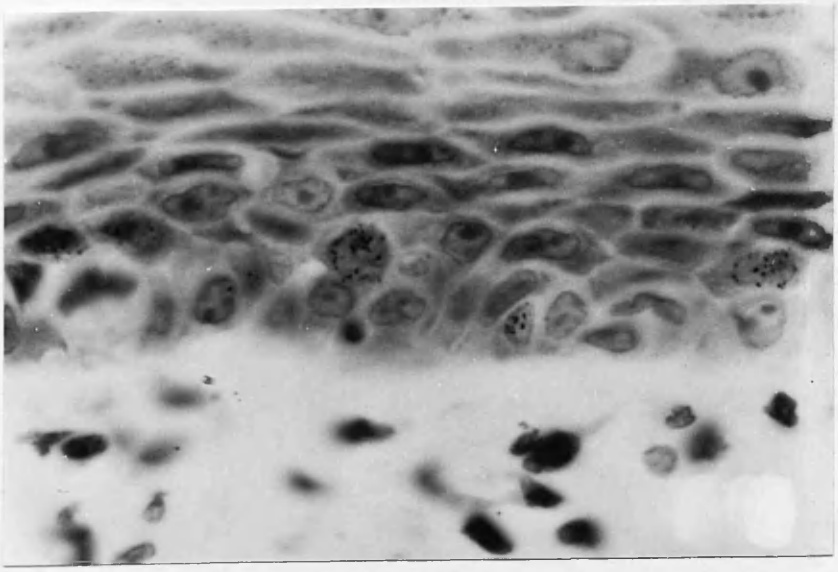


Fig. 6.6 Autoradiograph prepared from a leukoplakia biopsy following ^{3}H thymidine labelling in vitro (single pulse of 1 μCi/ml of medium). Five labelled cells are shown.

Patient No.	P.C.	M.C.	T.N.C.	L.C.	I_s	I_{bm}	S.L. (mm)	B.M.L. (mm)
P1	2685	3262	5947	179	78.33	121.50	6.157	9.550
P2	1243	1980	3223	61	52.83	56.33	4.153	4.428
P3	2289	1996	4283	73	65.17	109.00	5.122	8.567
P4	1429	1461	2855	137	49.67	86.67	3.904	6.969
P5	1454	1173	2627	98	53.67	65.50	4.218	5.148
P6	1500	1938	3438	116	52.17	66.67	4.100	5.240
P7	1145	964	2109	98	52.50	60.83	4.127	4.781
P8	1289	1628	2917	80	53.17	62.33	4.179	4.899

Table 6.3 Estimations of reference units in the epithelium from control biopsies by cell counting and stereology.

Patient No.	P.C.	M.C.	T.N.C.	L.C.	I_s	I_{bm}	S.L. (mm)	B.M.L. (mm)
P1	1963	2214	4177	189	51.67	99.33	4.061	7.808
P2	1252	1390	2642	137	78.33	78.16	6.157	6.144
P3	1266	1389	2655	148	43.16	66.83	3.392	5.253
P4	1206	1050	2256	171	51.16	77.00	4.021	6.052
P5	1217	898	2115	150	53.50	66.83	4.205	5.253
P6	997	1059	2056	88	68.33	59.67	5.370	4.690
P7	1772	1447	3219	189	50.50	121.33	3.969	9.537
P8	1023	921	1994	109	53.33	54.12	4.199	4.256

Table 6.4 Comparable data as in Table 6.3 for Leukoplakia biopsies.

Patient No	L C.% Total Cells		L.C.% Prog. Cells		L.C./mm Surface		L.C./mm B.M.	
	Control	Leuko	Control	Leuko	Control	Leuko	Control	Leuko
P1	3.01	4.52	6.67	9.63	29.07	46.54	18.74	24.21
P2	1.89	5.19	4.91	10.94	14.69	22.25	13.78	22.30
P3	1.70	5.57	3.19	11.69	14.25	43.63	8.52	28.17
P4	4.80	7.58	9.59	14.18	35.09	42.53	19.66	28.26
P5	3.73	7.09	6.74	12.32	23.23	35.67	19.04	28.56
P6	3.37	4.28	7.73	8.83	28.29	16.39	22.14	18.76
P7	4.65	5.87	8.56	10.66	23.78	47.62	20.50	19.82
P8	2.74	5.60	6.21	10.66	19.14	26.01	16.33	25.61
$\bar{X}$	3.24	5.71	6.70	11.11	23.44	35.08	17.34	24.46
SD	1.14	1.15	2.02	1.65	7.28	12.03	4.39	3.87

Table 6.5 Estimated labelling indices for control and leukoplakia biopsies by the four reference index units.

Patient No.	$P.C._{ND}$	$C.F._{p}$	$M.C._{ND}$	$C.F._{m}$	$C_{P.C.}$	$C_{M.C.}$	$C_{L.C.}$	$C_{T.N.C.}$	$C_{LC/TNC}$%
P1	6.09	.365	7.15	.329	980	1073	65.34	2053	3.18
P2	6.62	.345	6.43	.352	429	697	21.05	1126	1.87
P3	4.65	.429	7.16	.328	982	655	31.32	1637	1.91
P4	6.63	.346	7.46	.319	494	452	47.40	946	5.01
P5	5.83	.375	6.64	.345	545	405	36.75	950	3.87
P6	7.44	.320	8.45	.293	480	568	37.12	1048	3.54
P7	6.30	.357	7.26	.325	409	313	34.99	736	4.75
P8	5.06	.409	6.55	.348	527	567	32.72	1094	2.99
$\bar{x}$	6.08	$\bar{x}$	7.14						$\bar{x}$ 3.39
SD	0.90	SD	0.65						SD 1.16

Table 6.6 Control biopsies - Derivation of correction factors for the progenitor and mature cells using the estimated nuclear diameters in control biopsies. The corrected values and the labelling index by corrected cell index is shown.

Patient No	$P.C_{ND}$	$C.F._{p}$	$M.C_{ND}$	$C.F._{m}$	$C_{P.C.}$	$C_{M.C.}$	$C_{L.C.}$	$C_{T.N.C.}$	$C_{LC/TNC}$%
P1	6.05	.368	7.36	.322	722	713	69.55	1435	4.85
P2	6.69	.343	9.19	.276	429	384	46.99	813	5.78
P3	6.66	.344	7.64	.314	436	436	50.91	872	5.84
P4	6.53	.349	6.78	.340	421	357	59.68	778	7.67
P5	6.50	.350	9.10	.278	426	250	52.50	676	7.77
P6	6.28	.358	8.25	.298	355	316	31.32	671	4.67
P7	6.33	.356	6.60	.347	631	502	67.28	1133	5.94
P8	9.34	.273	9.11	.278	279	257	29.75	538	5.53
$\bar{x}$	6.80	$\bar{x}$	8.00						$\bar{x}$ 6.01
SD	1.05	SD	1.06						SD 1.15

Table 6.7 Leukoplakia biopsies - Derivation of correction factors for progenitor and mature cells using the estimated nuclear diameters. The corrected total cell labelling index is shown.

Patient No	^{c}L.C.% ^{c}Total Cells		^{c}L.C.% ^{c}Prog. Cells		^{c}L.C./mm Surface		^{c}L.C./mm B.M.	
	C	L	C	L	C	L	C	L
P1	3.18	4.85	6.67	9.63	10.61	17.13	6.84	8.91
P2	1.87	5.78	4.91	10.95	5.07	7.63	4.75	7.65
P3	1.91	5.84	3.19	11.67	6.11	15.01	3.66	9.69
P4	5.01	7.67	9.59	14.18	12.14	14.84	6.80	9.86
P5	3.87	7.77	6.74	12.32	8.71	12.49	7.14	9.99
P6	3.54	4.67	7.73	8.82	9.05	5.83	7.08	6.68
P7	4.75	5.94	8.56	10.66	8.48	16.95	7.32	7.05
P8	2.99	5.53	6.21	10.66	7.83	7.09	6.68	6.99
$\bar{X}$	3.39	6.01	6.69	11.11	8.50	12.12	6.28	8.35
SD	1.16	1.15	2.02	1.65	2.27	4.62	1.33	1.41

Table 6.8 Corrected labelling indices for control and leukoplakia biopsies following the correction for nuclear fragments.

PATIENT NO.	LABELLING INDEX CONTROL	LABELLING INDEX LEUKOPLAKIA	d	RANK OF d	RANK WITH LESS FREQUENT SIGN
1	3•18	4•85	+1•67	3	
2	1•87	5•78	+3•91	7	
3	1•91	5•84	+3•93	8	
4	5•01	7•67	+2•66	5	
5	3•87	7•77	+3•90	6	
6	3•54	4•67	+1•13	1	
7	4•75	5•94	+1•19	2	
8	2•99	5•53	+2•54	4	
					T = 0
					P < 0•005

Table 6.9 Comparison of labelling index data for controls and leukoplakias. Wilcoxon matched-pairs signed-ranks test (One Tailed)

Siegel (1956).

Patient No.	^{3}H - Cells in Controls				^{3}H - Cells in Leukoplakia			
		Suprabasal				Suprabasal		
	Basal	2 - 3	3 +	Total	Basal	2 - 3	3 +	Total
P1	29	137	13	179	45	110	34	189
P2	20	40	1	61	42	93	2	137
P3	27	38	8	73	74	59	15	148
P4	38	85	14	137	65	94	12	171
P5	24	67	7	98	84	60	6	150
P6	24	89	3	116	34	52	2	88
P7	19	73	6	98	71	99	19	189
P8	36	40	4	80	53	55	1	109

Table 6.10 Comparison of sites of labelled cells in controls and leukoplakia.

	BASAL	SUPRABASAL		TOTAL
	1	2 - 3	3 +	
CONTROL	217 (25.7%)	569 (67.6%)	56 (6.7%)	842
LEUKOPLAKIA	468 (39.6%)	622 (52.7%)	91 (7.7%)	1181

Table 6.11 Comparison of sites of labelled cells in control and leukoplakia.
Table shows distribution of labelled cells in each category and the percentages.
Chi square test.

Site of labelled cell	Basal	Suprabasal
Control	217	625
Leukoplakia	468	723

$x^2 = 40{\cdot}37$ $P = 0{\cdot}016$

Site of labelled cell	Deepest 3	Superficial to 3rd
Control	786	56
Leukoplakia	1090	91

$x^2 = 0{\cdot}81$ $P = 0{\cdot}567$

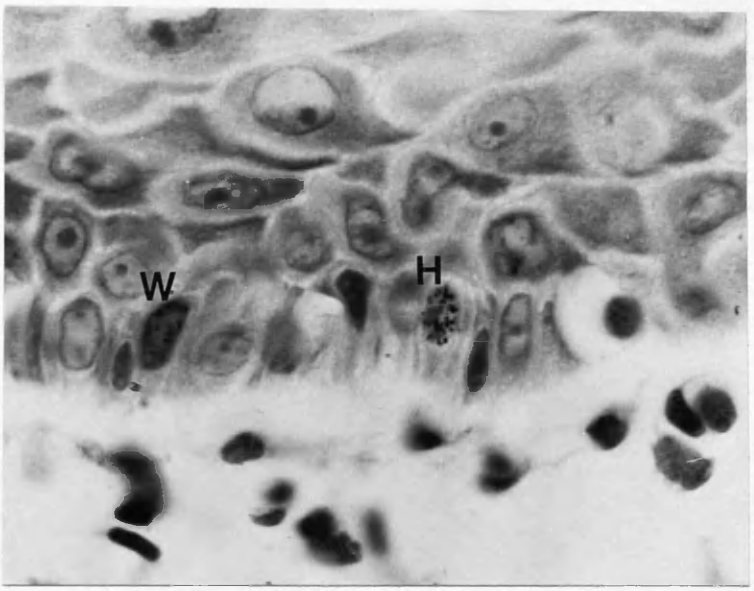


Fig. 6.7 An autoradiograph following double labelling in a leukoplakia lesion H. and E., x 1250.
H - Heavily labelled cell; W - Weakly labelled cell shows evidence of dense nuclear staining suggestive of being in G_2 phase.

Patient No.	CONTROL			LEUKOPLAKIA		
	H.L.C.	W.L.C.	T_s	H.L.C.	W.L.C.	T_s
P1	248	35	7.1	379	61	6.2
P2	58	11	5.3	53	7	7.6
P3	167	21	8.0	350	49	7.1
P4	121	22	5.5	183	29	6.3
P5	147	24	6.1	123	20	6.2
P6	158	28	5.6	118	19	6.2
P7	75	17	4.4	121	21	5.8
P8	141	24	5.9	118	23	5.1
			$\bar{x}$ 6.0			$\bar{x}$ 6.3
			SD 1.1			SD 0.8

Table 6.12 Counts of heavily and weakly labelled cells in control and leukoplakia lesions. T_s estimations are shown.

Patient No.	P.C.	M.C.	T.N.C.	HLC + WLC TLC	$L.I._2$	$L.I._1$	$L.I._2 - L.I._1$	Rate of Cell Entry % per Hour
P1	2724	3032	5756	283	4.92	3.01	1.91	1.53
P2	754	1461	2215	69	3.12	1.89	1.23	0.98
P3	1749	1666	3415	188	5.51	1.70	3.81	3.05
P4	1314	1341	2655	143	5.38	4.80	0.58	0.46
P5	1127	1006	2133	171	8.01	3.73	4.28	3.42
P6	1154	1178	2332	186	7.97	3.37	4.60	3.68
P7	1058	976	2034	92	4.52	4.64	- .12	- .10
P8	1324	1338	2662	165	6.20	2.74	3.46	2.77

Table 6.13 Rate of cell entry into S phase in controls.

Patient No.	P.C.	M.C.	T.N.C.	HLC + WLC TLC	$L.I._2$	$L.I._1$	$L.I._2 - L.I._1$	Rate of Cell Entry % per Hour
P1	2785	3096	5881	440	7.48	4.53	2.95	2.36
P2	603	698	1301	61	4.69	5.18	- .49	- .39
P3	3024	1686	4710	399	8.47	5.57	2.90	2.32
P4	1418	1435	2853	212	7.43	7.58	- .15	- .12
P5	1103	1024	2127	143	6.72	7.09	- .37	- .30
P6	800	1112	1912	137	7.16	4.28	2.88	2.30
P7	1264	1391	2655	142	5.35	5.87	- .52	- .42
P8	1139	1003	2142	141	6.58	5.61	.98	.78

Table 6.14 Rate of cell entry into S phase in leukoplakia specimens.

PAIR	RATE-CELL ENTRY % PER HOUR		d	RANK OF d	RANK WITH LESS FREQUENT SIGN
	Control	Leukoplakia			
1	1•53	2•36	−0•83	4	4
2	0•98	−0•39	+1•37	5	
3	3•05	2•32	+0•73	3	
4	0•46	−0•12	+0•58	2	
5	3•42	−0•30	+3•72	8	
6	3•68	2•30	+1•38	6	
7	−0•10	−0•42	+0•32	1	
8	2•77	0•78	+1•99	7	

T = 4

P < 0•05

Table 6.15 Comparison of rate of cell entry in leukoplakia with matched controls. Wilcoxon matched-pairs signed-ranks test (Two-Tailed) P < 0•05.
Siegel (1956).

Patient No.	CONTROL					LEUKOPLAKIA				
	P_t	A_t (mm^2)	Columns of each .48 mm	Estimated S.L. (mm)	Thickness (um)	P_t	A_t(mm^2)	Columns of each .48 mm	Estimated S.L. (mm)	Thickness (um)
P1	1125.0	2.813	12	5.76	488	525.7	1.314	8	3.84	342
P2	411.0	1.028	8	3.84	268	357.3	.893	12	5.76	155
P3	942.8	2.357	10	4.80	491	352.2	.880	9	4.32	203
P4	560.0	1.400	8	3.84	365	472.2	1.180	8	3.84	307
P5	459.2	1.148	8	3.84	299	287.8	.720	8	3.84	188
P6	923.7	2.309	8	3.84	601	692.3	1.731	8	3.84	451
P7	316.5	.791	8	3.84	206	538.7	1.347	8	3.84	351
P8	369.7	.924	8	3.84	241	370.3	.926	8	3.84	241
					$\bar{x}$ 369					$\bar{x}$ 279
					SD 142					SD 100

Table 6.16 Mean epithelial thickness of control and leukoplakia biopsies dervived from total point counts (P_t) and column width.

Patient No.	CONTROL				LEUKOPLAKIA				
	P_p	M_p	P_t	P_p/P_t%	P_p	P_m	P_k	P_t	P_p/P_t%
P1	145.3	979.7	1125.0	12.92	105.3	356.2	64.2	525.7	20.03
P2	62.2	348.8	411.0	15.13	54.3	178.7	124.3	357.3	15.20
P3	114.3	828.5	942.8	12.12	67.2	219.7	65.3	352.2	19.08
P4	96.0	464.0	560.0	17.14	72.7	297.7	101.8	472.2	15.40
P5	72.0	387.2	459.2	15.68	72.5	141.7	73.6	287.8	25.19
P6	75.5	848.2	923.7	8.17	56.6	162.2	473.5	692.3	8.18
P7	62.3	254.2	316.5	19.68	115.2	348.5	75.0	538.7	21.38
P8	72.0	297.7	369.7	19.48	57.3	195.5	117.5	370.3	15.47
			$\bar{x}$	15.04				$\bar{x}$	17.49
			SD	3.90				SD	5.12

Table 6.17 Proportions of epithelium formed by progenitor compartment in control and leukoplakia biopsies.

		RANK			
	Patient	Atypia Score	Labelling Index	di	di^2
Control	P1	8	4	4	16
	P2	7	1	6	36
	P3	2	2	0	0
	P4	5•5	10	4•5	20•25
	P5	3•5	6	2•5	6•25
	P6	1	5	4	16
	P7	3•5	8	4•5	20•25
	P8	9	3	6	36
Leuko.	P1	15	9	6	36
	P2	5•5	12	6•5	42•25
	P3	11•5	13	1•5	2•25
	P4	10	15	5	25
	P5	11•5	16	4•5	20•25
	P6	13	7	6	36
	P7	14	14	0	0
	P8	16	11	5	25
					$\Sigma d_i^2 = 337•5$

$$r_s = 1 - \frac{6 \sum_{i=1}^{N} d_i^2}{N^3 - N}$$

$$= 1 - \frac{6\ (337•5)}{16^3 - 16}$$

$$= 0•51$$

correction for ties:-

$$r_s = \frac{\Sigma x^2 + \Sigma y^2 - \Sigma d^2}{2\sqrt{\Sigma x^2\ \Sigma y^2}}$$

$$= \frac{338•5 + 340 - 337•5}{2\sqrt{338•5 \times 340}}$$

$r_s = 0•503$

$t = 2•64 \qquad df = 14$

$P = 0•019$

Table 6.18 Spearman rank correlation co-efficient test for atypia scores and labelling index (P = 0•019). Siegel (1956).

ABBREVIATIONS

A	Area of section
A_m	Area of maturation compartment in section
A_p	Area of progenitor compartment in section
B.M.L.	Basement membrane length
C.F.	Correction factor (Abercrombie's Correction)
$C.F._p$	Correction factor for progenitor cells
$C.F._m$	Correction factor for mature cells
$C_{L.C.}$	Corrected labelled cells
$C_{M.C.}$	Corrected mature cells
$C_{P.C.}$	Corrected progenitor cells
$C_{T.N.C.}$	Corrected total nucleated cells
$C_{L.C./T.N.C.\%}$	Corrected labelling index by total cell index
G_1	Post-mitotic pre-synthetic gap in cell cycle
G_2	Post synthetic pre-mitotic gap in cell cycle
H.L.C.	Heavily labelled cell or cell count
I	Number of intersection points
I_{bm}	Intercept points along basement membrane of epithelium
I_s	Intercept points along epithelial surface
L	Length of structure
L.C.	Labelled cell or labelled cell count
L.I.	Labelling index
$L.I._1$	Labelling index by a single pulse
$L.I._2$	Labelling index by double labelling

M	Mitosis
M.C.	Mature cell or mature cell count
M.C. (size)	Mature cell size
$M.C._{ND}$	Nuclear diameter of mature cells
M.K.	Mixed keratinization
N.D.	Nuclear diameter
N.K.	Non-keratinization
O.K.	Orthokeratinization
P	Number of point counts
P_p	Point counts in progenitor compartment
P_m	Point counts in mature compartment
P_t	Point counts in total epithelium
P.C.	Progenitor cells or progenitor cell count
P.C. (size)	Progenitor cell size
$P.C._{ND}$	Progenitor cell nuclear diameter
P.K.	Parakeratinization
S (phase)	DNA synthesis phase
S.D.	Standard deviation
S.L.	Surface length
T_c	Total cell cycle time
T_{g1}	Duration of G_1 phase in the cell cycle
T_{g2}	Duration of G_2 phase in the cell cycle
T_m	Mitotic duration
T_p	Thickness of progenitor compartment
T_s	Duration of S phase
T_t	Total epithelial thickness
T.L.C.	Total labelled cells
T.N.C.	Total nucleated cells
W.L.C.	Weakly labelled cells
$\bar{x}$	Arithmetical mean