

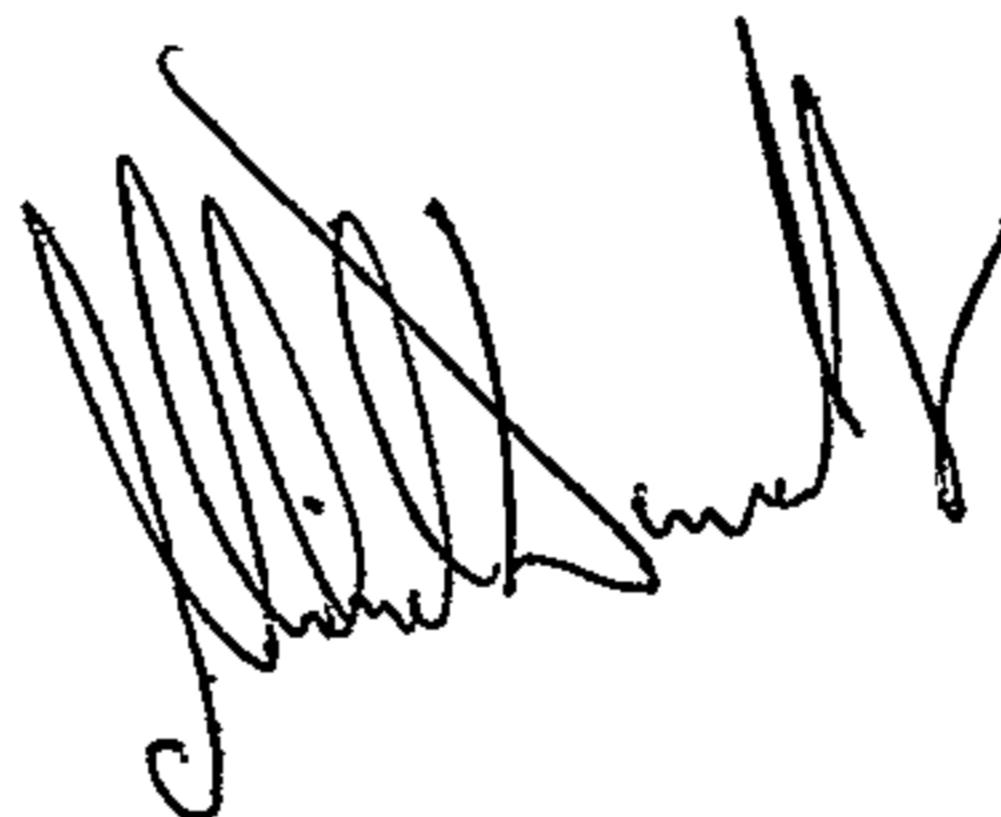
A STUDY OF THE EXFOLIATIVE CYTOLOGY  
OF NORMAL HUMAN BUCCAL MUCOSA WITH  
SPECIAL REFERENCE TO THE EFFECTS OF  
AGE AND SEX

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This thesis, based upon an original study conducted by the author, is submitted in partial fulfillment of the requirements for the Degree of Master of Dental Surgery of the University of Sydney.

A handwritten signature in black ink, appearing to read 'M.H. Dowsett', written in a cursive style.

M.H. Dowsett

February, 1964

"One should not lose sight of the fact that the cytological method - which has met with universal favour within the past few years because of its successful application in cancer detection and diagnosis - is an outgrowth of fundamental biological studies.

"In this we may find the elements of another noteworthy example of the all-important role of basic research in opening new and ever-widening avenues of investigation leading to accomplishments of broad significance for the advancement of science and the benefit of mankind."

George N. Papanicolaou

1883-1962

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## PREFACE

Interest in this study was prompted by the widespread adoption of the techniques of exfoliative cytology in the detection of oral carcinoma. In addition the morphologic differences in buccal cells are being used in the determination of sex, the diagnosis of anemias and other disorders, while recent studies point to useful applications of oral cytology in the estimation of the radio-curability of carcinomas and in the monitoring of the oral manifestations of toxicity in patients receiving chemotherapy for malignant disease.

A review of the literature related to the normal cytology of the oral cavity revealed considerable confusion as to what constituted the normal cytology of that region.

This study was therefore undertaken to determine whether there were variations in the cellular activity of the stratified squamous epithelium of human buccal mucosa which could be detected by stained smears made from surface scrapings, and whether such variations could be correlated with different ages.

The smear technique and the Papanicolaou stain were adopted as it was considered that they constitute an effective method for studying the cellular content of epithelia.

The buccal mucosa was selected as the site for study in the oral cavity as this area is the least influenced by side effects such as toothbrushing or the wearing of dental prostheses. In general, abnormalities of oral mucous membranes are usually found on the buccal mucosa although the probability for carcinoma is highest in lesions of the floor of the mouth and tongue.

Keratinization varies in different parts of the mouth, being most marked on the palate and at the gingival margin and least on the buccal mucosa. It is in this latter area that any pathological increase in keratinization can be most easily seen.

## AN INTRODUCTION TO EXFOLIATIVE CYTOLOGY

The essential difference between exfoliative and other branches of cytology is that it deals with desquamated cells which, because of their separation from their site of origin and the ensuing degenerative changes, acquire specific morphologic characteristics. Such cells are free from the pressure of surrounding cells and often assume distinctive forms differing markedly from those of the same type as they appear in tissue sections or those obtained by micro-dissection.

The study of exfoliated cells dates back to the middle of the nineteenth century. As early as 1843 Walsh took notice of small tissue fragments expectorated from malignant growths of the respiratory tract. <sup>(1)</sup> In 1847 Pouchet, in his book concerning ovulation and other related phenomenon, reported his observations on the cellular make-up of human vaginal smears. <sup>(2)</sup>

The earliest references on studies of desquamated cells for the purpose of diagnosis of cancer are those of Lebert in 1851 <sup>(3)</sup> and Beale in 1859. <sup>(4)</sup> Beale reported the finding of malignant cells in the sputum from a case of cancer of the pharynx. Numerous contributions have since been made by other workers, who have studied the cytology of various organs of

the body, chiefly for establishing criteria for the diagnosis of cancer.

Originally the vaginal smear technique was used as a means of analyzing the sexual cycle in the guinea pig. (6) Its value as a method for determining the morphologic and functional state of the female reproductive organs resulted in its general adoption as a standard method for the study of the sexual cycle of female mammals and of problems related to sex physiology and endocrinology.

The scope and significance of the vaginal smear were widened considerably by its application to the human. (6) The study of the normal cellular forms characteristic of the various phases of the sexual cycle was subsequently expanded to include the many aberrant types found in pathologic conditions and, more particularly, in cancer.

Although the primary application of cytology had been directed toward the detection of cancer of the uterus, it became and is now increasingly applied to the problems of occult cancer of the lung, stomach, breast, nasopharynx and rectum. (7)

It has been recognized that one of the salient features of the cytologic approach is the possibility which it offers for the recognition of cancer in its incipency. This has been demonstrated by the large number of pre-invasive carcinomas of the cervix detected in recent years by the use of the smear method. A new interest has thus been created in the study of histologic and cytologic changes in early carcinogenesis. Much progress has been made in this field but, in addition, the techniques of exfoliative cytology are contributing to a great extent to our knowledge of fundamental concepts of the morphologic and physiologic properties of the cell.

The observed characteristics of cells shed from oral carcinomas conform with established cytologic criteria for malignancy (8)(9) in other parts of the body. These features include enlarged nuclei, variation in nuclear size and shape, increased nuclear-cytoplasmic ratio, multiple and prominent nucleoli, hyperchromatism, abnormal chromatin pattern and distribution and a discrepancy in maturation in groups of malignant cells.

Until recently, relatively few studies had been undertaken in the field of intra-oral exfoliative cytology,

mainly because it was felt that the accessibility of oral lesions ruled out the need for techniques other than biopsy. (10) However, the large number of non-specific lesions that occur in the oral cavity, along with the inconsistency of signs and symptoms, suggested the need for the adoption of this technique as a means of cytological screening of such lesions. The accuracy and value of this technique has been confirmed by a number of workers. (11)(12)(13)(14) and it is now conceded, even by those who were sceptical, that popularization of the cytologic investigation will go a long way toward the early recognition of oral cancer. (15)

## THE HUMAN BUCCAL MUCOSA - GENERAL DESCRIPTION

Urban and Sicher classify the buccal mucosa as a lining mucosa as distinct from a masticatory type, being a covering of muscles - the change in volume and surface area of which in their different functional phases necessitating a relatively loosely fixed lining. (16)

The mucous membrane consists of two components, the lamina propria and a layer of stratified squamous epithelium. The latter is separated from the former by a distinct boundary called the basement membrane. This membrane lacks a definite organized structure, but has a fibrillar appearance resulting in indefinite margins from which fine filaments may extend to the basal cells above or the connective tissue fibrils below. The mechanism of attachment of the epithelium to the basement membrane is not well understood. (17)

The layer of stratified squamous epithelium is divided into more or less distinct layers. The deepest layer consists of cuboidal cells which are neatly aligned and constitute the basal cells or stratum germinativum. Next to the basal layer the epithelium consists of a number of layers of large,

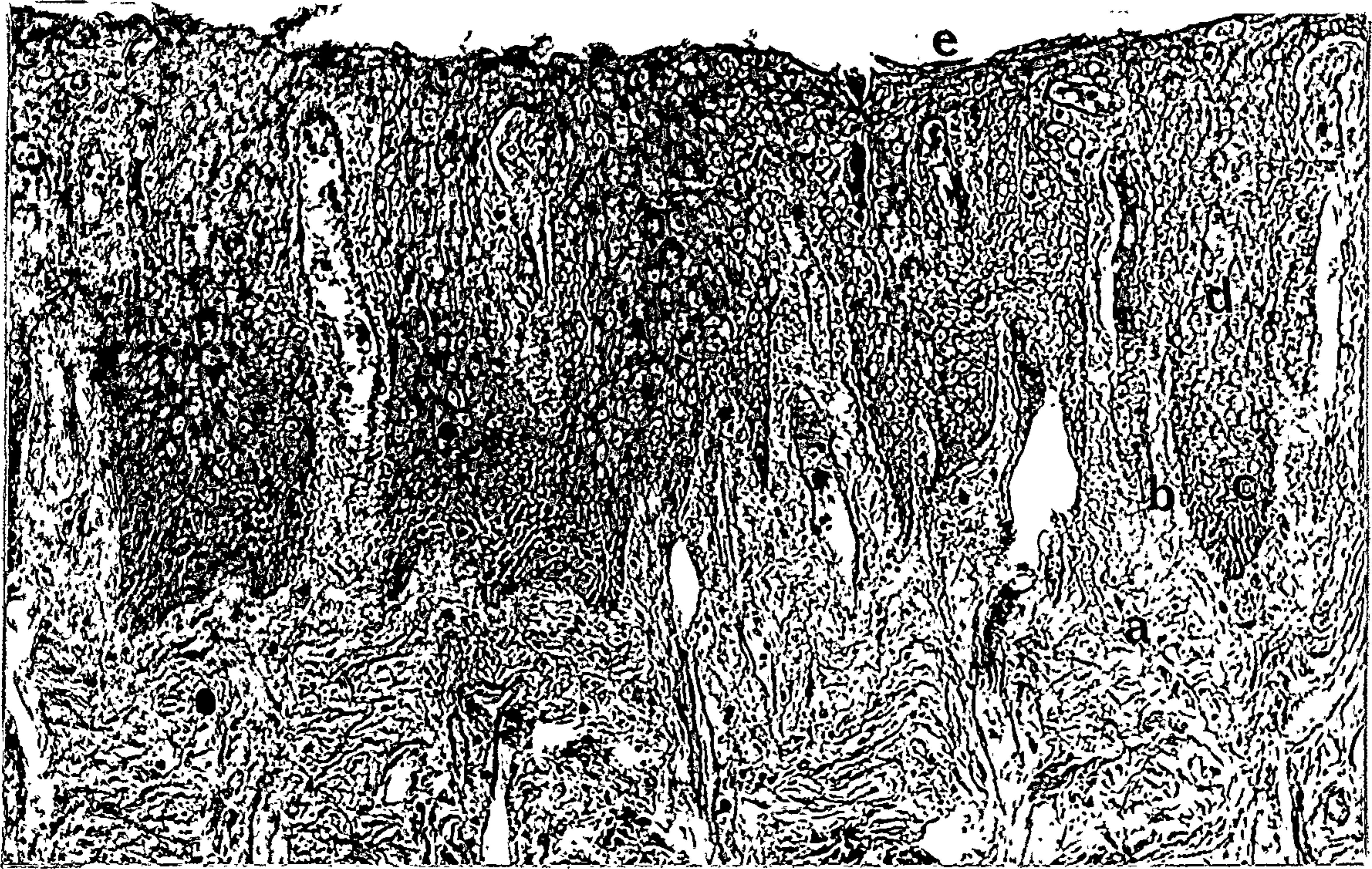


FIG. 1 -- HISTOLOGIC SECTION OF BUCCAL MUCOSA  
Female, 42 years. H. & E., (x 42)

A. Lamina propria  
B. Basement membrane  
C. Stratum germinativum

D. Stratum spinosum  
E. Keratinized cells.

polyhedral cells constituting the stratum spinosum or prickly cell layer. This term is applied to these cells because it was thought (18) that these cells were connected to each other and with the basal cells by means of small inter-cellular bridges. Actually, there is no direct connection between the cells, but a modification of the adjoining areas of cytoplasm has been demonstrated, forming what is termed a desmosome. (19)

Desmosomes are found on the epithelial cells in all layers. The electron microscope shows that desquamation occurs along the proximal light layers of the desmosomes of the detached cells so that remnants of the desmosomes are found lined up along the superficial border of the cell which is still connected to the underlying epithelium. (20)

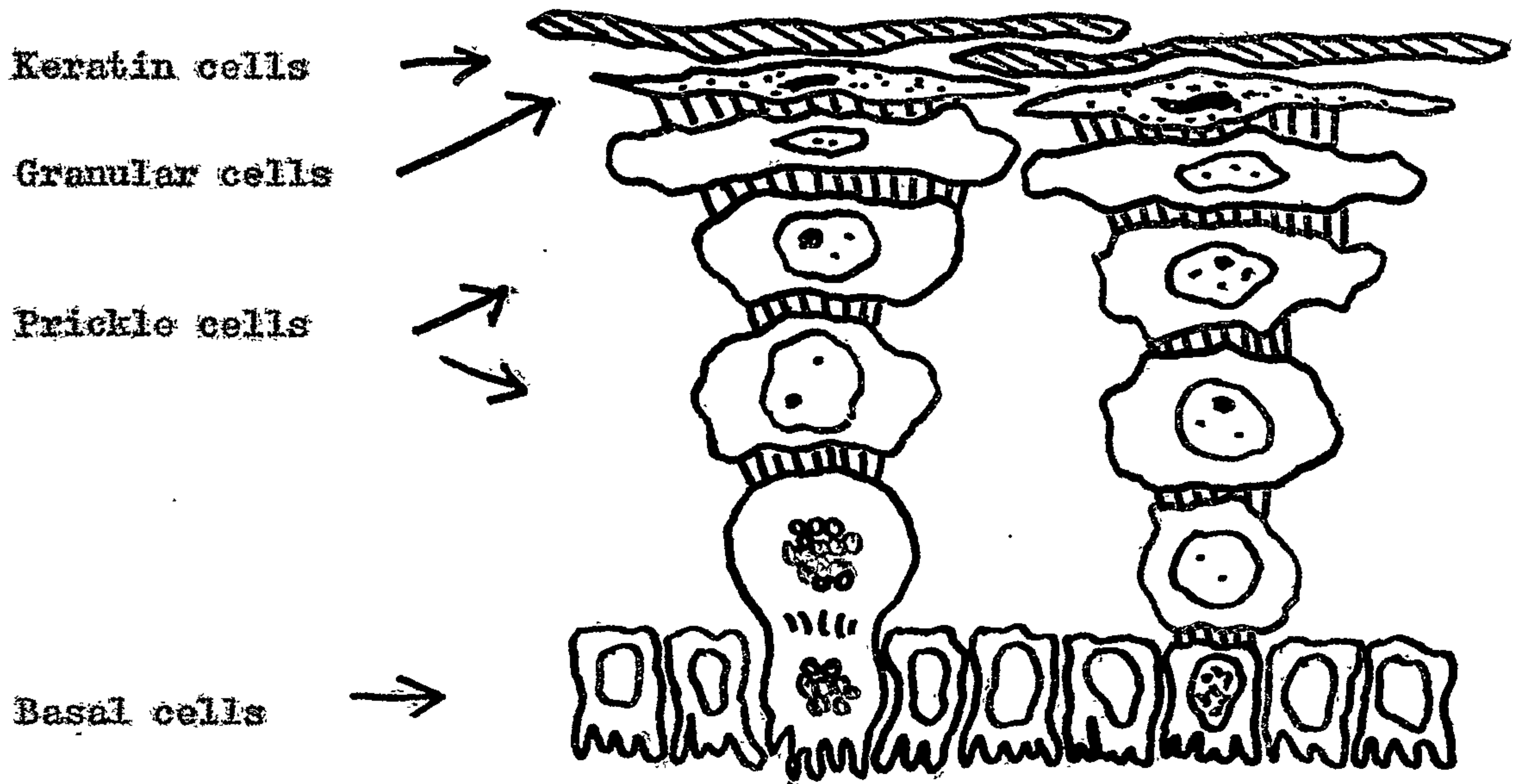
The stratum spinosum and the basal cell layer are together termed the rete papillae. It is in these two layers that most of the mitoses occur. There is evidence that ordinarily one daughter cell of a basal cell moves up into the prickly cell layer whereas the other retains its position at the basement membrane. It is generally conceded that young prickly cells also can undergo mitosis (differentiating intermitotics) thus adding to the supply of superficial cells. (21)

Studies purporting to demonstrate greater mitotic activity in the higher layer of prickle cells than in the basal layer did not exclude a number of technical sources of error and a re-evaluation is required. (22)

On the surface of the stratum spinosum the epithelial cells gradually become flattened and contain fine, basophilic granules, believed to be precursors of keratin and called keratohyaline granules. The actual composition and role of these granules is not clearly understood. (23) The layer of cells which contains them is called the stratum granulosum.

The surface of the buccal mucosa is formed by a layer of parakeratotic cells, that is a layer of epithelial cells which are incompletely keratinized and retain their nuclei. There is some degree of complete keratinization which may increase markedly in some pathologic states.

In general, it may be assumed that (i) a cell follows a straight path upward in the mucosa, or (ii) the progeny of one basal cell form an inverted cone (as illustrated) with the small basal cell at the basement membrane and the broad superficial fluke at the external surface. It follows



Diagrammatic illustration of the life cycle of epidermal cells, indicating the changes in size and shape of the cells as they move towards the surface. Two clones, descendants of individual basal cells, are sketched in order to illustrate the cone-shaped bodies formed by them, and the resulting intercalation of surface cells.

- after Pinkus (24)

that the progeny of neighbouring basal cells mingle to some degree, but this does not alter the general concept. (24)

The mitotic rate of the human buccal mucosa has not been determined. The mitotic rate of various tissues seems to be dependent on an inborn, that is hereditary, feature of the cells, at least partially conditioned by the prevailing environment. (25)

Epithelia that are continuously exposed to powerful enzymatic media, mechanical stress or infectious agents display often considerably rapid renewal rates. The mitotic index of the human epidermis has been reported as between 1:10,000 and 1:1,000. (26) The turnover time for the buccal mucosa of the rat has been estimated as 4.3 days, and can be compared with that of body surface epithelium, 2.9 days, and inferior surface of the tongue, 7.7 days. (25)

### TERMINOLOGY

The terminology used in this thesis is in conformity with that of the International Academy of Gynecological Cytology, having been adopted by the Academy in 1953.

#### KERATINIZATION

The research and general understanding in cytology and histology is complicated by the fact that English-speaking authors use the terms keratinization and cornification, or keratinized and cornified cells. The authors adopting such terminology sometimes apply these names in entirely different ways so that what one author means by keratinization is just the contrary of the meaning proposed by another.

A review of the literature to date has failed to reveal any clear description as to what should be understood by cornification and keratinization of epithelial cells, terms indicating for some authors two different processes, while for others, identical processes.

French histology does not use the term cornification and only uses the term keratinization, in French "keratinisation". (27)(28) German histology uses the same terminology as the French without any important difference, the German terms being

"keratinisierung" or "vorhornung". The superficial layer of squamous epithelium is sometimes called the stratum corneum, but the appearance of keratohyaline granules which characterize this layer is considered only an antecedent of actual keratinization. (29)

Fawcett and Bloom (30) use the general term, cornification, which is applied to the vagina and the epidermis as well as to the nails and hair. The term, keratinization, is not registered. The only term used by Ham (31) is keratinization for the vagina as well as for the epidermis, nails and hair.

In this thesis the term, cornification, is not used since it is considered that it describes exactly the same process which other authors call keratinization.

Keratinization is thus the process by which the protein of epidermal cells is transformed into keratin. The exact nature of the change is not known. (32)

**ANUCLEATE SQUAME**

A superficial cell with no visible nucleus

**SUPERFICIAL CELL**

A large cell with squared-off edges containing a completely pyknotic nucleus. It is usually eosinophilic but may be cyanophilic. It is distinguished from the intermediate cell by its pyknotic nucleus.

**INTERMEDIATE CELL**

Cell presenting a definite differentiation of the cytoplasm, a beginning retraction of the nuclear diameter, but without complete karyopyknosis.

**PARABASAL CELL**

A round or oval cell, small in size, with a dense cytoplasm deeply staining in green or purple. Quite often no specific vacuoles are present. The nucleus, the size of which is about one third of the size of the cell, may sometimes show irregular borders. It is centrally located and has a well defined chromatin arrangement.

**BASAL CELL**

A cell which derives, apparently, from deeper layers than the parabasal cell.

**KARYOPYKNOTIC**

Degenerative retraction or condensation of the nucleus.

with the loss of all nuclear structure, so that the nucleus shrinks to a dense, structureless mass of chromatin. Quantitatively, a pyknotic nucleus is one having a diameter of less than 6  $\mu$ .

A REVIEW OF PREVIOUS INVESTIGATIONS  
RELATING TO THE NORMAL ORAL MUCOSA

Interest in the exfoliative cytology of the oral cavity dates back as far as 1898 when Miller (33) described epithelial cells and leucocytes in human saliva.

Orban and Weimann (34) in a study of the cellular elements of saliva in 1939 stained saliva samples from the buccal fold with Wright's stain. The mucous membrane was not scraped but epithelial cells were observed in the stained smears. They noted that these cells showed large cytoplasmic bodies and oval nuclei, the cell bodies being round, elliptical or irregular, sometimes folded and sometimes containing coarse granules. Nuclei were deeply stained by the basic dyes. Binucleate and multinucleate cells were occasionally seen.

In 1940 Weimann studied the process of keratinization of the oral mucosa with various cytologic methods. (35) He selected the Ernst-Graen and Wright methods as the most suitable and demonstrated the various stages of keratinization of oral epithelial cells. Smears were taken from five male and five female patients, ranging in age from 25 to 40 years.

The smears from the region of the cheek showed cells with well preserved nuclei. Neither the cytoplasm nor the nucleus was stained by Gram's method. In only a few cases were cells observed in which the nucleus and sometimes parts of the cytoplasm showed affinity to Gentian violet. The nucleus was entirely or partly stained by Gentian violet when the cytoplasm was stained. However, there were cells where only the nucleus showed affinity to this dye.

This observation suggested that, in the course of changes leading to keratinization, the nucleus is the first part of the cell that exhibits changes in staining characteristics. If the process progresses further, then the cytoplasm is also affected.

No specimen from the cheek indicated a complete keratinization in this region. The majority of the cells studied from the cheek showed no changes that could be interpreted as a stage preceding the stage of keratinization. Weinmann assumed that the buccal epithelial cells were sloughed off before the cells were able to produce keratin.

The smear technique and Ernst-Gram staining was used by Zlotkin, Kamen and Littay in 1941 to study the oral mucosa. (36) Six stages of keratinization were disclosed, differentiated on the basis of the affinity of the cellular structures to Gentian violet or safranin and the presence or absence of a nucleus. They noted that the incidence of leucocytes varied inversely with the degree of keratinization.

Because of the known effect of oestrogenic hormones on keratinization, tests were made to determine whether or not cyclical variation occurred which could be correlated with oestrogenic levels during menstruation. Variations in keratinization levels were noted, but could not be synchronised with those purported in systemic oestrogen. Application of this method to the study of pregnancy, when the oestrogen level falls markedly, disclosed that a low degree of keratinization and a high incidence of leucocytes exist in the normally keratinized palate and gingival areas.

In a series of 15 climacteric cases, gross oral manifestations, subjective symptoms and biopsies of the buccal mucosa were correlated before, during and after therapy with various oestrogens. (37) The investigators,

Richman and Abarbanel, concluded that, as a result of declining ovarian activity, manifested clinically by the cessation of menstruation, atrophic changes may occur in the oral mucosa. Histologically, the first stage consists of atrophy of the germinal layer in general and the prickle cells in particular. With time and irritation, the superficial layer, under an abnormal growth stimulus, may become the site of hyperkeratosis or, eventually, leukoplakia.

Ziskin and Moulton in a later study, which compared oral and vaginal smears from patients with certain gynecologic disorders, indicated a certain degree of parallelism in the process of keratinization between oral and vaginal mucosa. (33)

The first attempt to use the Papanicolaou technique in this type of study was made by Henegonory in 1950. (39) A group of 75 individuals with clinically normal mouths was studied, the group comprising 25 children between the ages of 3 and 10 years, 25 adults between the ages of 20 and 40 years, and 25 individuals above 63 years of age.

Differential counts of the cells contained in the smears were made, the cells being divided according to

their staining qualities into red, yellow and blue varieties. He showed that definite cytologic patterns existed in various regions of the mouth and further concluded that age and sex did not significantly influence the cellular patterns.

In addition, Montgomery studied the effect of the menstrual cycle and the pH of the environment in two separate experiments and concluded further that no significant differences in the results could be noted. Rather drastic deviations from the normal cytologic patterns were observed occasionally without explanation for their occurrence.

Juscum (40) noted a similar correlation as demonstrated by Ziskin and Moulton in smears from the buccal mucosa and ventral aspects of the tongue in a study of three women conducted over 35 days, the smears being processed according to the Papanicolaou technique.

Taking gingival smears from 215 females and relating the date to the menstrual cycle in each case, Foyie and Glickman (41) concluded that such a correlation does not exist. They did, however, demonstrate a trend toward diminished keratinization with increasing age.

The absence of cyclical trends in the degree of keratinization in the gingival mucosa in their study did not necessarily contradict other investigations referred to earlier, which point to a relationship between oestrogenic activity and the gingival mucosa. The former studies dealt either with experimental conditions in which the hormonal picture was methodically altered or with cases of advanced gingival disease. (42)(43) Poppe and Glickman, however, dealt with a normally keratinized region of the gingiva under physiologic conditions. There might very well be an influence of oestrogenic hormones upon the keratinized portion of the gingival mucosa under the physiologic conditions of the experiment. However, alterations in the oestrogenic hormones during the menstrual cycle may not constitute a physiologic variation of sufficient magnitude to produce cyclical changes consistently in the normal keratinization process.

The possibility that keratinization of the gingival mucosa is influenced by oestrogen activity under physiologic conditions is suggested by their findings of decreased keratinization with increasing age and in the menopause. Since diminished oestrogen activity is associated with both increasing age and menopause, the reduction in keratinization

observed in both these conditions may be a correlated physiologic, atrophic phenomenon.

Miller, Sobornan and Stahl (44) compared the degree of keratinization of the oral epithelium of fifty young males, ranging in age from 20 to 30 years, utilizing the technique of Papanicolaou, Trout and Marchetti. (45) They found the degree of keratinization to be highest at the gingivae, followed by the dorsum of the tongue, the cheek, and the ventral surface of the tongue in the order named.

Stone (46) examined smears taken from fifty males ranging in age from 60 to 100 years, using the same sites in the mouth and employing the same technique as Miller, Sobornan and Stahl. In comparing their results with his own, he concluded that there was a definite decrease in gingival keratinization with age. However, he did not extend his comparison any further, a comparison which indicates an increase in keratinization in the three other sites examined. (see tables)

Podreln, in a similar study of fifty males aged between 60 and 80 years and using the original Papanicolaou method, concluded that in old age there is a marked tendency toward

A COMPARISON OF PREVIOUS STUDIES RELATING TO THE  
REGENERATION OF THE ORAL MUCOSA OF MALES

	TONGUE - DORSAL	TONGUE - VENTRAL	BUCCAL MUCOSA	LOWER GINGIVAE
<u>Age 20-30</u>				
(Miller, et al)				
Blue cells	14	79	27	-
Red cells	66	21	73	10
Yellow cells	-	-	-	89
<u>Age 60-100</u>				
(Stone)				
Blue cells	21	60	32	26
Red cells	48	65	61	57
Yellow cells	31	5	7	17
<u>Age 60-80</u>				
(Paucoira)				
Blue cells	32	69	66	26
Red cells	50	28	30	31
Yellow cells	18	3	4	43

a decrease in the keratinization of the oral mucosa. (47)

Pedreira noted " a great similarity for all areas except the lower gingivae " in a comparison of his figures with those of Stone. An examination of their results shows that this is not the case. (see table)

In a later study by Miller, Seberman and Stahl smears were taken from 300 males ranging in age from 8 to 80 years. (48) Using the same sites as their previous study (44) they observed a constant pattern of keratinization which remained constant throughout ageing. The general environment (i.e. supervised medical and nutritional care as compared to patients chosen at random from private homes) as well as progressive ageing did not seem to alter the keratinization pattern. In one group of aged males a lesser degree of gingival keratinization was noted. This was attributed to inadequate gingival massage rather than the effects of ageing.

Mulleromann in 1950 demonstrated, by means of gingival biopsies, that there is a fall in keratinization during menstruation. (49)

A year later, Trott, using the same technique in 140 patients, found no relationship between the variations

in the degree of keratinization of the gingivae and the phases of the menstrual cycle. (50)

Using smears made from scrapings from the attached gingivae and the vestibular mucosa, Trott observed 39 cycles and graded the smears according to the degree of keratinization. (51) He concluded that continual changes in the pattern of keratinization do occur but that these would seem to be due more to the functional stimuli supplied by food and saliva, rather than being oestrogenic in nature.

One hundred apparently healthy subjects of each sex, with an age range of 20 to 50 years, were examined by Jacobs. Smears were taken from the buccal mucosa, stained by the standard Papanicolaou technique, and the percentage of acidophil and orangeophil cells (cornification index) was calculated. Daily smears were also obtained from four healthy women over a period of five weeks. Jacobs concluded that in buccal smears the effect of age, sex and menstrual phase can be ignored under normal conditions. (52)

Instead of the direct smear technique Shiraishi (53) examined the cytology of mouth washings and garglings, the cells being collected by means of centrifugation. Normal, healthy subjects used in this study were composed of 25 newborns and sucklings, 30 adults and 28 edentulous elderly persons. The specimens were stained by the Papanicolaou technique.

With the progress of age keratinized cells or cells with keratinizing tendencies showed an increase. Fewer white blood cells were noticed in newborns, sucklings and old persons, while more were noticed in adults. This may be due to the presence of gingival pockets in this group.

McMillan and Tonge studied serial histologic sections of the oral cavity in representative areas of a range of human embryos and fetuses. (54) Nuclear loss among the superficial cells was seen at all stages. No clearly defined prickle cell layer or stratum granulosum could be detected. Keratinization was seen between 120 days and 150 days.

Smears from the buccal mucosa have also created interest as a means of diagnosing anaemia and as a method of chromosomal sex detection.

A COMPARISON OF PREVIOUS STUDIES RELATING

TO THE CYTOLOGY OF THE BUCCAL MUCOSA

	Blue cells	Red cells	Yellow cells	
Montgomery (1950)	51.5	43.8	4.7	All ages 75 subjects, M & F
Miller, et al. (1951)	27.0	73.0	-	20-30 years 50 males
Podreira (1951)	65.7	30.4	3.9	60-80 years 50 males
Miller, et al. (1952)	38.0	48.0	9.0	8-90 years 300 males
Stone (1953)	32.0	61.0	7.0	60-100 years 50 males

Increase in proportion of keratinized cells with age reported by:

Stone (1953)

Shiraishi (1959)

Decrease in proportion of keratinized cells with age reported by:

Papic and Glickman (1950)

Podreira (1951)

No change in proportion of keratinized cells with age reported by:

Montgomery (1950)

Miller, et al. (1952)

Jacobs (1958)

Interest has been centred mainly in patients suffering from megaloblastic anaemia in which recognizable changes occur in epithelial cells lining mucosal surfaces. The principal variation noted has been an increase in the mean nuclear diameters of cells exfoliated from mucous surfaces and this change is most consistent in specimens taken from the oral cavity. (55) Boddington does not confirm this and suggests that in iron deficiency anaemias a smaller cytoplasmic size is common. (56)

Farrant examined smears from 95 normal persons of varying ages. (57) By relating nuclear size to age, a surprising increase of the shorter axis of the nuclei became evident. The effect is obvious only over the age of 60, and if normal controls of 60 years and above are compared with the rest a significant difference arises.

When a sex difference can be recognized in mammalian cells, it is seen to consist of an excess of chromatin in the female nucleus. This excess, which stains with basic dyes and gives positive tests for D.N.A., is visible as a discrete particle, "sex chromatin". This is larger than other chromatin particles in the nuclear space, stains more deeply, and in the majority lies

adjacent to the nuclear membrane and follows its outline.  
A smaller and similar particle can be made out in a  
small percentage of males. (58)

Human oral epithelium has been shown to be similar  
to epidermal epithelium in showing such a distinct sex  
difference both in sections (59) and smears. (60)

A DISCUSSION RELATING TO PREVIOUS STUDIES  
OF THE CYTOLOGY OF THE BUCCAL MUCOSA

A review of previous investigations clearly demonstrates that there is considerable confusion as to the cytological and histological evaluation of the human oral mucosa, especially in regard to the effect, if any, of age.

The present investigation is concerned solely with the buccal mucosa and the accompanying table summarises the results obtained previously in studies of this region.

None of these studies has produced an overall estimation of the effect of age as each has been somewhat restrictive in its content.

Montgomery studied a group of 75 individuals, composed of 25 children between the ages of 3 and 10 years, 25 adults between the ages of 20 and 40 years, and 25 elderly individuals above the age of 63. He did not state the proportion of male and female subjects in each group.

Miller, Sobornan and Stahl in their first study,

Pedreira as well as Stone, restricted themselves to small groups of males, their individual results being subsequently compared. In their second study, Miller, Soberman and Stahl extended the range to include 300 subjects ranging in age from 8 to 80 years but again restricted the study to male subjects.

Jacobs investigated a group of 100 aged between 20 and 70 years and comprising both sexes. His results were restricted to a determination of "the cornification index - the percentage of acidophil and orangeophil cells".

These previous studies have also used criteria which can no longer be considered accurate for such estimations.

Colour changes, while of undoubted value, are not sufficiently consistent from individual to individual to permit of their accurate use in comparative studies of the degree of keratinization of the oral cavity. (61)  
They are certainly subject to variation in the technical processes, unless great care is taken in the selection of the stains and the timing of the various stages in the complicated process.

Osborn (62) concluded that "whether cytoplasm stains pink, green or brown or some other colour depends so largely on the clinician who prepares the smear and the technician who stains it that ( a study depending on such changes)\*\*\* is not a reliable index of the degree of cornification in buccal epithelium."

Asscher, Turner and de Boer (63) stated that they had confirmed the value of Papanicolaou staining in the demonstration of keratinized cells, although red colourations (stated to be indicative of keratinization) were produced in two specimens from a total of twenty eight, in regions in which no keratin could be detected histochemically or by polarized light.

Staining techniques to study exfoliative cytology have been used in a more or less empiric way. Using the technique of Papanicolaou and Shorr (64) the "acidophilic" cells are those cells, the cytoplasm of which takes on a red or orange stain. This term is technically in error because the affinity of the cytoplasm of some epithelial cells for red stains has nothing to do with the real acidophilic in the histological sense of the word, (65) nor has the blue or green staining of the cytoplasm

a real basophilic etiology.

When intense "acidophilic colour reaction" was observed by Papanicolaou and Shorr in the superficial cells, it naturally followed that cells of the same smear that did not appear pink were called basophilic, and from then on the cells were termed either acidophilic or basophilic. Since all the stains in the routine Papanicolaou procedure are on the acid side (Harris' Haematoxylin; pH 2.75: EA-50; pH 5.80: GG-5; pH 5.75), it is not correct to speak of basophilia. (66)

For this reason the terms "acidophilia" and "basophilia" have been abandoned by most authorities (67)(68) and the terms "eosinophilia" and "cyanophilia" adopted, so that the terms indicate only the colour staining reaction, and are descriptive rather than interpretative.

Furthermore, Papanicolaou and Corre (69) have shown that by modification of the staining technique, the same stain in one formula is fixed by the superficial cells which generally take the red stains and in another formula only by the usually cyanophilic cells.

## THE METHOD OF THE INVESTIGATION

### A. Selection of Subjects

The subjects used in this study consisted of persons attending the United Dental Hospital of Sydney, staff and students of the Faculty of Dentistry, Sydney University, and naval personnel attending the dental department, H.M.A.S. NIEHBA. In addition, five smears were obtained from the maternity section, Queen Elizabeth Hospital, Woodville, South Australia.

In each case a brief medical history was obtained from each subject, the subject's age and sex noted and the oral cavity examined. In this way, only subjects with a normal medical history and a clinically normal buccal mucosa were used in this study.

### B. Method of collection

The buccal mucosa on the right side just anterior to the opening of Stenson's duct was scraped with a wooden spatula. The spatulas used had rounded ends and had dimensions of 10 cm. x 1 cm. Ten separate scraping movements were made for each subject, each movement being a downward one using firm pressure.

Prior to the scraping being made, each subject was instructed to rinse thoroughly with tap water. The spatulas were allowed to stand in a solution of physiological saline which softened the spatulas and aided in preventing cells from sticking to them. This procedure assists in obtaining more cellular material on the slide and facilitates spreading as well as protecting the morphology of the cells.

The slides (3 x 1", 0.9 mm.) were cleaned in the following way. The slides were stored in chromic acid for at least 24 hours, followed by washing in tap water until all traces of colour had disappeared. They were then stored in distilled water for 24 hours and absolute alcohol for 24 hours and rubbed with paper tissues before use.

The cells obtained on each spatula were immediately smeared onto a glass slide with a circular motion and covering a region on the slide about 15 mm. in diameter.

### C. Method of fixation

Immediately following smearing each slide was placed in a fixative consisting of a solution of 5% glacial acetic acid in 95% ethyl alcohol. Although Papanicolaou recommended the use of a mixture of equal parts of 95% alcohol and ether <sup>(70)</sup> alternative fixatives are used with satisfactory results.

Cahn <sup>(71)</sup> and Klinger <sup>(72)</sup> suggested the use of 95% alcohol alone, the addition of 5% glacial acetic acid being advocated by Carpentier to enhance the adhesion of the cellular material to the slide. <sup>(73)</sup>

The coating of slides with Meyer's albumin prior to smearing, although advocated by some, <sup>(74)</sup> was considered unnecessary. This view was supported by Quisenberry <sup>(75)</sup> and Powell, <sup>(76)</sup> the latter stating that the fluid portion of the specimen contains sufficient protein material to induce good adhesion to the slide.

Fixation does not require more than a few minutes but a minimum of 15 minutes is advisable for proper adherence of the smear to the slide. <sup>(77)</sup> The slides

were kept in the fixative for a period not exceeding 30 hours nor less than 24 hours. Lengthy storage in the fixative may result in an alteration in the staining reaction of the cells.

#### D. Staining of smears

Staining of smears is directed towards three main objectives: i. definition of nuclear details (Because of the widespread nuclear abnormalities and their diagnostic significance in the determination of malignancy, good staining of the nucleus is of primary importance.)  
 ii. transparency - this is of particular importance in view of the varying thickness and the frequent overlapping of cells, iii. differences in the staining reaction such as that between eosinophilic and cytophilic cells.

#### E. Staining procedure

The staining procedure employed was that of Papanicolaou and Traut. (78) The procedure is as follows.

1. After fixation, transfer slides, without drying, directly into 80% alcohol and run through 70% and 50% alcohols to distilled water.
2. Stain in Harris' Hematoxylin for 1 minute.
3. Rinse three times in distilled water, using three

separate containers.

4. Rinse in 50% alcohol.
5. Place in solution of 1.5% ammonium hydroxide in 70% alcohol for 1 minute.
6. Rinse in 70% alcohol and run through 80% and 95% alcohols.
7. Stain in OG-6 for 1½ minutes.
8. Rinse in 3 containers of 95% alcohol.
9. Stain in EA-50 for 3 minutes.
10. Rinse 3 times in 95% alcohol, using three separate containers.
11. Dehydrate and clear by running through 2 containers of absolute alcohol, a mixture of equal parts of absolute alcohol and xylol and 2 containers of xylol.
12. Mount in Canada Balsam.

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The stains used were obtained from Ortho Pharmaceutical Company, 23 Ridge Street, North Sydney.

EA-50 is a multiple polychrome stain. OG-6 is a single Orange G stain. Harris Haematoxylin (Ortho Modification) is a specially prepared acidified nuclear stain.

## F. Examination of smears

Each smear was examined with the aid of a Leitz Laborlux microscope at a magnification of 1,000. A random cell count of one hundred cells was made on each slide and the cells were classified into the following six groups.

- (a) Anucleate squames
- (b) Eosinophilic superficial cells
- (c) Cyanophilic superficial cells
- (d) Eosinophilic intermediate cells
- (e) Cyanophilic intermediate cells
- (f) Parabasal - basal cells.

Some cells showed a gradual transition in colour tones and in such cases the predominant colour determined the final classification of the cell.

Only those areas on the slides where the cells were spread thinly were considered suitable for examination. The heavy clusters of cells, resulting from a mechanical effect during preparation of the smear, were omitted from the study. Only cells with definite morphology and distinct staining characteristics were evaluated.

## RESULTS

Examination of the smears showed essentially four types of cells - cyanophilic and eosinophilic intermediate cells, eosinophilic superficial cells and anucleate squames, with intermediate cells predominating.

The cells showed a gradual transition in colour from blue-green to red and orange depending upon the depth from which they originated. This gradual transition between the various colour tones has been commented upon by Montgomery and von Haam who stated that this is a prominent feature of the normal smear, but is often absent in smears from patients with carcinoma of the oral mucosa. (79)

The overall size of the cells varied, depending upon the layer of epithelium from which they originated. The cells from the basal layer were the smallest, anucleate cells the next smallest followed by those from the superficial layer and those in the intermediate layer, the largest. This observation has also been made by Weathers (80) but is contrary to the popular conception of the clonal growth of cells.

The figure obtained from the differential counts of cells were grouped in table form according to age groups and sex. A total of 245 individual smears were examined, made up of 15 males and 15 females from each ten year age group and 5 smears from premature infants who were delivered after 32 to 37 weeks in utero.

These data were tested according to the type of the cells in a certain age group. The mean of the items in each group can be considered as a random variable.

The material was treated by calculating the confidence interval. A confidence interval is an interval computed in such a manner that it would contain the true mean of a group of items a certain fixed per cent of the time ( in this instance, 95 per cent). If the confidence intervals of two particular groups do not overlap, the means of these groups are significantly different on the 5 per cent level.

It can be seen from examination of the accompanying tables, that in any one age group when comparing the male and female figures, no significant differences occur between cells of the same type. It can thus be concluded that sex does not significantly influence the cellular

pattern as explored by this method.

The mean values of cell types from all age groups of both males and females was then determined. The confidence intervals of these values were then compared with the confidence intervals of cell types of individual age groups.

It was observed that the confidence intervals of individual cell types overlapped in each case and it is therefore concluded that there is no significant variation between age groups within the range examined.

Differential counts of cell types were made in the same manner from a group of five smears obtained from infants at Queen Elizabeth Hospital. These infants were delivered prematurely after 32 to 37 weeks in utero, their birth weights being less than 5 pounds. Smears were taken at the ages of 1, 3, 5, 7 and 17 days from two males and three females. Large variations in the differential counts were noted which were attributed to fluctuations in the oestrogen level due to variations in feeding and age. The distribution of cells was, however, within the limits previously determined.

PERCENTAGE DISTRIBUTION OF CELLSMales

AGE GROUP	CELL TYPE	MEAN %	STANDARD DEVIATION	CONFIDENCE INTERVALS
0-10	A.S.	2.0	4.3	0.00 - 4.32
	E.S.	3.5	2.8	2.02 - 4.88
	C.S.	0.5	0.6	0.18 - 0.82
	E.I.	42.8	15.5	34.6 - 51.0
	C.I.	50.5	18.6	40.7 - 60.3
	P.B.	0.5	1.0	0.48 - 0.54
11-20	A.S.	0.6	1.05	0.08 - 1.16
	E.S.	2.7	3.2	1.00 - 4.40
	C.S.	1.8	2.04	0.06 - 2.88
	E.I.	31.5	28.7	16.3 - 46.7
	C.I.	62.8	25.6	49.2 - 76.4
	P.B.	0.2	0.39	0.00 - 0.48
21-30	A.S.	1.0	1.22	0.38 - 1.64
	E.S.	2.0	1.60	0.16 - 2.84
	C.S.	0.5	0.24	0.38 - 0.62
	E.I.	42.0	28.6	26.8 - 57.2
	C.I.	54.8	29.4	38.5 - 70.1
	P.B.	0.3	0.15	0.24 - 0.36
31-40	A.S.	1.0	1.14	0.40 - 1.60
	E.S.	5.0	4.80	2.40 - 7.60
	C.S.	0.9	1.10	0.32 - 1.48
	E.I.	37.5	24.3	24.5 - 50.5
	C.I.	55.2	25.9	41.4 - 69.0
	P.B.	0.25	0.19	0.13 - 0.37
41-50	A.S.	1.5	1.92	0.48 - 2.52
	E.S.	3.2	2.14	2.06 - 4.94
	C.S.	0.25	0.15	0.15 - 0.36
	E.I.	27.8	12.8	21.0 - 34.6
	C.I.	67.5	15.6	59.2 - 75.8
	P.B.	0.3	0.16	0.22 - 0.38
51-60	A.S.	0.6	0.99	0.00 - 2.55
	E.S.	1.7	1.89	0.68 - 2.82
	C.S.	0.6	0.97	0.08 - 1.12
	E.I.	37.6	18.3	28.0 - 47.2
	C.I.	58.7	16.1	50.1 - 67.8
	P.B.	0.5	1.08	0.00 - 1.64

Males (cont).

AGE GROUP	CELL TYPE	MEAN %	STANDARD DEVIATION	CONFIDENCE INTERVALS
61-70	A.S.	0.64	0.78	0.14 - 1.14
	E.S.	1.0	1.01	0.42 - 1.58
	C.S.	0.4	0.27	0.26 - 0.54
	E.I.	28.4	20.0	17.8 - 38.0
	C.I.	69.3	19.6	49.7 - 88.9
	P.B.	0.15	0.14	0.07 - 0.23
71-80	A.S.	1.0	1.10	0.38 - 1.62
	E.S.	1.3	1.08	0.64 - 1.76
	C.S.	0.6	0.22	0.50 - 0.70
	E.I.	44.4	18.5	34.6 - 54.2
	C.I.	51.9	20.7	40.9 - 62.9
	P.B.	0.3	0.4	0.10 - 0.50

A.S. - Anucleate squame

E.S. - Eosinophilic superficial

C.S. - Cyanophilic superficial

E.I. - Eosinophilic intermediate

C.I. - Cyanophilic intermediate

P.B. - Parabasal

PERCENTAGE DISTRIBUTION OF CELLSFemales

AGE GROUP	CELL TYPE	MEAN %	STANDARD DEVIATION	CONFIDENCE INTERVALS
0-10	A.S.	1.0	2.6	0.00 - 2.58
	E.S.	2.2	2.0	1.12 - 3.28
	C.S.	0.6	1.0	0.02 - 1.18
	E.I.	50.7	19.6	40.3 - 61.1
	C.I.	48.4	21.6	32.0 - 54.8
	P.B.	0.0	0.14	0.00 - 0.06
11-20	A.S.	0.9	1.27	0.59 - 1.95
	E.S.	2.0	1.18	1.38 - 2.62
	C.S.	0.6	0.95	0.10 - 1.10
	E.I.	38.8	9.98	34.6 - 43.0
	C.I.	56.8	10.6	51.2 - 62.4
	P.B.	1.0	1.9	0.00 - 2.02
21-30	A.S.	0.5	0.85	0.46 - 0.54
	E.S.	1.4	1.02	0.86 - 1.94
	C.S.	0.9	0.15	0.09 - 0.41
	E.I.	35.3	19.8	25.7 - 46.9
	C.I.	61.8	20.4	51.0 - 72.6
	P.B.	0.4	0.31	0.35 - 0.44
31-40	A.S.	1.2	1.04	0.66 - 1.74
	E.S.	3.9	4.70	1.50 - 6.30
	C.S.	1.0	1.54	0.10 - 1.84
	E.I.	42.2	21.9	30.6 - 53.8
	C.I.	51.0	24.1	38.2 - 63.8
	P.B.	0.4	0.31	0.38 - 0.42
41-50	A.S.	1.2	2.01	0.14 - 2.26
	E.S.	2.1	1.83	1.12 - 3.08
	C.S.	0.7	1.18	0.08 - 1.32
	E.I.	31.0	12.4	25.8 - 38.5
	C.I.	66.5	18.4	59.5 - 73.5
	P.B.	0.15	0.14	0.07 - 0.23
51-60	A.S.	1.0	1.03	0.46 - 1.54
	E.S.	4.8	3.70	2.82 - 6.70
	C.S.	0.7	1.18	0.08 - 1.32
	E.I.	36.4	12.1	26.3 - 46.0
	C.I.	58.9	17.3	47.5 - 68.3
	P.B.	0.22	0.15	0.14 - 0.30

Females (cont.)

AGE GROUP	CELL TYPE	MEAN %	STANDARD DEVIATION	CONFIDENCE INTERVALS
61-70	A.S.	1.0	1.48	0.22 - 1.78
	B.S.	2.0	1.78	1.05 - 2.04
	C.S.	0.5	0.17	0.42 - 0.58
	E.I.	39.7	21.4	23.3 - 51.1
	G.I.	42.2	25.7	23.6 - 55.8
	R.B.	0.22	0.15	0.14 - 0.30
71-80	A.S.	1.0	1.14	0.40 - 1.60
	B.S.	3.2	2.95	1.64 - 3.76
	C.S.	1.6	2.17	0.44 - 2.76
	E.I.	44.4	21.1	33.2 - 55.6
	G.I.	51.0	21.5	40.5 - 63.3
	R.B.	0.8	0.4	0.10 - 0.50

PERCENTAGE DISTRIBUTION OF CELLSMean Values of All Age Groups

SEX	CELL TYPE	MEAN %	STANDARD DEVIATION	CONFIDENCE INTERVALS
Male	A.S.	1.03	0.50	0.07 - 1.39
	E.S.	2.72	1.23	1.84 - 3.72
	C.S.	0.72	0.57	0.35 - 1.10
	E.I.	36.5	6.13	31.9 - 41.1
	C.I.	58.7	6.51	48.7 - 68.7
	P.B.	0.20	0.22	0.04 - 0.36
	Female	A.S.	0.92	0.20
E.S.		2.45	1.19	1.55 - 3.35
C.S.		0.57	0.31	0.35 - 0.79
E.I.		40.1	5.14	36.3 - 43.9
C.I.		53.8	7.68	48.0 - 59.6
P.B.		0.28	0.28	0.06 - 0.50

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PERCENTAGE DISTRIBUTION OF CELLSPremature Infants

The following group is that of five smears taken from premature infants who were delivered after 32 to 37 weeks in utero.

CELL TYPE	MEAN %	STANDARD DEVIATION	CONFIDENCE INTERVALS
A.S.	1.40	1.34	0.00 - 2.94
E.S.	8.00	5.70	1.42 - 13.7
C.S.	1.20	0.84	0.24 - 2.16
E.I.	37.6	15.9	19.3 - 55.9
C.I.	50.4	19.6	27.0 - 73.2
P.B.	1.30	1.30	0.00 - 2.70

It was noted that there was a general pattern of distribution of the various types of cells which remained constant in all age groups and in both sexes, except in one instance. In general, the cyanophilic intermediate cells made up the largest group of cells, followed by the eosinophilic intermediate cells, the eosinophilic superficial cells, the anucleate squames, the cyanophilic superficial cells and the parabasal types.

The one exception to this pattern occurred in the 0-10 year old female group where the mean values for the eosinophilic intermediate cells exceeded the value for the cyanophilic types. The range, as evidenced by the confidence intervals, would still permit conformation with the general pattern.

Binucleated cells were occasionally observed and were mainly in cell types derived from the deeper layers of the epithelium. The presence of these cells did not seem to have any relation to either age or sex.

The staining reactions of the cells would seem to indicate that, in the course of changes leading to keratinization, the nucleus is the first part of the cell that exhibits changes. If the process progresses

farther, than the cytoplasm is also affected.

The study of a smear from the buccal mucosa would be incomplete if attention was not paid to the other details found in the smears aside from the epithelial cells. The most constant finding was the many types of bacteria which occurred in varying numbers. Leucocytes were present in some of the smears and appeared to be mainly polymorphonuclear neutrophils. A varying amount of mucous material and debris was always present.

In summary, it can be stated that within the age limits of this study and using the method outlined, age or sex does not significantly influence the cellular pattern of the buccal mucosa.

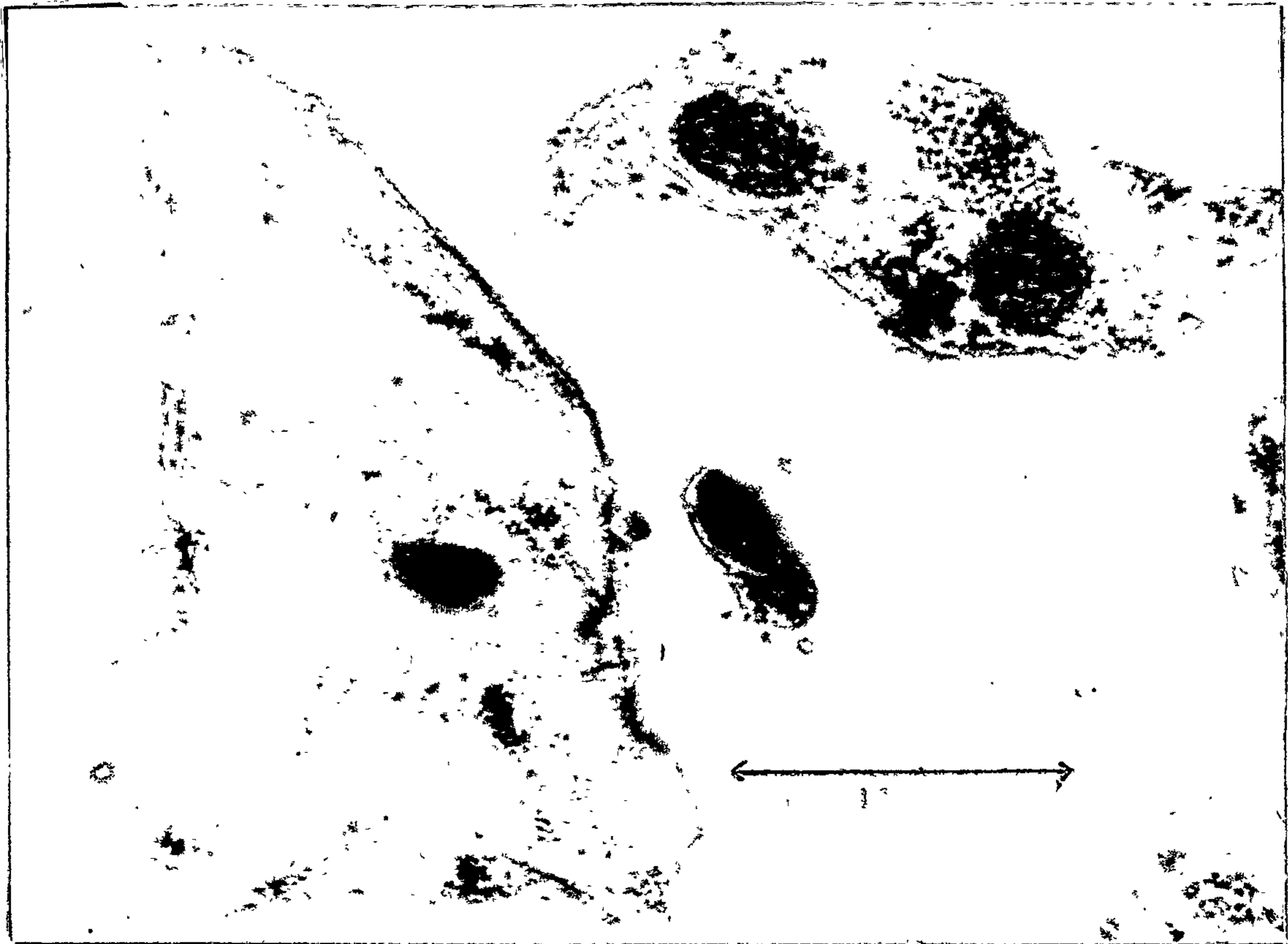


FIG. 2 - BUCCAL SMEAR OF A SIX YEAR OLD FEMALE.  
(10  $\mu$  scale imprinted)

Cell on left hand side is an Eosinophilic Superficial Cell.

Cell on upper right is a binucleated Cyanophilic Intermediate type.

Remaining cell to the right of centre of the photomicrograph is a Parabasal Cell.

The relative size of the different types of cells seen in smears is well demonstrated.

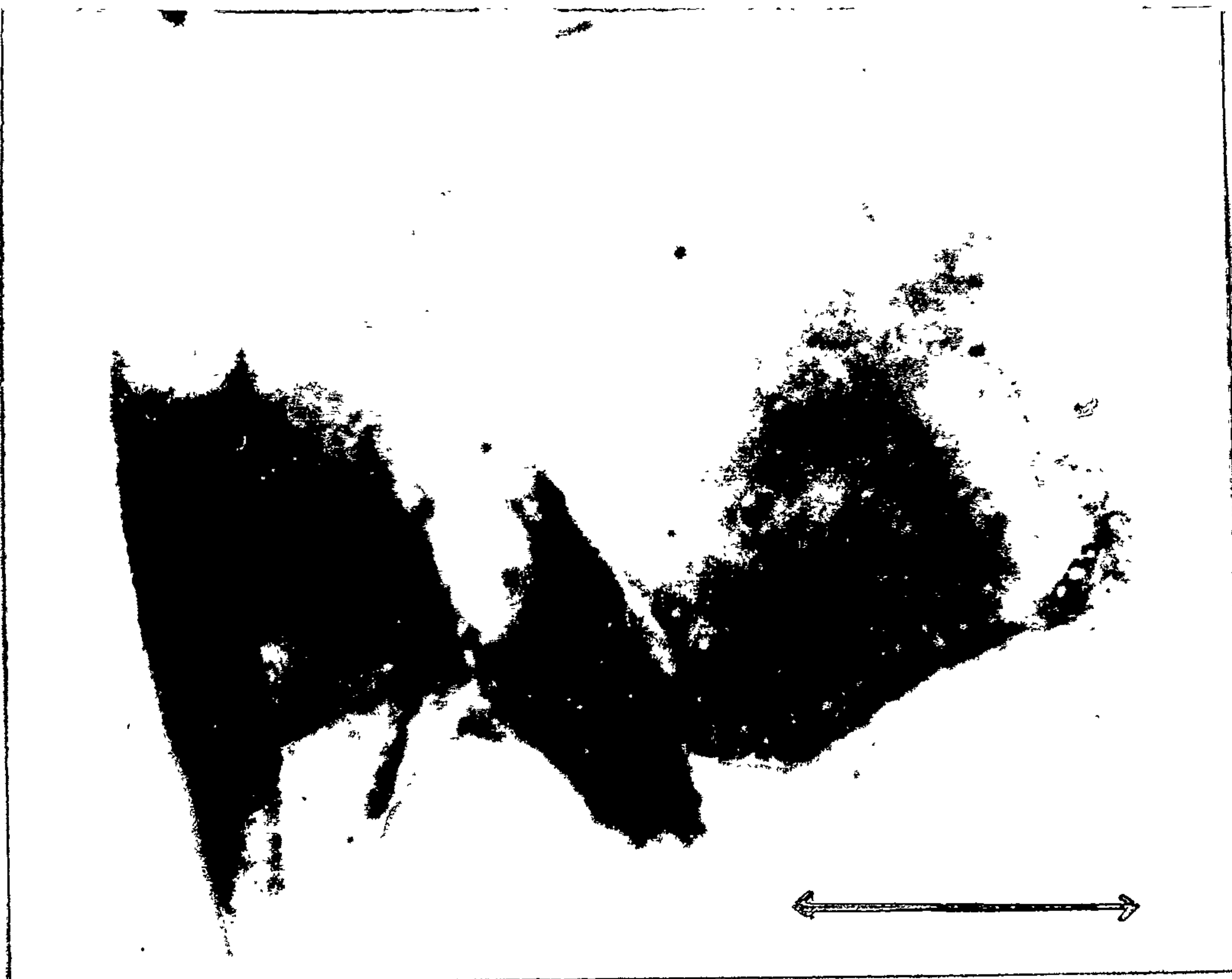


FIG. 3 - BUCCAL SMEAR OF A 36 YEARS OLD MALE  
(10  $\mu$  scale imprinted)

The photomicrograph shows several anucleate squames, in some instances overlapping each other. No evidence of a nucleus is visible although in some cases a "halo effect" is noted. (not in this view)

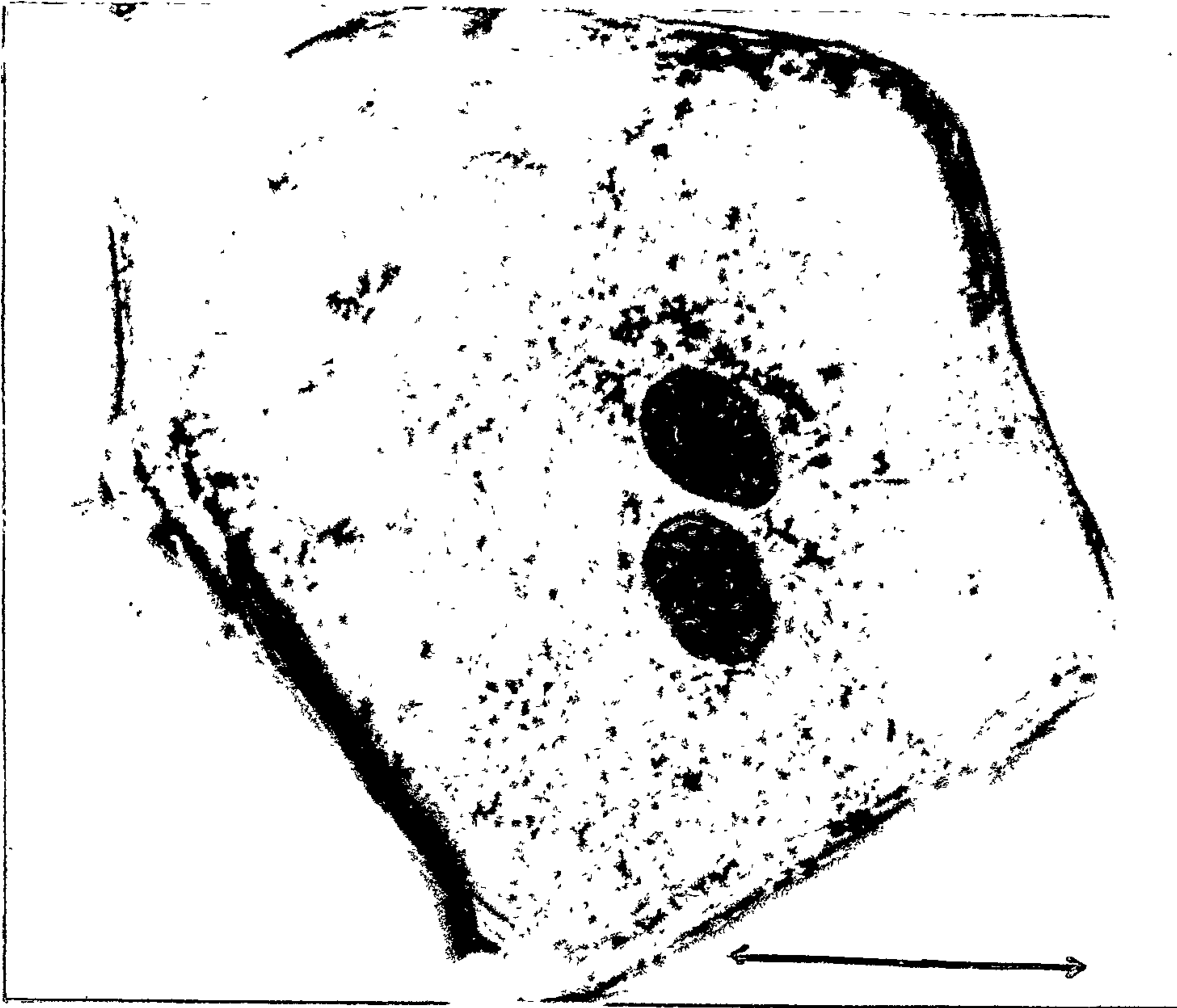


FIG. 4 - BUCCAL SMEAR OF A 76 YEAR OLD FEMALE  
(10  $\mu$  scale imprinted)

The photomicrograph shows a binucleated cyanophilic Intermediate cell. Note the presence of granules and vacuoles within the nuclei.

Such binucleated cells are rare in normal smears, but no special significance has been attached to their occurrence.

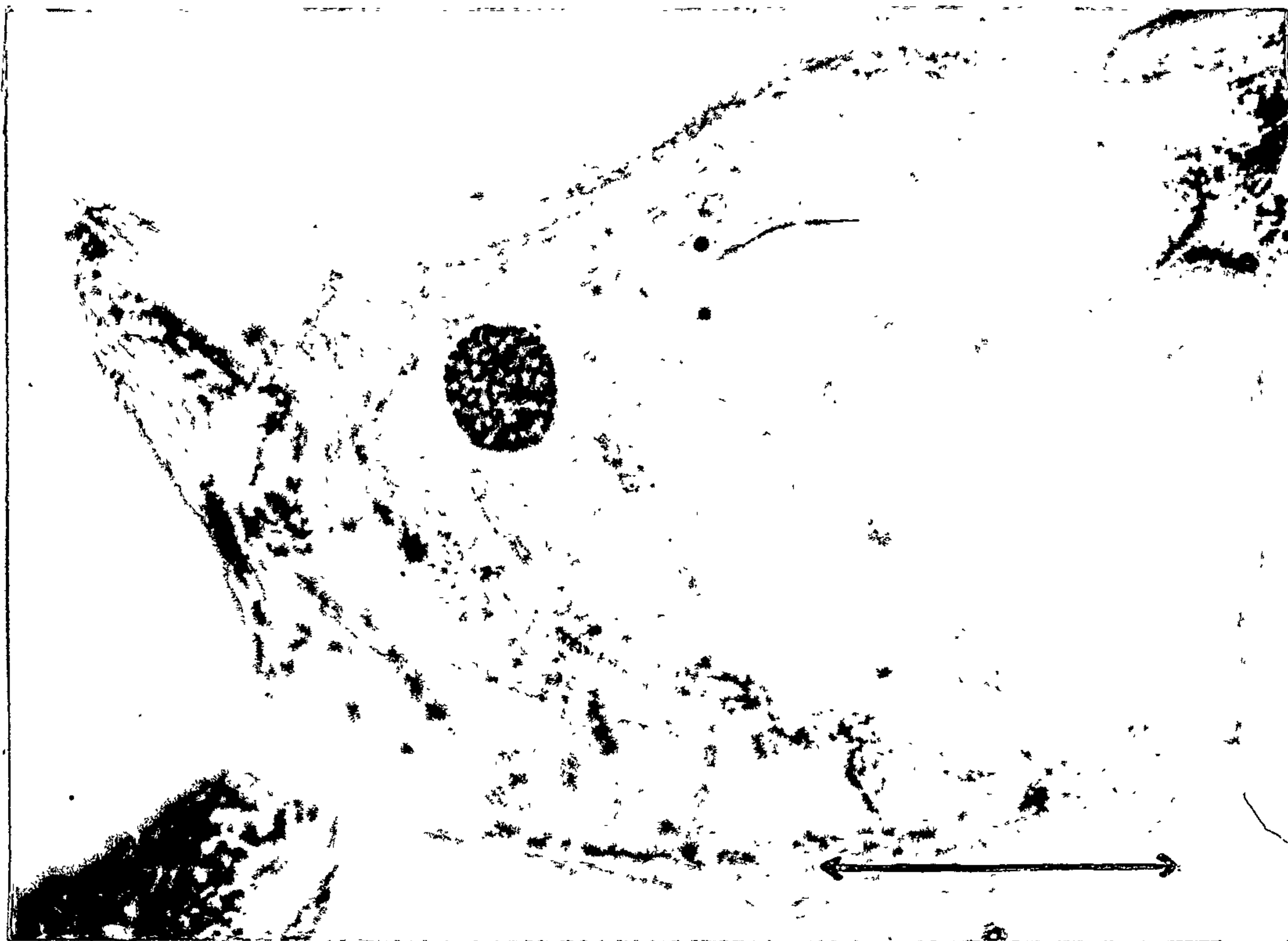


FIG 5.- BUCCAL SMEAR OF A 22 YEAR OLD MALE  
(10 u scale imprinted)

The photomicrograph shows an eosinophilic superficial cell.

The nuclear changes are not as far advanced as in most superficial cells but are suggestive of early degenerative changes in the nucleus. It is clearly shown that the measurement of the nucleus of the cell shows a diameter less than 6 u, which is the micrometric criterion for pyknosis.

## DISCUSSION

Most clinicians consider atrophy of the oral epithelium occurs with increasing age. Burket (81) describes these changes in the following manner: "The lips, the oral mucosa and the tongue in senescence present changes which are comparable with those observed in other tissues. They become atrophic; there is a loss of elasticity and a decrease in the tunica propria; the characteristic stippling of the healthy gingival and oral tissue seen in adult life is absent in the aged. The tissues frequently lack a protective layer of keratinized cells which render them more susceptible to mechanical, chemical or bacterial irritation."

Massler (82) reports a loss of elasticity in the oral mucosa with dryness and atrophy together with a tendency to hyperkeratosis. He states that there is a progressive thinning of the epithelial layer as age advances.

The findings of this study, together with others, contradicts the common concept of ageing, according to which bodily processes slow down and the

regenerative capacity of the tissues diminishes with age.

Meyer, Marwah and Weimann (83) studied age differences in the mitotic index of the epithelium of the attached gingiva in 30 male subjects aged 25 to 35 years and 30 aged 50 to 75 years. The older group was found to have, on the average, 50% more mitotic activity than the younger one. Their study was stimulated by Bullough's finding that the mitotic frequency in mouse epidermis is considerably higher in middle-aged than in young animals even before they have reached full size. (84)

The first author to raise the question whether physiologic regeneration of epithelia actually slows with age was Picon (85) who studied the epidermis of abdomen and back of mice from birth to 33 months of age. Since this study was made in 1933, it is not surprising that no attention was paid to the effect of diurnal variation and strain differences. At six months the rate of division was twice as high as at one month and remained higher than at one month for the entire period studied.

A study on human epidermis by Thuringer and Cooper (86) for which quantitative findings were not reported, showed age changes in the same direction. Katzberg (87) reported a doubling of mitotic frequency from the first to the eighth decade in human abdominal epidermis.

Before generalizing such observations too sweepingly, it would obviously be desirable to extend them. Meanwhile, however, it appears that the concept of ageing as a general slowing down of physiologic regenerative processes cannot be maintained for epithelia. Whether it should be exchanged for the opposite, that is, whether there is a general acceleration of epithelial proliferation, is doubtful.

It may be worth mentioning that the cases cited above are examples of accelerated rates of regeneration, but not of hyperplasia. Absence of age changes in the thickness of epithelium and length and configuration of the epithelial ridges of the gingiva was noted by Wentz, Meier and Urban (88), and no increase in the number of cell layers was observed by Dullough (84) or Thuringer. (86) Thus shedding at the surface keeps

pace with the increased rate of proliferation. This distinguishes such cases from cases like the benign hypertrophy so frequently seen in the prostate of older individuals and from the proliferation of the human cardiac glands in old people observed by Lendrum. (89)

It is considered that useful information could be obtained by extending the study of normal buccal exfoliated cells to include investigation by modern cytochemical staining methods in the estimation of sulphhydryl activity, DNA and RNA content using acridine orange and fluorescence microscopy.

Enzymatic and histochemical examination of normal exfoliated cells is necessary to amplify on studies which have demonstrated the usefulness of monitoring the oral manifestations of toxicity in patients receiving chemotherapy for malignant disease. (90)

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