CYTOPLASMIC GRANULES IN HUMAN ORAL AND VAGINAL EPITHELIUM

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SUMMARY

A survey of the literature reveals controversy concerning the occurrence, origin, and function of keratohyalin granules in the stratified squamous epithelium of the human mouth and vagina.

The aims of the present study were to establish the fact that so-called keratohyalin granules are present in the epithelium of the mucosal surfaces of the mouth and vagina, to suggest a possible origin of the granules, and to investigate any relationship between the incidence of the granules and degree of keratinization.

The subjects used in the investigation were human adults, infants, and foetuses.

Histological sections and mucosal smears of oral epithelium were examined with the light microscope following histochemical treatment. Oral smears were also examined by phase contrast microscopy and investigated with microbiological culture methods. Statistical analysis was carried out on the results of differential counts concerning the incidence of keratohyalin granules and the degree of keratinization in buccal smears.

Vaginal smears were examined with the light microscope following staining by the Papanicolaou method and by phase contrast microscopy.

The results proved that keratohyalin granules occurred in both oral and vaginal epithelium, and that the appearances were not due to pigments, staining artefacts, or micro-organisms.

Granules appeared to have their origin in the nucleus of the epithelial cell and their incidence bore no relationship to degree of keratinization. This last finding supports the concept that the keratohyalin granules are not directly involved in the process of keratinization but ultimately become an interfibrillary component of the keratinized cell.

This thesis is submitted in fulfilment of the requirements for the degree of Master of Dental Surgery, University of Adelaide. A year of full-time study, leading to the attainment of the Honours degree of Bachelor of Dental Surgery in 1961, fulfilled the requirements for the entry to candidature for the degree of Master of Dental Surgery.

The thesis contains no material previously submitted by me for a degree in any University, and to the best of my knowledge and belief it contains no material previously published or written by another person, except when due reference is made in the text.

Robert V. Blanden.

April, 1964.

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GENERAL INTRODUCTION

Stratified squamous epithelium is a protective tissue which occurs in the skin (a dry surface), and the wet mucosal linings of the oral cavity, oropharynx, oesophagus, and vagina. Its protective function is attained through a multi-layered structure in which the cells of the deepest layer, nearest the underlying connective tissue, undergo mitotic division to replace cells lost at the surface. As the cells progress from the deepest layer to the superficial layer they undergo a course of differentiation which changes them morphologically and chemically so that they function effectively as a mechanical protection. Their course of differentiation in normal epithelium appears to vary according to the demands of the situation, and its ultimate is the formation of a cell which is essentially fibrous protein (keratin). These dead, keratinized cells then form the surface layer of the tissue.

Histology.

Epidermis.

In the epidermis of human skin the course of differentiation is fully developed and the following layers can be identified in histological sections of the epithelium, cut at right angles to its surface and

examined with the light microscope (Montagna 1962).

1. Basal layer (stratum basale), the deepest single layer of epithelial cells, cuboidal or columnar in shape, arranged on a basement membrane.

2. Prickle-cell layer (stratum spinosum), lying superficial to the basal layer and composed of polyhedral cells which appear to be connected by intercellular bridges from which the name was derived. These cells become more flattened as they approach the succeeding layer.

3. Granular layer (stratum granulosum), composed of flattened cells containing basophilic cytoplasmic granules.

4. Hyalin layer (stratum lucidum), composed of flattened cells which do not stain readily by histological techniques.

5. Cornified or keratinized layer (stratum corneum). The surface layer, composed of flattened, scale-like cells containing the fibrous protein keratin.

Cells in the basal layer and the immediately adjacent cells in the prickle-cell layer are the only epithelial cells capable of reproduction by mitosis. These cells are known collectively as stratum germinativum.

Cells from the basal layer, prickle-cell layer and granular layer contain nuclei and are vital. They are known collectively as stratum Malpighii.

The stratum lucidum and stratum corneum are composed of cells without nuclei which are non-vital.

Epithelium with an anuclear, keratinized surface layer (as in epidermis) is defined as keratinized epithelium.

Oral epithelium.

Text-books of Oral Histology (Orban, 1962; Noyes, 1960) classify the normal stratified squamous epithelium of the mouth into four categories, depending on the degree of keratinization, or, more accurately, the degree of development of the course of differentiation undergone by the epithelial cells.

1. Keratinized epithelium (fig. 1.)

Composed of (i) Basal layer,
(ii) Prickle-cell layer,
(iii) Granular layer,
(iv) Keratinized layer.

There is no stratum lucidum, and stratum corneum is never as thick as that of epidermis.

2. Parakeratinized epithelium (fig. 1.)

Composed of (i) Basal layer,
(ii) Prickle-cell layer,
(iii) Parakeratinized surface layer.

which consists of flattened cells which stain similarly

to keratinized cells but still retain their nuclei. This fact, plus the absence of a granular layer are the two essential features of parakeratinization.

3. _Incompletely parakeratinized epithelium._

This is similar to parakeratinized epithelium except that the staining of the surface cells with Mallory's technique differs. The deeper part of the parakeratinized surface stains like keratin but this staining is lost in the superficial cells, perhaps due to the influence of the oral fluids on incompletely formed keratin in the nucleated cells.

4. _Non-keratinized epithelium_ (fig. 1.)

Composed of (i) Basal layer,
(ii) Prickle-cell layer,
(iii) Non-keratinized surface layer, consisting of one or two layers of flattened cells containing nuclei and not staining like keratin.

Oral mucosa is divided into three categories depending on function.

1. _Masticatory mucosa_: gingiva and hard palate.
2. _Lining mucosa_: soft palate, buccal and labial mucosae, alveolar mucosa, ventral surface of tongue, and floor of mouth.
3. _Specialized mucosa_: dorsum of tongue.
   (i) Anterior part - masticatory.

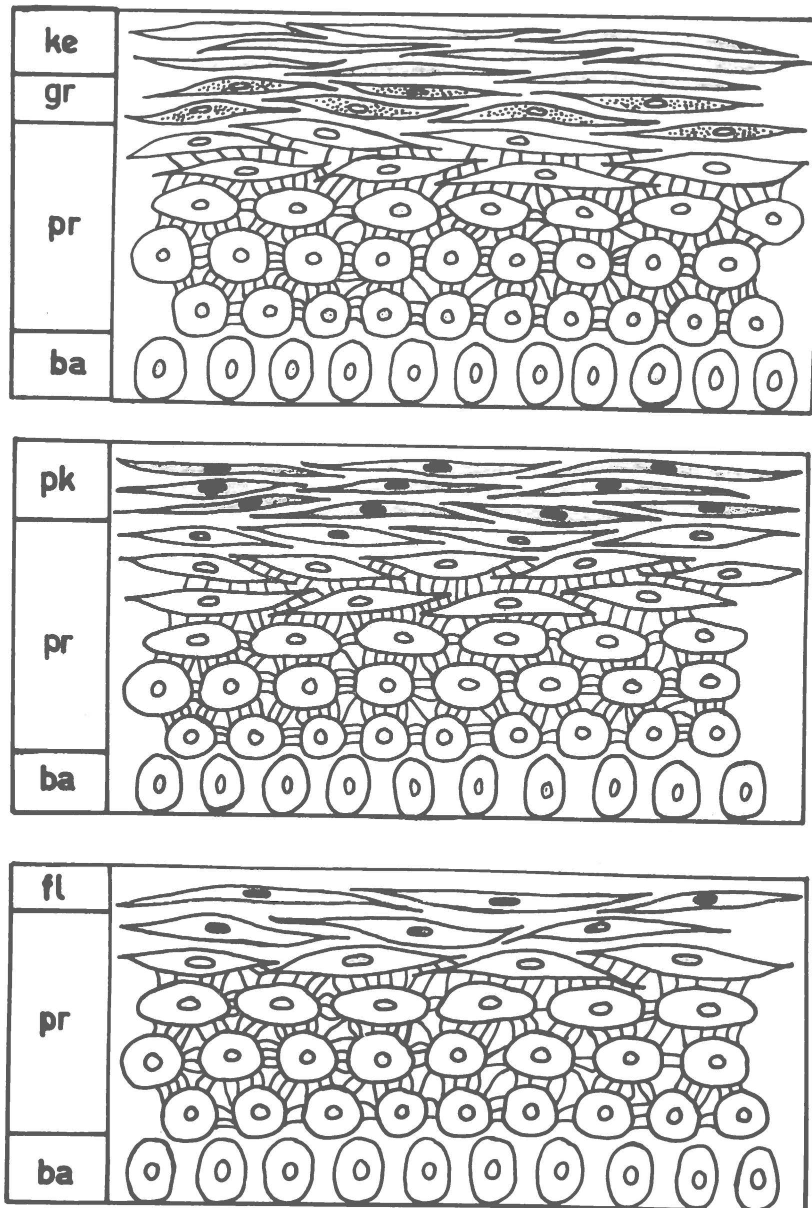

Fig. 1. Diagrammatic representation of histological sections of oral epithelium.

Top: keratinized.
Middle: parakeratinized.
Bottom: non-keratinized.

Legend: keratinized layer (ke), granular layer (gr), prickle-cell layer (pr), basal layer (ba), parakeratinized layer (pk), flattened surface layer (fl).

(ii) Posterior part - lining.

As a general rule, masticatory mucosa is keratinized and lining mucosa is non-keratinized but either may be parakeratinized.

Vaginal epithelium.

There is some variation in text-book descriptions of normal vaginal epithelium. In "The Cytologic Diagnosis of Cancer" by the Vincent Memorial Hospital Staff (1958) vaginal squamous epithelium is said to be composed of three layers.

1. Germinal layer, adjacent to the basement membrane.
2. Transitional or basal layer.
3. Superficial layer, consisting of so-called cornified cells with small nuclei.

On the other hand, Papanicolaou (1954), in his "Atlas of Exfoliative Cytology" describes five zones of cells in vaginal epithelium.

1. Basal zone, a single layer of cuboidal or columnar cells.

2. Parabasal zone, several layers of round, oval, or polyhedral cells with inter-cellular bridges.

3. Intermediate zone, several layers of moderately flattened cells with inter-cellular bridges.

The fourth and fifth zones very depending on the type of epithelial surface, which, Papanicolaou states, may be

either fully keratinized (which is rare and pathological) or "cornified" which is normal.

If the surface is fully keratinized the fourth and fifth layers are:-

4. Independent zone, which corresponds to the stratum granulosum of epidermis, as the flattened cells contain basophilic granules.

5. Superficial zone, composed of several layers of flattened anuclear cells.

If the surface is cornified the fourth and fifth layers are:-

4. A narrow dense zone, not always present, composed of flattened, densely packed, acidophilic cells which may represent densification and collapse of the deeper layers of the superficial zone.

5. Superficial zone, consisting of several layers of flattened but nucleated cells.

Histological Definitions.

There appears to be universal accord in the definitionsdescribing keratinized epithelium and non-keratinized epithelium. However, "cornification" as used by Papanicolaou and "parakeratinization" as used by oral histologists appear to describe similar types of epithelium. "Cornification" and "keratinization" are used with

confusion in the literature, and at times are undoubtedly synonymous. In future sections of this text, "cornification" and "cornified" will only be used in quotations from other writers, and where the meaning implied by a particular writer is ambiguous in that "cornified" could mean either "parakeratinized" or "keratinized".

Cytology.

The epithelial cell.

Examination under the light microscope of sections of stratified squamous epithelium stained by haematoxylin and eosin shows that the cells usually have one nucleus (rarely two, and no nucleus in the keratinized surface). The nucleus has a basophilic border (the nuclear membrane), finely reticulated basophilic chromatin and sometimes a basophilic nucleolus. As the cell nears the superficial zone, the nucleus may become smaller so that it appears as a dark mass of basophilic material. Such a nucleus is described as pyknotic.

The cytoplasm of the cells of the deeper layers is basophilic. The basal cells may contain melanin pigment granules and the cells of the granular layer (if present) contain basophilic cytoplasmic granules which are known as "keratohyalin granules".

Mitochondria can be found in the epithelial cells under the light microscope but only if special techniques

are used. They have been defined by Montagna (1962) as:-

1. Intra-cytoplasmic elements demonstrable in living cells with dark field or phase contrast microscopy as motile filaments, rods or granules with a definite polarity.

2. These elements that are stained selectively supra-vitally with Janus green.

3. Those pleomorphic cytoplasmic entities in appropriately fixed tissue which can be stained with Regaud's and Heidenhain's haematoxylin or with Altmann's technique.

Ultra-structure.

Studies of epithelial cells with the electron microscope have elucidated the structure, distribution and possible function of cytoplasmic organelles not readily demonstrable with the light microscope.

Mitochondria appear in basal epidermal cells as long, narrow bodies (Montagna, 1962). As the cells become flattened nearing the granular layer, normal mitochondria are absent, but small spherical granules believed to be fragmented mitochondria are discernable.

An abundance of mitochondria in the basal layer of oral epithelium has been described by Sognnaes and Albright (1958). Normal mitochondria become increasingly

hard to find in the superficial oral epithelial cells, where they appear to be vesiculated. These altered mitochondria may be seen in complexes of finer cytoplasmic elements.

The Golgi apparatus, another cytoplasmic organelle, occurs in epithelial cells from the deeper layers of epidermis and oral epithelium. Like mitochondria, it is difficult to define in more superficial cells.

Smaller bodies termed ribosomes are present on the endoplasmic reticulum in the deeper cell layers.

The structure of the intercellular bridges of the prickle-cell layer has been investigated with the electron microscope. They are termed desmosomes.

Basal cells of epidermis have fewer, smaller desmosomes which caused Hibbs and Clark (1959) to believe them better adapted to divide and glide over each other during mitosis. Cells in the layers superficial to the prickle-cells have smaller desmosomes.

Fine protein filaments or tonofibrils occur in the cytoplasm of epidermal cells and are associated with the structure of the desmosomes.

Histochemistry.

Some cytoplasmic constituents of the epithelial cell can be demonstrated by special histochemical techniques.

They include lipids, polysaccharides and enzymes.

Lipids occur as sudanophilic perinuclear granules in epidermal cells.

Glycogen is often present in squamous epithelial cells of mucosal surfaces but rarely in epidermis (Montagna, 1962). Most investigators agree that where keratinization occurs, glycogen is absent, and vice versa. In a study of white lesions of the oral mucosa, Cahn, Eisenbud, and Blake (1961), found that the basal layer is always free of glycogen, which is usually confined to the outer two-thirds of the epithelium. A further paper (Cahn, 1961) stated that glycogen is not found in invasive cancer, carcinoma in situ, or in keratinized lesions. (This is the basis of the Schiller iodine test (1933) used in the clinical diagnosis of oral lesions, and in suspected cases of carcinoma of the uterine cervix). The occurrence of glycogen appears to be related to the degree of epithelial hyperplasia (Turesky, Glickman, and Litwin, 1961), and epithelial glycogen has been found to increase during inflammation and repair (Dewar, 1955).

A large number of enzymes have been demonstrated in the epidermis and the epithelium of the mouth and vagina. Some of those more relevant to the mucous membranes are reviewed below.

Alkaline phosphatase has been demonstrated in oral epithelium, although not conclusively. Ring and Levy (1950) found that in the rat, oral epithelium exhibited varying activity of alkaline phosphatase during the oestrus cycle; this could be correlated with similar changes in the vaginal epithelium. A study on the levels of alkaline phosphatase in human vaginal epithelium (Herovici, 1960) showed that the enzyme reached a maximum concentration at the time of ovulation. It occurred mainly in the superficial layers. A curve of alkaline phosphatase activity was symmetrically opposed to that of glycogen. On the other hand, Kirkland (1964) found that the vaginal epithelium demonstrated no alkaline phosphatase activity if an azo-dye technique were used. He considered that results obtained with the Gomori technique were unreliable. A relationship between alkaline phosphatase and keratinization has been suggested by some writers.

The presence of acid phosphatase in the superficial layers of oral epithelium, with the exception of the keratinized zone was noted by Cabrini and Carranza (1958). A similar distribution was reported in skin (Moretti, Adachi, and Ellis, 1960). Examination of vaginal epithelium revealed acid phosphatase activity in the basal and intermediate

layers but not in the superficial layer (Kirkland, 1964).

Esterase activity has been observed in the superficial layers of oral epithelium, including the keratinizing zone.

A group of enzymes capable of hydrolysing the beta-glycoside linkage of a number of naturally occurring and synthetic glucuronides are known as beta-glucuronidases. They occur in all animal tissues but mainly in liver, kidney and spleen. Fishman (1950) has suggested three possible roles for these enzymes:

1. Conjugation of steroid hormones.
2. Hydrolysis of conjugated glucuronides.
3. A role in cellular proliferation.

The activity of such enzymes in the basal layer of oral epithelium in both humans and rats was described by Cabrini and Carranza (1960) suggesting a possible role in cellular proliferation.

Cytochrome oxidase has a low activity in oral epithelium but is found in the basal cells in some areas. Inflammation appears to increase its activity (Burstone, 1960). Iron deficiency may reduce cytochrome oxidase activity (Beutler, 1959), and Waldenstrom (1958) believed that changes occurring in the oral and oesophageal mucosae in iron deficiency were due to lack of the enzymes.

Eichel (1960) described the distribution of succinic

dehydrogenase in the gingival epithelium. Most activity was found in the basal layer. This was confirmed by Mori and Kishiro (1962) who stated that in the oral epithelium, the basal and adjacent prickle-cells exhibit succinic dehydrogenase activity. On the other hand, triphospho-pyridine nucleotide diaphorase was found to be equally active in all the deep layers of the epithelium but the superficial layer was inactive. The distribution of both succinic dehydrogenase and TPN diaporase was found to be in the peri-nuclear cytoplasm. They, along with cytochrome oxidase are believed to be located in close relationship to mitochondria,and their distribution supports this view.

Amino-peptidase, a proteolytic enzyme, has a low activity in the oral epithelium (Orban, 1962).

Biochemistry and Physiology.

The biochemistry of the individual cell of stratified squamous epithelium is the basis of its final physical structure, which is related to the function of the single cell, and to the epithelial tissue as an entity.

An epithelial cell consists essentially of a cell membrane surrounding protoplasm which consists of a solution of inorganic and organic compounds in water.

The nucleus is separated from the cytoplasm by a

nuclear membrane. Nuclear chromatin contains desoxyribonucleic acid (DNA). In females, a distinctive "sex chromatin" body can be identified in the nucleus of stratified squamous epithelial cells. The nucleolus contains ribonucleic acid (RNA). Nucleoproteins (DNP and RNP) are the structural elements of the nucleus.

The cytoplasm is a solution of mineral salts and organic compounds such as enzymes, polysaccharides and mucopolysaccharides, ribonucleic acid, ascorbic acid, sulphydryl and di-sulphide groups and other acid groups. Lipids occur as small droplets.

Keratolyalin granules are surrounded by a capsule of ribonucleoprotein (RNP) and contain dense mineral material (probably containing calcium).

Mitochondria are thought to contain protein fibrils and are surrounded by small protein granules.

The Golgi apparatus may contain lipids and phospholipids.

Ribosomes contain ribonucleic acid.

A concept of the physiology of the epithelial cell as it undergoes its course of differentiation from the basal layer to the surface of fully keratinized stratified squamous epithelium can now be presented.

1. Daughter cells originate in the basal layer of

the epithelium as a result of a mitotic division of a basal cell. It is thought that this "squeezes" cells out of the basal layer and into the prickle-cell layer. The rate of mitosis appears to be at a level to meet the need for the replacement of cells which are lost from the surface layers. There are fluctuations in mitotic rate which are related to periods of rest and activity e.g. the rate increases during sleep, and decreases during muscular exercise, hunger and stress. Further fluctuations may be caused by the oestrus cycle in the female, and by the rate at which cells are lost from the epithelial surface. A higher oestrogen level induces more mitoses as does a higher rate of surface loss.

2. As the cell progresses through the prickle-cell layer it accumulates a number of basophilic granules, the so-called "keratohyalin granules", and its cytoplasm becomes more expansive and flattened in the plane of the epithelial surface. It is then a part of the granular layer.

3. It is believed that the granular layer is the seat of the major changes in the epithelial cell. Keratin formation occurs and the cell loses its nucleus and its keratohyalin granules to become an anuclear squame composed of fibrous protein, lipid and organic salts. This change involves water loss and must require high

metabolic activity in which enzyme systems are no doubt essential. There is much diversity of opinion concerning the roles played by the numerous components of the epithelial cell during this process of keratinization.

Montagna (1962) states, "Mitochondria are the seat of all important biological syntheses, and the process of keratinization must be mediated by them". In stratified squamous epithelium, however, intact, normal mitochondria cannot be found in the granular layer. This, along with electron microscopic evidence, has led a number of workers to believe that keratin formation begins in the basal layer, where mitochondria are abundant, and that the granular layer is the site of wholesale macromolecular rearrangements.

A simple biochemical concept of protein synthesis was presented by Datta and Ottaway (1962). Protein biosynthesis involves the polymerization of amino-acids with the formation of peptide bonds, an energy consuming process. It is thought to require the presence of ribonucleic acid (RNA), a source of regenerate adenosine triphosphate (ATP), some sort of cytoplasmic organelle, and ground cytoplasm. A series of steps leading to the formation of a simple protein is outlined below.

1. Amino acids are activated by a specific enzyme and ATP to form an (aminoacyl-AMP)-enzyme complex and

pyrophosphate.

2. The activated amino acid is then transferred to soluble RNA in the cytoplasm. There is probably a specific RNA for each amino acid.

3. The amino acid, now attached to RNA, is transferred again to an appropriate site on an RNA particle on a ribosome. This particulate RNA acts as a template for protein synthesis, controlling the sequence in which the amino acids appear. The code of control is ultimately determined by the DNA of the nucleus which controls the synthesis and varies the structure of ribosomal RNA.

4. When the polypeptide chain of amino acids has been completed, the primary structure of the protein has been determined. The protein is then detached from the RNA and the secondary and tertiary structures are formed.

It may be that a simple protein fibril, a keratin precursor, is formed in the layers deep to the granular cells by such a process. There is abundant evidence in the literature of the presence of the requirements such as the nucleic acids, cytoplasmic organelles and enzyme systems, and of the presence of such fibrils. This may be followed, in the granular layer, by the addition of secondary and tertiary structures, involving other biologically active substances, ascorbic acid and sulphydryl groups. (Ascorbic acid may mediate transformation of

sulphydryl groups to disulphides.)

The keratin molecule was investigated by Swanbeck (1959) using X-ray diffraction patterns. He suggested a hypothetical model of the fibril unit as being made up of protein cylinders surrounded by a lipid layer with lipid chains arranged radially on the protein cylinder.

The part played by keratohyalin granules, once thought to be keratin precursors, is not clear, but there is no doubt that they become part of the fully-keratinized squame in stratum corneum.

5. With the loss of keratohyalin granules, the cell enters the lower part of stratum corneum or stratum lucidum where loss of the nucleus occurs (Santoianni and Rothman, 1962). Degradation of nuclear material is probably an enzymatic process. Animal experiments have demonstrated an association between desoxyribonuclease II activity and nuclear breakdown,and histochemistry reveals ribonuclease activity in the lower portion of stratum corneum.

Factors Affecting the Epithelium.

With the loss of the nucleus the cell becomes the dead mass of fibrous protein, lipids and organic salts which constitute stratum corneum.

It is probable that the different types of histolog-

ical appearance of stratified squamous epithelium (viz. keratinized, parakeratinized and non-keratinized) represent different degrees of activity of the processes outlined above. Thus keratinized epithelium would represent the highest degree of epithelial metabolic activity and non-keratinized epithelium the lowest, with parakeratinized epithelium occupying an intermediate position in the scale.

If the epithelial mitotic rate increases, or outstrips the functional demand, a hyperplastic epithelium results. This may be confined to the basal layer as basal cell hyperplasia or may result in a thickening of the prickle-cell layer which is known as acanthosis. A thickening of the keratinized surface layer is denoted hyper-keratinization.

Conversely,hypoplasia produces a thinner epithelial tissue which may be referred to as atrophic.

The factors effecting mitotic rate have been briefly discussed previously; they can be related to the factors which produce clinical changes in stratified squamous epithelium.

1. <u>Genetic factors</u>.

It has been suggested by Montagna (1962) that different areas of epidermis have a different genetic character. This is supported by experimental grafting of

plantar epidermis to areas such as the chest where the epithelium is normally thin. Such plantar epidermis retains its thickness.

2. Local external environmental stimuli.

Vaginal and uterine cervical epithelium, in cases of uterine prolapse, may become fully keratinized. This is a protective measure against the abnormal trauma and drying produced by the prolapse. Trauma to the gingivae, due to toothbrushing, may induce hyperkeratinization.

3. Hormonal factors.

Normal vaginal epithelium is influenced more by hormone levels than by any other factors. Similar studies on the effect of oestrogens on the oral epithelium have given conflicting results. Although parallel changes in degree of keratinization have been demonstrated in oral epithelium and vaginal epithelium during the menstrual cycle, it appears that local environmental factors have more influence on the oral epithelium than do hormones.

4. Nutrition.

Nutritional disturbances may greatly influence the state of stratified squamous epithelium. Vitamin A deficiency causes keratinization of normally non-keratinized epithelium and overdose of Vitamin A may cause parakeratinization of normally keratinized epithelium.

Vitamin B complex deficiency produces atrophic

changes in the oral epithelium, particularly in the specialized epithelium of the dorsum of the tongue. Iron deficiency anaemias, pernicious anaemia and sprue may also affect the epithelium of the tongue.

These changes are thought to occur due to upsets in the enzyme systems, and in the synthesis of nucleic acids.

5. Age.

In older people atrophy of the stratified squamous epithelium occurs, along with the other body tissues. As a result,the integrity of the epithelium is more easily destroyed and its protective function is less efficient.

There is some controversy, it appears, as to the presence of keratohyalin granules in epithelium which is classified histologically as non-keratinized or para-keratinized. The origin of such granules and the part they play in the process of keratinization is similarly controversial.

The series of experiments described in this thesis have the following aims.

1. To establish that keratohyalin granules do occur in the non-keratinized and parakeratinized epithelium of the mouth and vagina.

2. To suggest a possible origin of these granules.

3. To investigate the relationship, if present, between the incidence of the granules and degree of keratinization.

4. To offer comment on the role of the keratohyalin granules in the light of the present concepts of epithelial cell function.

CHAPTER I

CYTOPLASMIC BODIES OF ORAL EPITHELIUM IN HISTOLOGICAL SECTIONS

Introduction.

The generally accepted picture of the stratified squamous epithelium of the mouth as seen in stained histological sections examined with the light microscope, and the cytoplasmic bodies present, are described below.

1. Basal layer.

Granules of melanin pigment sometimes occur in the basal layer of normal oral epithelium. These deposits are most common in races with pigmented skin. Pathological deposits of melanin may be present in Addison's disease. Melanin pigmentation in the gingiva has been found to be restricted to the basal layer with occasional granules in the adjacent prickle-cells (Dummett and Bolden, 1963). The under-lying connective tissue may also contain melanin; it is produced in melanoblasts which are thought to inject the formed pigment into the basal epithelial cells.

Very fine granules of basophilic material, identified histochemically as containing RNA, are present in the basal layer of the oral epithelium. Ribonucleic acid (RNA) is said to occur in the cells of the germinative layers, and not in the superficial strata, (Cahn, 1961). Ribonucleic acid occurring in all the epithelial layers

is thought to indicate malignant potential. These fine RNA granules are unquestionably involved in protein synthesis and cell proliferation, and are presumably constituents of ribosomes. Montagna (1962) described similar basophilic material in the basal layer of epidermis, and Herovici (1960) found that the basal layer of vaginal epithelium also contained "very fine pyroninophilic granulations".

2. Prickle-cell layer.

Apart from occasional melanin granules and some fine granules containing RNA in cells immediately adjacent to the basal layer, the prickle-cell layer is almost completely free of any cytoplasmic bodies demonstrable by light microscopy. The cytoplasmic basophilia and pyroninophilia of the basal layer fades perceptibly into the stratum spinosum. This is true for oral epithelium, epidermis and vaginal epithelium.

3. Granular layer.

The granular layer is usually described as being two to five cells thick, with the cytoplasm containing large basophilic keratohyalin granules up to three microns in diameter. In the mouth and vagina this layer is not usually present. The complete absence of a granular layer when the surface of the epithelium is not keratinized appears to be taken for granted in most texts. Only

Sognnaes and Albright (1958) make any suggestions to the contrary, and the particles they describe are demonstrable only with the electron microscope.

4. Superficial layers.

The cells of the surface of keratinized epithelium are devoid of cytoplasmic bodies, and contain keratin. In the case of parakeratinized or non-keratinized epithelium no cytoplasmic bodies are described as occurring in surface layers in histological texts.

The aim of the present study was to determine whether cytoplasmic granules occurred in histological sections of oral epithelium.

Materials and Methods.

Oral epithelium was obtained in specimens from excised oral lesions. These lesions were from various sites, the labial or buccal mucosa, the tongue, gingiva, and palate. They had often been present for many months, and there was no clinical evidence of acute inflammation, ulceration or epithelial hyperplasia. Histopathological examination had resulted in a diagnosis of benign fibrous hyperplasia in each case.

Although epithelium from these specimens could not be classed strictly as completely normal, it was considered to approximate to the normal so nearly that the inconvenient business of obtaining biopsy specimens of normal tissue from volunteers was not undertaken.

In addition, specimens of the buccal oral and epidermal epithelium of two male foetuses were obtained. The approximate crown-rump measurements of the foetuses were 95 mm. and 150 mm., and their intra-uterine ages were assessed as about 15 weeks and 19 weeks respectively (Noyes, 1960).

Details of the specimens used are contained in Table I.

After fixation in 10 per cent formol saline all specimens were processed routinely, and embedded in paraffin. The sections cut were six microns thick.

Table I

Details of specimens for preliminary observations on cytoplasmic bodies in oral epithelial cells.

The known duration was taken as the time elapsed since the patient first noticed the lesion.

The measurements given are from the fixed specimens.

The comments on histology and diagnosis are those contained in the histopathological reports from the department of Oral Pathology, Dental School, University of Adelaide.

Specimen number	Age (years)	Sex	Known Duration (months)	Shape P= Pedunculated S= Sessile	Size in Mm.	Site	Histology		Diagnosis
							Epithelium	Connective tissue	
1	33	F	120	P	3x6x2	Lower labial sulcus	Hyperplastic	Mature fibrous	Denture hyperplasia
2	36	F	1	P	2x3x3	Lateral border tongue	Hyperplastic	Vascular	Fibro-epithelial polyp
3	85	F	2	S	3x4x2	Lower buccal sulcus	Normal	Dense fibrous	Denture hyperplasia
4	47	M	3	S	5x5x2	Lower buccal sulcus	Acanthotic	Dense fibrous	Denture hyperplasia
5	57	F	96	S	5x8x4	Lower buccal sulcus	Parakeratotic	Mature fibrous	Denture hyperplasia
6	77	F	72	S	6x10x4	Lower buccal sulcus	Acanthotic	Fibrous	Denture hyperplasia
7	69	F	96	S	7x8x5	Lower buccal sulcus	Hyperplastic	Mature fibrous	Denture hyperplasia
8	61	F	24	S	5x6x2	Lower buccal sulcus	Acanthotic	Fibrous; chronic inflammation	Denture hyperplasia
9	15	F	2	P	3x3x2	Lower anterior gingiva	Acanthotic	Vascular; chronic inflammation	Fibrous epulis
10	43	F	96	P	2x5x2	Maxillary tuberosity	Keratinized	Fibrous	Denture hyperplasia

Adjacent sections from each block were stained by the following methods.

1. Routine iron haematoxylin and eosin.

This was used to obtain an overall picture of the histology of the epithelium. The haematoxylin gives an indication of cytoplasmic basophilia and stains any basophilic granules present, although this was not intended as a valid histochemical method for the indication of basophilia.

2. Methyl green - pyronin (Culling 1957)

Alcohol fixation is generally recommended for use with this stain but the morphology is then poor. As this was only a preliminary study with morphology most important, formol saline fixation was used. The purpose in using this stain was to determine whether cytoplasmic bodies containing nucleic acids or nucleo-proteins exist in the layers superficial to the prickle-cell layer. RNA stains red and DNA stains blue. Ribonuclease digestion was not used, and was not necessary, as more thorough investigation was to be carried out subsequently if warranted.

3. The Feulgen reaction (Culling 1957)

This stain is specific for DNA and depends on the release of aldehyde groups from the desoxypentose sugar of DNA by acid hydrolysis. Ten minutes of acid

hydrolysis by the standard technique was used. DNA stains red.

4. Ferric-ferricyanide reduction (Lillie 1954)

This method stains the keratohyalin granules of stratum granulosum an intense blue colour (Chevremont and Frederic, 1943). It demonstrates reduction sites in tissue. The concept of the chemistry involved in the case of the so-called keratohyalin granules appears to be in a confused state, but is irrelevant to the issue here. In this study, the reaction was used to determine whether cytoplasmic bodies, staining blue (as do keratohyalin granules), occur in the non-keratinized and parakeratinized oral epithelium.

All of the stained sections were examined with the light microscope at magnifications up to 450 diameters.

Results.

Observations are summarized in Table II. All types of oral epithelium were present in the specimens, keratinized, parakeratinized and non-keratinized. Cytoplasmic granules were present in the epithelial cells superficial to the prickle-cell layer in all three types in all of the specimens. Most of the granules were between one and three microns in diameter as assessed by comparison with red blood cells. Such granules stained blue in the haematoxylin and eosin stained sections, red in the methyl green - pyronin stained sections, blue in the sections stained by the ferric-ferricyanide reduction test, and were Feulgen negative. The only Feulgen positive granules were tiny and occurred only in cells with degenerative "empty-looking" nuclei. They were thought to be composed of extruded nuclear chromatin and to be a manifestation of nuclear degeneration or karyorrhexis. The appearances of the epithelium and granules in the adult specimens were as follows.

1. Haematoxylin and eosin.

Keratinized epithelium usually comprised a basal layer, prickle-cell layer, granular layer, and an anuclear keratinized surface layer. The stratum granulosum was from one to four cells thick and composed of flattened

Table II

Staining reactions of granules present in cell layers superficial to the prickle-cells in specimens of oral epithelium.

Specimen number	Epithelial type Keratinised = K Parakeratinized= PK Not keratinized= NK	Stain			
		H and E Basophilic= B Acidophilic= A	M.G. - P. Methyl green= MG Pyronin = P	Feulgen	Ferric-Ferri-cyanide reduction
1	PK	+B	+P	-	+
	K	+B	+P	-	+
2	PK	+B	+P	-	+
	NK	+B	+P	-	+
3	PK	+B	+P	+	+
	K	+B	+P	-	+
4	PK	+B	+P	+	+
	K	+B	+P	-	+
5	PK	+B	+P	-	+
6	NK	+B	+P	+	+
7	PK	+BA	+P	+	+
	K	+B	+P	-	+
8	NK	+B	+P	-	+
9	PK	+B	+P	+	+
10	K	+B	+P	-	+
Foetus A	NK	+B	+P	+	+
Foetus B	NK	+B	+P	+	+

cells with fine blue-staining cytoplasmic granules. (fig. 2).

Parakeratinized epithelium was usually without a distinct granular layer, and its surface was of nucleated cells. Blue-staining granules occurred in the cytoplasm of cells between the prickle-cell layer and the surface, however, and in the surface layer itself (fig. 3). Sometimes considerable difficulty was encountered in classifying the epithelial type according to the histological text-book definitions. In some specimens a distinct granular layer occurred under a surface containing nucleated cells; others with an anuclear keratinized surface contained no distinct granular layer (figs. 4, 5).

Non-keratinized epithelium never contained a granular layer, but blue-staining granules were present in cells between the prickle-cell layer and the surface (fig. 6).

As a general rule, the less keratinized epithelium contained the larger granules. These cells with larger granules usually contained fewer than cells with smaller granules (e.g. cells in the granular layer of keratinized epithelium).

Granules occurred in the actual surface layer in non-keratinized epithelium and also in parakeratinized

Fig. 2. Photomicrograph of keratinized oral epithelium with a well-defined granular layer. H and E. X1200.

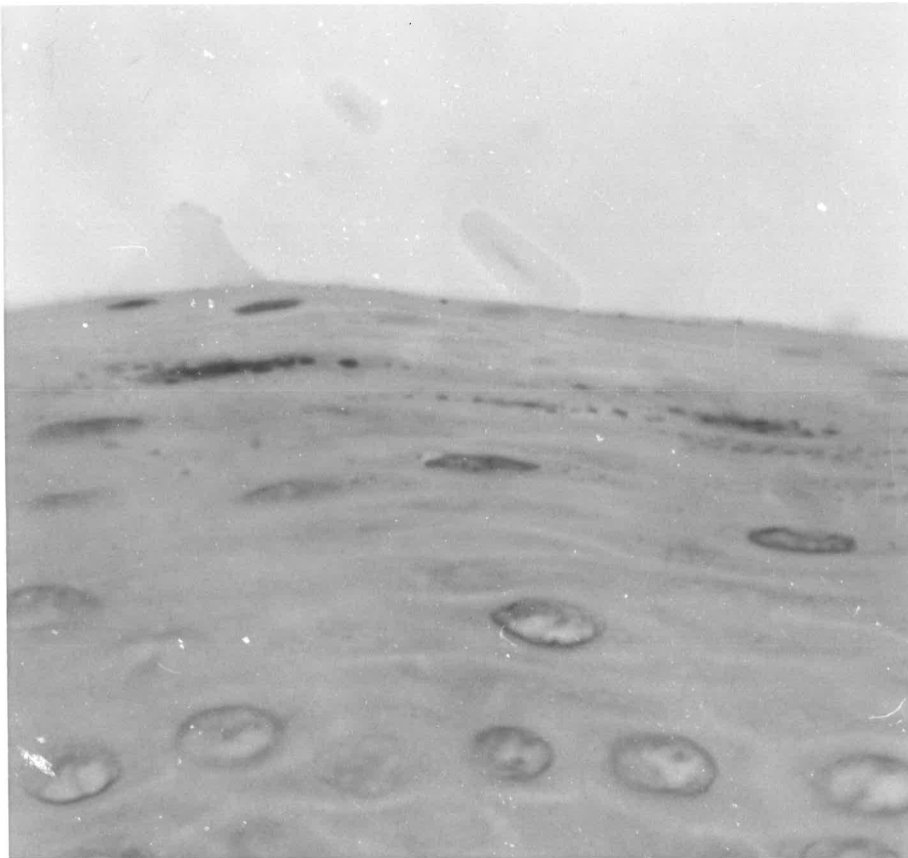

Fig. 3. Photomicrograph of parakeratinized oral epithelium with cytoplasmic granules in cells superficial to the prickle-cell layer. H and E. X1200.

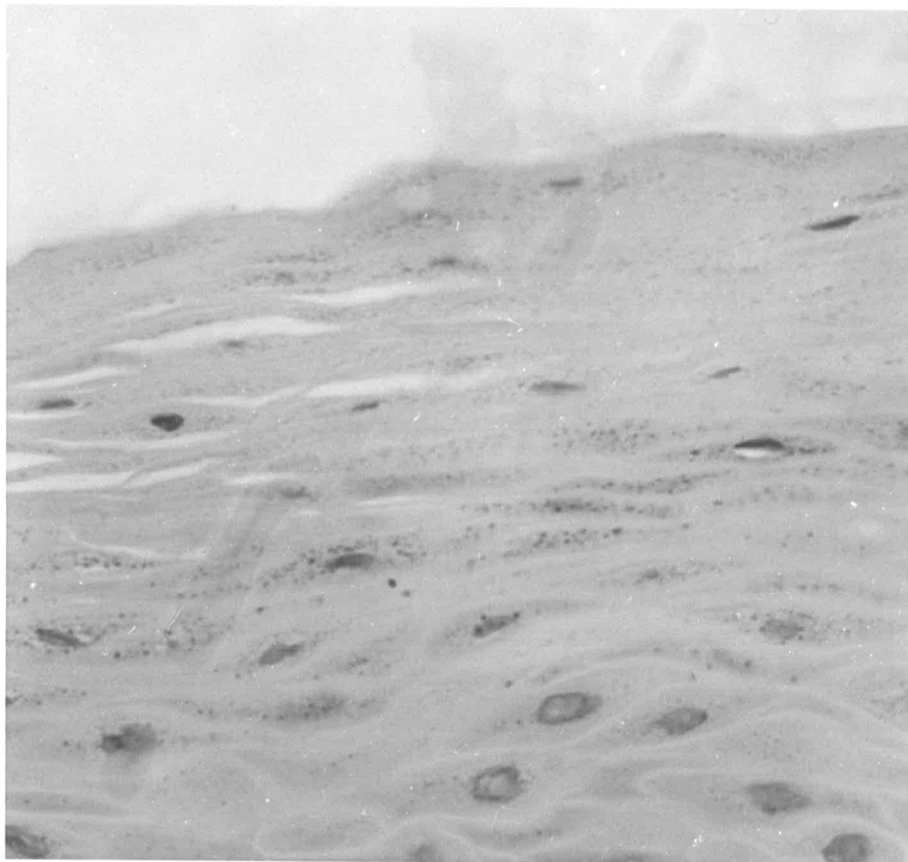

Fig. 4. Photomicrograph of oral epithelium with an extensive granular layer beneath surface cells containing nuclei. H and E. X1000.

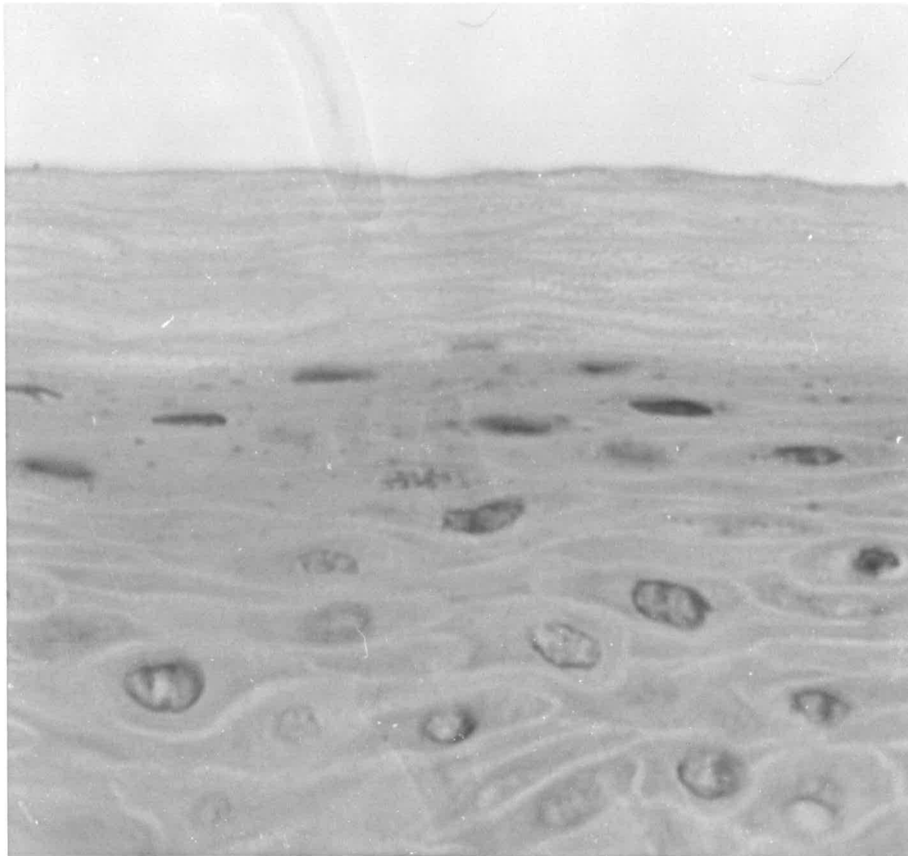

Fig. 5. Photomicrograph of oral epithelium with anuclear surface cells above a poorly defined granular layer. H and E. X1200.

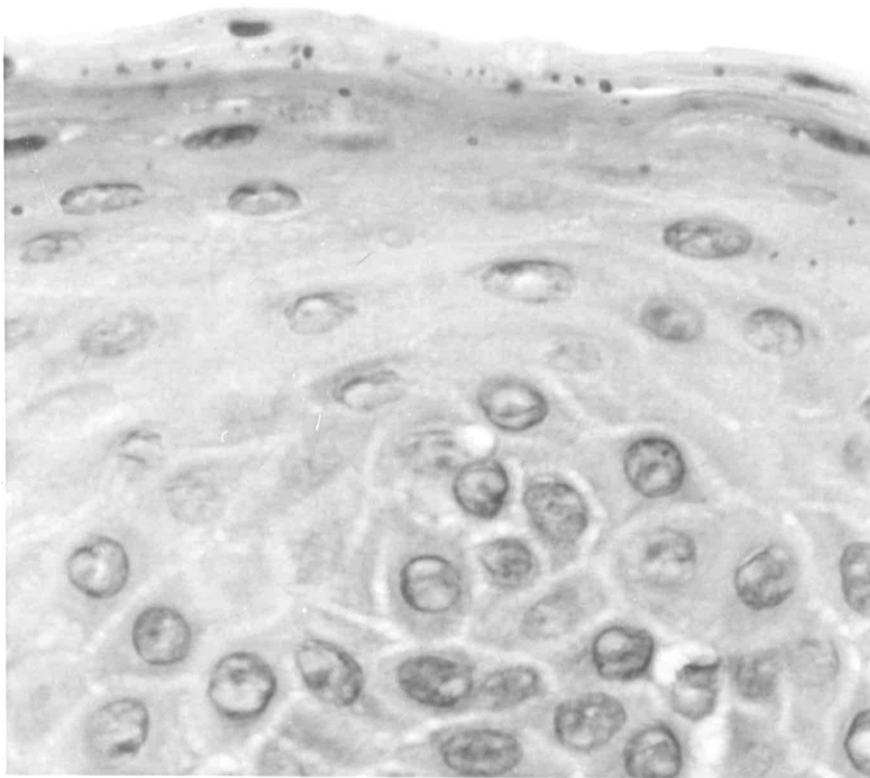

Fig. 6. Photomicrograph of non-keratinized oral epithelium with numerous cytoplasmic granules in cells superficial to the prickle-cell layer. H and E. X1200.

epithelium which had a thin surface zone. Where the parakeratinized surface was more than two cells thick, granules in the surface cells were rare. They never occurred in a keratinized surface layer.

In specimen seven blue-staining and red-staining granules occurred in the same cells. They were identical in size, morphology and distribution.

2. Methyl green - pyronin.

In keratinized epithelium red-staining granules occurred in situations similar to those staining blue with the haematoxylin. In the granular layer, the red-staining was diffuse, and not limited to individual visible granules. It was of about the same intensity as that in the basal layer. The prickle-cell layer and surface layers contained little red-staining material.

Parakeratinized, and non-keratinized epithelium also contained red-staining granules in similar situations to those staining blue with haematoxylin. Blue-staining material occurred only in the nuclei in all epithelial types. In general, the granules did not appear to stain as well with the pyronin as they did with the haematoxylin.

3. Feulgen reaction.

Red-staining granules occurred in some specimens,

usually in cells in the surface layer of parakeratinized and non-keratinized epithelium. They appeared to be fragments of nuclear chromatin (fig. 7).

4. Ferric-ferricyanide reduction.

In keratinized and parakeratinized epithelium there appeared to be a zone of activity beneath the surface layers; this was not as prominent in non-keratinized epithelium. Cells in the zone contained blue-staining granules of similar distribution and appearance to those staining with haematoxylin.

The appearances of both oral epithelium and epidermis in the foetal specimens were as follows.

1. Haematoxylin and eosin.

Both oral epithelium and epidermis were multi-layered but the epidermis was thinner. Blue-staining cytoplasmic granules were present in the more superficial cells of both oral epithelium and epidermis (fig. 8). There were fewer granules in epidermis and they were usually of smaller size than those in the oral epithelium which were up to three microns in diameter.

2. Methyl green - pyronin.

Red-staining granules occurred in situations similar to those staining blue with haematoxylin in both epidermis

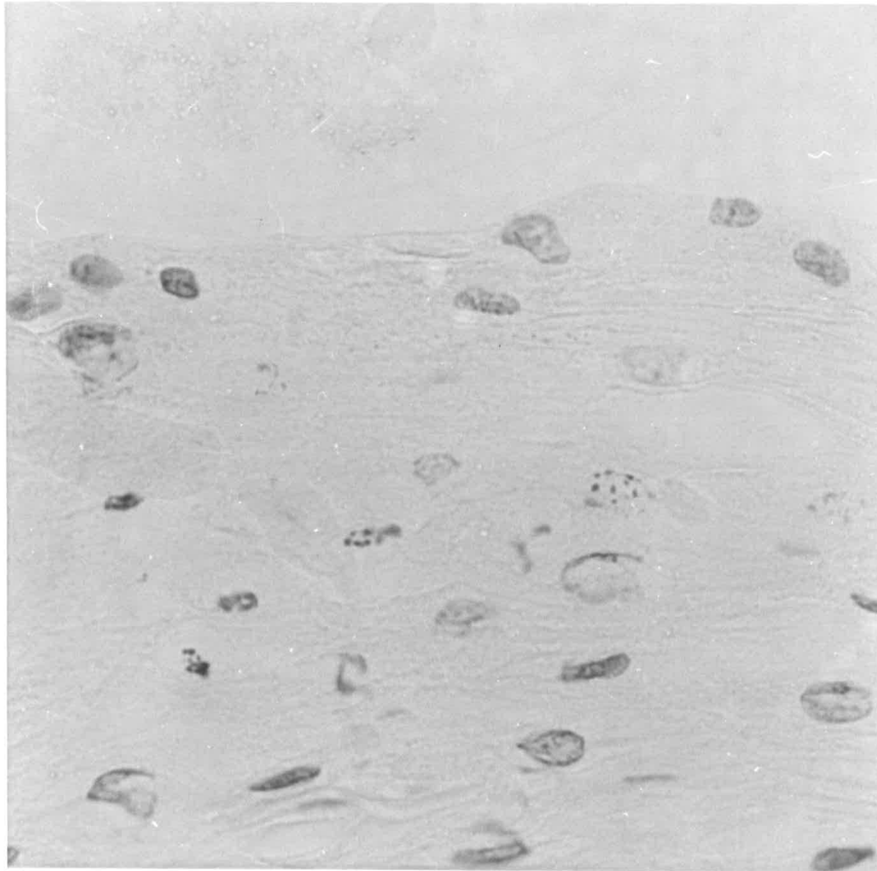

Fig. 7. Photomicrograph of non-keratinized oral epithelium. Two cells contain a number of fragments of nuclear chromatin. Feulgen stain. X1200.

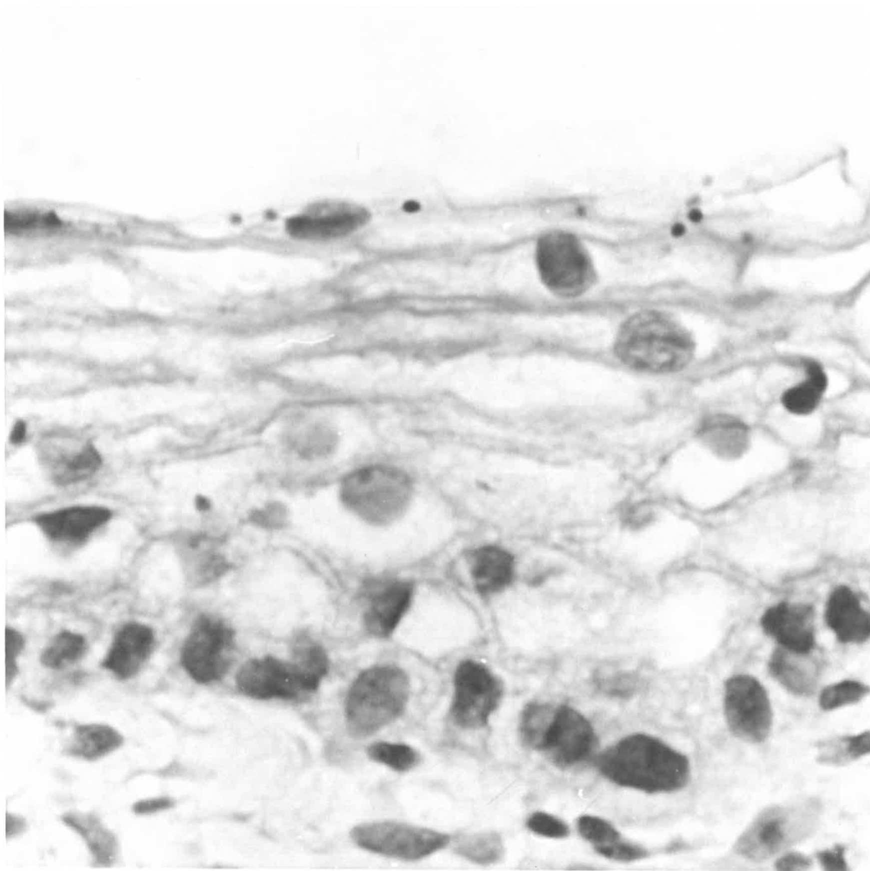

Fig. 8. Photomicrograph of oral epithelium from a 19 week foetus. Cytoplasmic granules are present in the superficial cells. H and E. X1200.

and oral epithelium.

3. Feulgen reaction.

A few tiny red-staining granules occurred in some superficial cells of both epidermis and oral epithelium.

4. Ferric-ferricyanide reductions.

Blue-staining granules occurred in situations similar to those staining blue with haematoxylin and red with pyronin.

Discussion and Conclusions.

The results suggest that there were two main types of cytoplasmic granules in the oral epithelial cells between the prickle-cell layer and the surface:-

1. Granules that stained blue with haematoxylin and with the ferric-ferricyanide reduction test, and red with the methyl green - pyronin stain, thus indicating that they contained RNA or ribonucleoprotein (RNP). These granules had the staining characteristics of keratohyalin granules (Montagna, 1962) yet they occurred in parakeratinized and non-keratinized epithelium as well as in the granular layer of keratinized epithelium. This suggested that either the concept of the keratohyalin granules being present only when the epithelial surface is completely keratinized is wrong, or that there are other bodies in the epithelium which are indistinguishable from keratohyalin granules by the staining methods used in this study.

2. Granules that stained red with the Feulgen reaction and contained DNA or DNP. They appeared to be composed of nuclear chromatin as a result of nuclear degeneration in cells of the epithelial surface.

The red-staining granules seen in the haematoxylin and eosin stained section of specimen seven suggested that the blue-staining granules may lose their apparent

basophilia (perhaps their RNA or RNP) in the surface layers.

Further investigation is necessary to clarify these conclusions. Possibilities that may warrant further consideration in relation to the constituents or nature of the granules are listed below.

1. The lobes of the nucleus of a neutrophil polymorphonuclear leucycyte are about the same size as the largest of the cytoplasmic granules seen in the epithelial cells. However, these nuclei invariably stain red with the Feulgen reaction, and differ in this respect to the large cytoplasmic granules.

2. Melanin is limited to the basal layer of the oral epithelium. None of the specimens in this study was pigmented. Melanin cannot be considered, therefore, as a possible cause for the appearance of cytoplasmic granules. However, haemoglobin, haemosiderin and bile pigments remain as possible, though unlikely, constituents of cytoplasmic granules.

3. Yeast cells could conceivably stain blue with haematoxylin and red with pyronin. Their size and shape could be similar to those of the larger cytoplasmic granules and they could occur inside or on the surface of oral squamous epithelial cells. This requires further investigation. Bacteria could conceivably be confused

with the smaller cytoplasmic granules, particularly on the epithelial surface.

4. Virus aggregates within oral epithelial cells have been described by many workers. They too, could conceivably simulate the appearance of cytoplasmic granules. This will be discussed subsequently.

5. Staining artefacts have often been the cause of incorrect conclusions.

It may be concluded that there are probably two main types of granules.

1. Large (up to three microns in diameter) basophilic granules containing RNA or RNP which may lose their basophilia and stain red with haematoxylin and eosin.

A. They are probably so-called keratohyalin granules even though they occur in parakeratinized and non-keratinized epithelium.

B. They may be:-

(i) Fragments of "polymorph" nuclei,

(ii) Pigments: haemoglobin, haemosiderin or bile,

(iii) Yeast cells,

(iv) Virus aggregates,

(v) Staining artefacts.

2. Small Feulgen positive granules containing DNA or DNP.

A. They are probably chromatin.

B. They may be bacteria.

CHAPTER II

CYTOCHEMISTRY OF ORAL SQUAMOUS EPITHELIAL CELLS

Introduction.

As a means of examining the cytoplasm of individual cells, the ordinary formalin-fixed, paraffin-embedded section, six microns thick, is inadequate. Usually the plane of the section is almost at right angles to the plane of the cytoplasm of the flattened surface cells, so that a cell of 40 microns diameter (or even up to 100 microns) is represented by a slice six microns thick, which could easily miss the granules in the cytoplasm. Formalin fixatives are not satisfactory for some histochemical work.

The simple smear method offers several advantages. It is a convenient method of obtaining specimens of normal epithelium without the necessity for biopsy. The whole of each cell's contents can be studied. Fixation is by alcohol, allowing better histochemical results.

The technique of exfoliative cytology has now been in use for many years. Papanicolaou, in America, is accepted as the pioneer in this field, although exfoliated cells have been studied for almost 120 years, particularly in sputum. In 1917, Stockard and

Papanicolaou used vaginal smears to study the oestrus cycle of the guinea pig, and on this biological basis was built the huge number of present-day applications of the technique in pathology. From about 1923, Papanicolaou was concerned with the use of the vaginal smear as a means of diagnosis of uterine cancer. In England, Dudgeon and Patrick (1927) published a paper concerning the wet-smear technique for cell examination, and the diagnosis of tumours. Today, the simple smear, and modifications of it, are widely used as an adjunct to diagnosis of various pathological conditions.

Many workers have used the oral smear in examining both physiological and pathological problems. Weinmann (1940) utilized staining reactions of cells in oral smears to investigate keratinization of the oral mucosa, and subsequently other workers have studied the effect of hormones on oral keratinization. The use of exfoliative cytology in oral diagnosis was described by Montgomery and von Haam (1951); they assessed its usefulness in the diagnosis of benign, pre-malignant and malignant lesions. Vitamin and mineral deficiencies have also been studied using oral smears, while Cooke (1958, 1960) utilized oral smears in the diagnosis of bullous lesions, including Herpetic infections. A

tuberculous ulcer was diagnosed from a giant-cell in a smear of the lesion by Cawson (1960). Buccal smears are commonly used as a reliable means of determining nuclear sex in suspected examples of intersex syndromes.

Of all the investigators using the smear technique, only Peters (1958) has described the cytoplasmic granules occurring in normal squamous epithelial cells from the mouth. The following observations were made.

1. The granules were round or oval in shape and up to two or three microns in diameter.

2. Frequently there were two large granules in close proximity to the nucleus.

3. Characteristically, the granules were so distributed that a clear peri-nuclear halo occurred.

4. They were arranged with smaller ones nearer the cell periphery, and larger ones nearer the nucleus.

5. There were rarely more than five granules in each cell, frequently only one or two, and sometimes none.

6. Irradiation, e.g. an airdose of 2000 to 3000 roentgens over a period of three weeks caused a marked increase in the number of granules so that up to 60 occurred in one cell. This increase occurred in 70 to 80 per cent of cells.

7. The increase was not immediate, but occurred approximately 10 days after irradiation.

8. Granules were found in normal cells, and in those from leukoplakic patches, but not in malignant cells.

9. They stained deeply with haematoxylin, did not yield the Feulgen reaction, and stained red with methyl green - pyronin. They did not stain after ribonuclease digestion, suggesting that they contained RNA.

10. There was no correlation between the occurrence of the granules, and sex,or menstrual cycle.

Similar bodies in vaginal epithelial cells were described by Papanicolaou (1954). They too stained deeply with the haematoxylin of the Papanicolaou stain, but faded and became acidophilic following ovulation. They were thought to emanate from the nucleus. Papanicolaou made a further statement: "Superficial squamous cells sometimes contain an unusually large number of chromatin granules, some of which are in direct contact with the nucleus. The granules may be seen in a variety of cases, and their significance for diagnostic purposes is not clear." Haemosiderin granules were said to occur occasionally in cells from cases where bleeding had occurred.

The Vincent Memorial Hospital Staff in their text-book "The Cytologic Diagnosis of Cancer" (1958) described an illustration of a vaginal cell containing

granules, stating that it was a "cornified cell with small dense nucleus, irregular cytoplasm with granules, and evidence of a perinuclear vacuole." They offered no explanation of the nature or significance of the granules.

Clearly, the observations made in Chapter I support those of Peters, but disagree with the implications of Papanicolaou that large granules (of two microns or more in diameter) containing DNA occur in squamous epithelial cells with intact nuclei.

The following study was designed to confirm the findings of Chapter I, utilizing the technique of exfoliative cytology which was more suitable than histological methods for this investigation.

Materials and Methods.

Superficial oral squamous epithelial cells were obtained from three sources.

1. The buccal mucosa of 10 premature infants with no oral lesions.

2. The normal buccal mucosa of 10 adults.

3. The buccal mucosa of five adults with so-called "occlusal plane keratosis". The cells were obtained only from the white lesions in such cases, and not from adjacent clinically normal epithelium.

The subjects were chosen to represent different degrees of keratinization of the buccal mucosa. By histological criteria, infant mucosa is non-keratinized, normal adult buccal mucosa is non-keratinized or parakeratinized, and epithelium from the areas of buccal keratosis is parakeratinized or keratinized.

Epithelial cells for examination were obtained as follows. The patient was instructed to rinse out the mouth with water; this was not possible in the case of infants. The buccal mucosa was scraped with a metal spatula until adequate numbers of epithelial cells had been removed. These cells were then spread evenly and quickly on a labelled, albuminized, flat glass slide. Fixation was by immersion in 96 per cent ethyl alcohol in a Coplin jar for a minimum of 30 minutes.

Fourteen specimens were obtained from each

individual. Care was taken to scrape a new area of buccal mucosa for each specimen to ensure a similar cell sample. Each specimen was treated in a different manner. The methods used are listed below.

1. <u>The Papanicolaou stain</u> (Papanicolaou, 1954).

This multichrome stain was used to demonstrate the morphology of the squamous epithelial cells, and to examine the staining of the cytoplasmic granules. Papanicolaou (1933) stated that in smears from the vaginal mucosa, red cytoplasmic staining of the squamous epithelial cells occurred in superficial, "cornified" cells, and that blue-staining cytoplasm occurred in cells from the deeper layers of the epithelium. Studies on the oral mucosa (using the Papanicolaou stain) by Miller, Soberman, and Stahl (1951) and Montgomery (1951) described similar findings in the staining of the oral epithelium. They found that the epithelial cells could be readily divided into groups according to the colour of their cytoplasmic staining. Blue or blue-green-staining cells came from the deeper layers of the epithelium, red-staining cells from the more superficial layers of "cornified" epithelium, and yellow-staining cytoplasm, in cells which were usually anuclear, indicated complete keratinization of the epithelial cell.

2. Methyl green - pyronin (Culling, 1957).

The validity of the assumption that red-staining was produced by RNA or RNP was tested by the use of ribonuclease. (See Appendix I.)

3. Azure A (Fullmer, 1962).

A basic dye, Azure A, was used to support the results obtained with the methyl green - pyronin stain, the intention being to stain material containing RNA or RNP with Azure A at pH 4. (See Appendix II.)

4. The Feulgen reaction (Culling, 1957).

The fixed smears were stained by exactly the same method as the sections used in Chapter I.

5. Ferric-ferricyanide reduction (Lillie, 1954).

The fixed smears were stained by exactly the same method as the sections used in Chapter I.

6. The Gram stain (Lillie, 1954).

Keratohyalin granules are said to occasionally stain positively with the Gram stain. This method may distinguish between keratohyalin granules staining blue, and Gram negative bacteria, staining red.

7. Periodic acid-Schiff method (Culling, 1957).

The primary purpose of this stain was to demonstrate the polysaccharide capsules of yeast cells, if they were

present. Keratohyalin granules should not stain by this method. Cytoplasmic glycogen is stained a magenta colour.

8. Perl's Prussian Blue reaction (Culling, 1957).

Ferric salts, and therefore pigments such as haemosiderin, can be stained blue by this method.

9. Benzidine stain for haemoglobin.

The method used was that of the Department of Pathology, University of Adelaide. (See Appendix III.)

10. Gmelin's reaction (Culling, 1957).

This method was used to investigate the smears for the presence of bile pigments.

The stained smears were examined by the light microscope at magnifications up to 450 diameters.

In addition to the oral smears, Papanicolaou-stained vaginal smears from the files of the Cytology Laboratory at the Queen Elizabeth Hospital were similarly examined microscopically.

Results.

1. The Papanicolaou stain.

(i) Oral smears.

The findings with respect to the contents of the smears and the colours of cytoplasmic staining were similar to those of Montgomery (1951) and Miller et al. (1951).

The smears contained predominantly squamous epithelial cells. As expected, most smears from the infants contained mainly blue-staining cells; red-staining cells were less common, and anuclear yellow-staining cells were a rarity.

Normal adults gave smears containing more red-staining cells than those of the infants; the remaining cells were blue-staining with occasional anuclear yellow-staining cells.

Smears from areas of buccal keratosis contained only red-staining and yellow-staining cells.

Smears from all the subjects contained cells with cytoplasmic granules similar to those described by Peters (1958) (fig. 9). These granules stained deeply with haematoxylin and occurred in both blue-staining and red-staining cells, but never in yellow-staining cells. Papanicolaou's (1954) observation that acidophilic

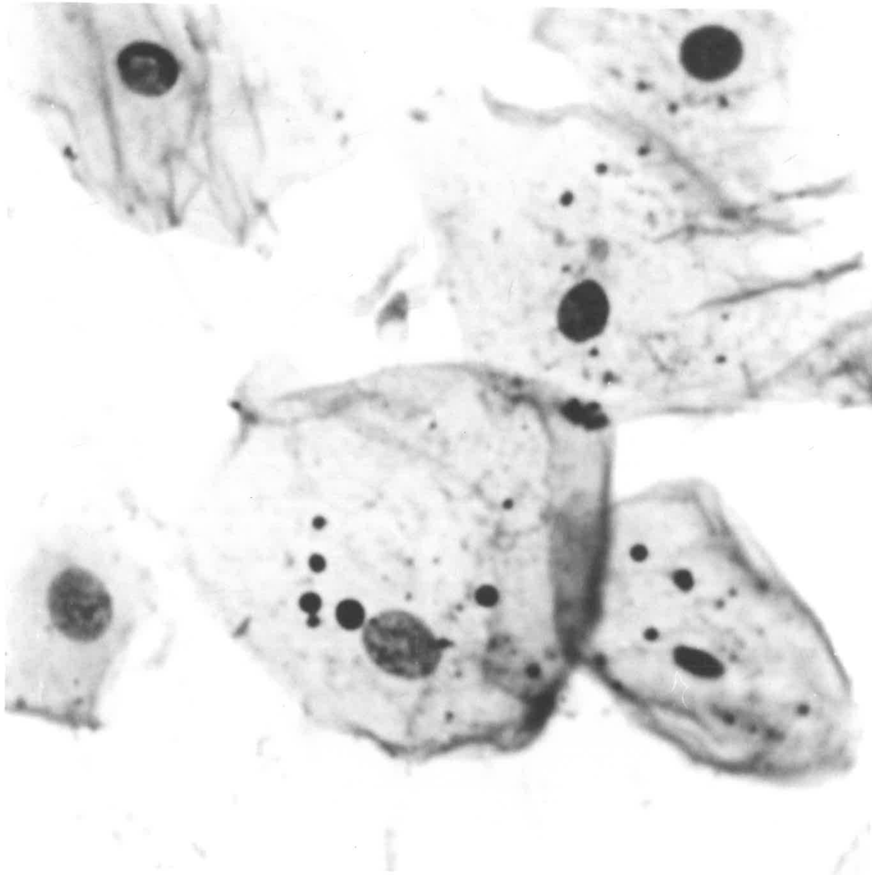

Fig. 9. Photomicrograph of oral epithelial cells in a buccal smear from an infant. Prominent cytoplasmic granules are present in four of the six cells. Papanicolaou stain. X1400.

(red-staining), faded granules sometimes occurred in vaginal epithelial cells was recalled by a parallel observation in the oral smears. The red-staining granules occurred almost invariably in red-staining cytoplasm, and rarely in blue-staining cells. The fact that indentations of the nucleus of some cells accomodated some granules, and that the granules came into focus concurrently with the nucleus, suggested that they were intracellular and not adherent to the cell membrane (fig. 10).

The granules were a much more prominent feature of the cells in the smears than they were in the sections described in Chapter I.

Polymorphonuclear leucocytes, histiocytes and bacteria also occurred in the smears. The bacteria often appeared to be in intimate relationship to the epithelial cells but were not in focus concurrently with the nucleus, suggesting that they were adherent to the cell membrane (fig. 11.). The "polymorph" nuclei and bacteria could be easily recognized so that no confusion with cytoplasmic granules in the epithelial cells occurred.

(ii) Vaginal smears.

The vaginal smears were similar to the oral smears from infants, in that their epithelial cell nuclei were

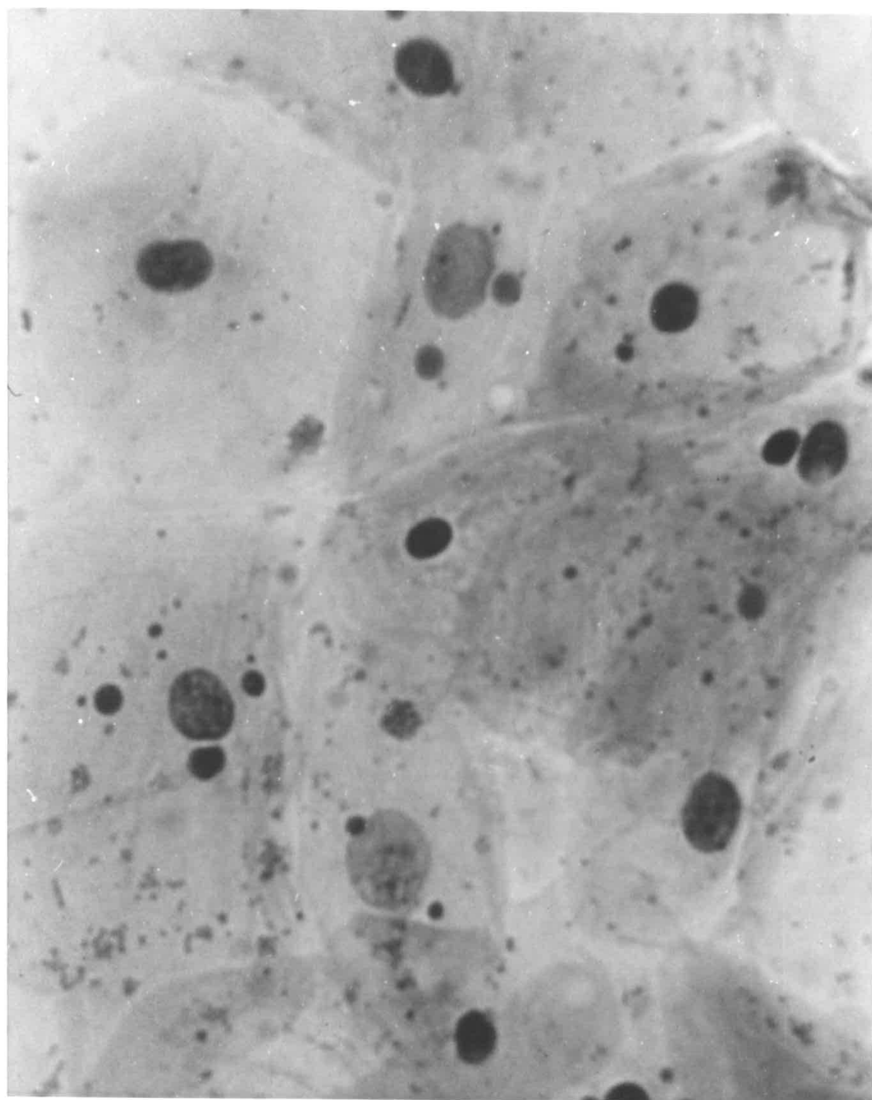

Fig. 10. Photomicrograph of a clump of oral epithelial cells in a buccal smear showing nuclear indentation by cytoplasmic granules. Papanicolaou stain. X1600.

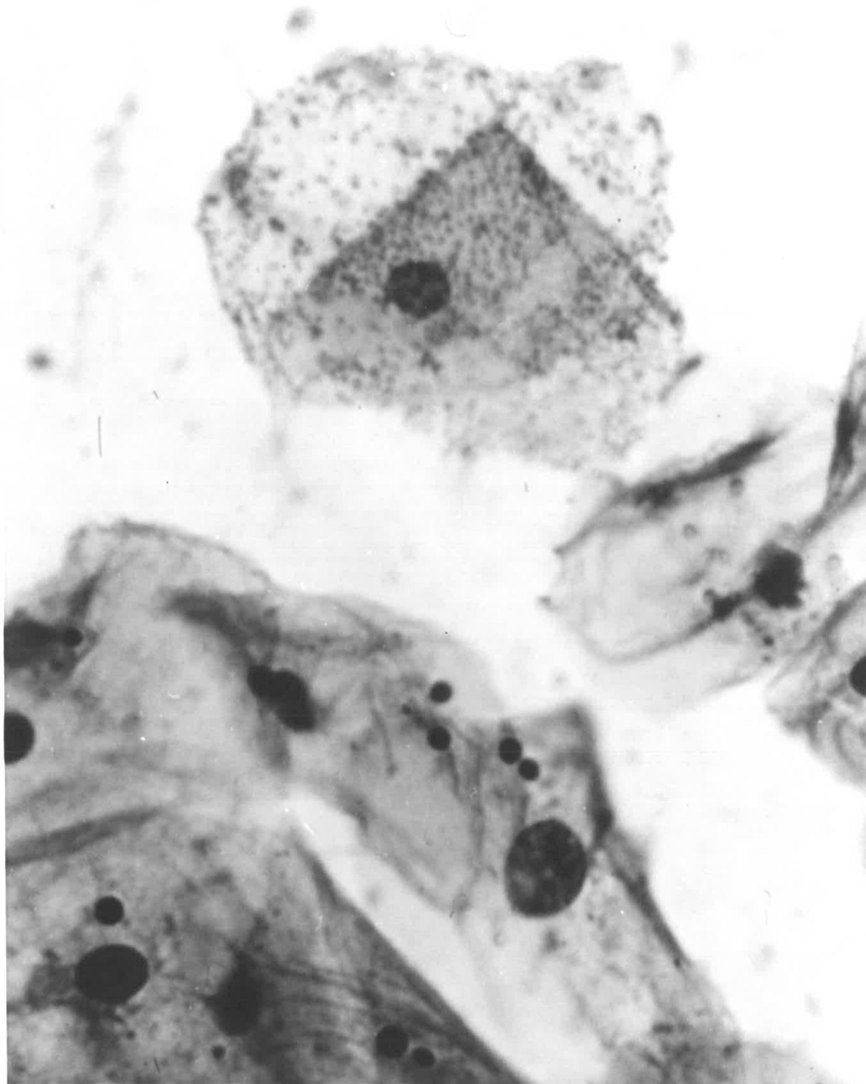

Fig. 11. Photomicrograph of oral epithelial cells in a buccal smear. The folded cell (top centre) has numerous bacteria adhering to its surface. Papanicolaou stain. X1600.

less often pyknotic than those in adult oral smears. Cytoplasmic granules were present in all the smears examined, although they appeared to be much less prevalent than in the oral smears. These granules were identical with those described in oral cells by Peters (1958), and those in vaginal cells described by Papanicolaou (1954).

2. Methyl green - pyronin.

(i) After digestion with ribonuclease.

No cytoplasmic granules were observed in any of the specimens.

(ii) After incubation with distilled water.

Red-staining cytoplasmic granules occurred in some epithelial cells in all of the specimens (fig. 12). Cell cytoplasm stained pink and the nucleus blue. There were more granules in some specimens than others, most being observed in the smears from the areas of buccal keratosis. There appeared to be fewer red-staining granules in these smears than there were blue-staining granules in the Papanicolaou-stained smears from the same subject.

(iii) After no incubation or pre-staining treatment.

Results were identical with those obtained after incubation in distilled water.

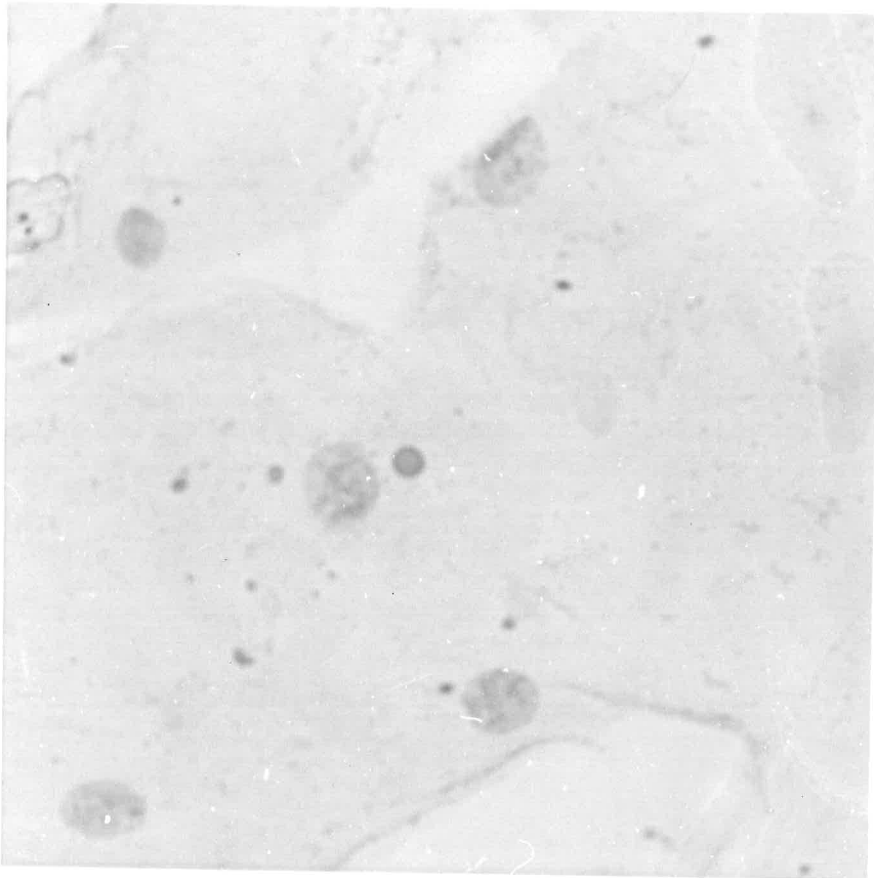

Fig. 12. Photomicrograph of oral epithelial cells in a buccal smear. Pyroninophilic cytoplasmic granules are present with one large granule in the centre of the field. Methyl green - pyronin stain. X1400.

3. Azure A.

(i) After digestion with ribonuclease.

No blue-staining cytoplasmic granules were observed in any of the specimens.

(ii) After incubation with distilled water.

Blue-staining granules were observed in the cytoplasm of some of the epithelial cells in all of the specimens (fig. 13). There were more in some specimens than others. The results in this respect were similar to those obtained with the methyl green - pyronin stain.

(iii) After no incubation.

The results were identical with those obtained after incubation with distilled water.

4. The Feulgen reaction.

In all the specimens there were rare examples of Feulgen positive (red-staining) granules occurring in the cytoplasm of the epithelial cells. They appeared to comprise small clumps of chromatin which resulted from nuclear breakdown as the nuclei in such cells never appeared intact (fig. 14).

5. Ferric-ferricyanide reduction.

Blue-staining granules occurred in the epithelial cells of all the specimens (fig. 15). They appeared to be similar in numbers and distribution to the granules

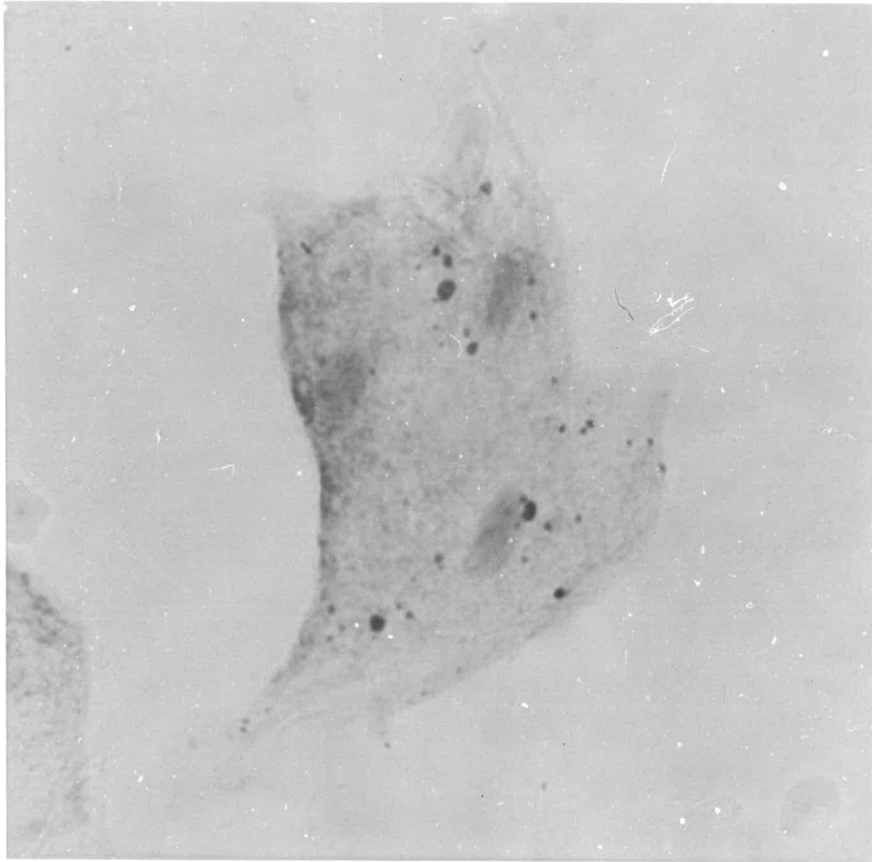

Fig. 13. Photomicrograph of a clump of oral epithelial cells in a buccal smear. Cytoplasmic granules are clearly shown although cell morphology is poorly demonstrated. Azure A stain. X1000.

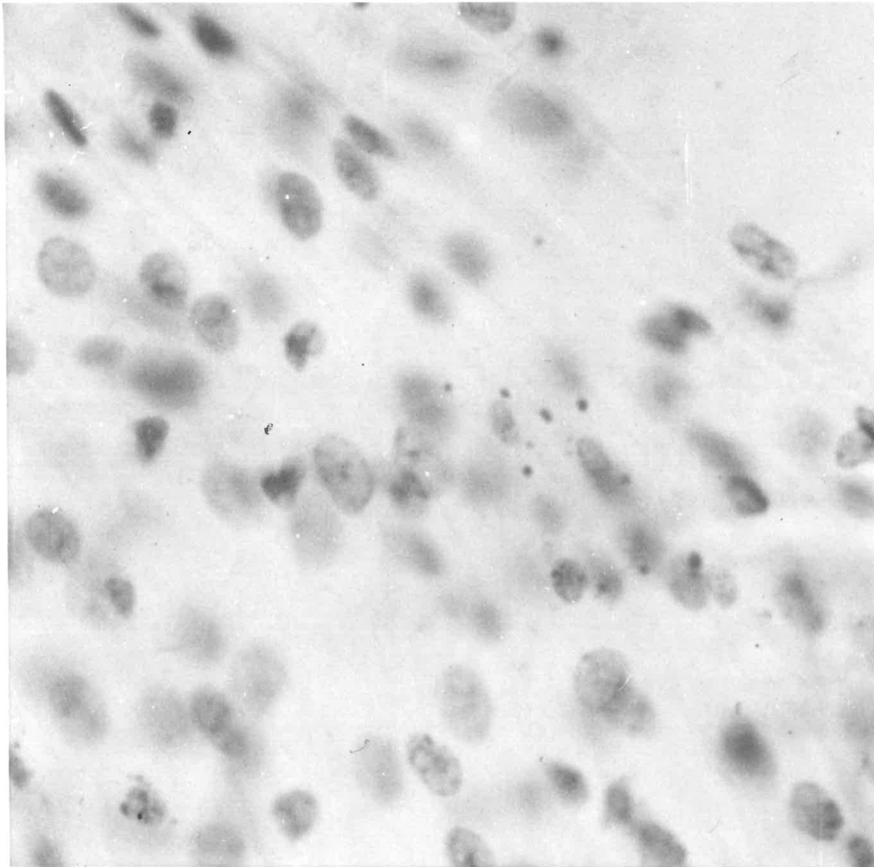

Fig. 14. Photomicrograph of a dense clump of oral epithelial cells in a buccal smear. Feulgen-positive cytoplasmic granules which appear to be associated with a small nucleus are present in the centre of the field. Feulgen stain. X1200.

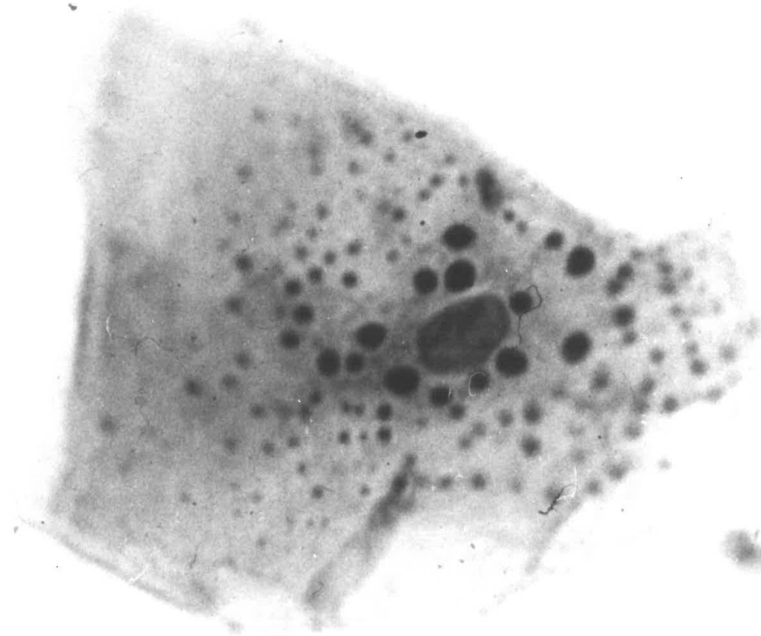

Fig. 15. Photomicrograph of an oral epithelial cell in a buccal smear. There are an abnormally large number of cytoplasmic granules in this cell. Ferric-ferricyanide reduction. X2000.

staining with the haematoxylin of the Papanicolaou stain.

6. The Gram stain.

Some cytoplasmic granules stained positively with the Gram stain in all of the specimens, but these appeared to be fewer than demonstrated by the Papanicolaou stain (fig. 16). Bacteria were often present, some of which could be identified as Neisseria by virtue of their negative staining, characteristic bean-shape, and diplococcal configuration.

7. The Periodic acid-Schiff method.

In some epithelial cells there was a diffuse cytoplasmic positive staining indicative of the presence of glycogen, but no bodies suggestive of yeast cells could be identified in any of the specimens.

8. Perl's Prussian Blue Reaction.

There were no positive reactions in the specimens examined, thus excluding the presence of haemosiderin.

9. Benzidine stain for haemoglobin.

There were no positive reactions in the specimens examined, thus excluding the presence of haemoglobin.

10. Gmelin's reaction.

There were no positive reactions in the specimens examined, thus excluding the presence of bile pigments.

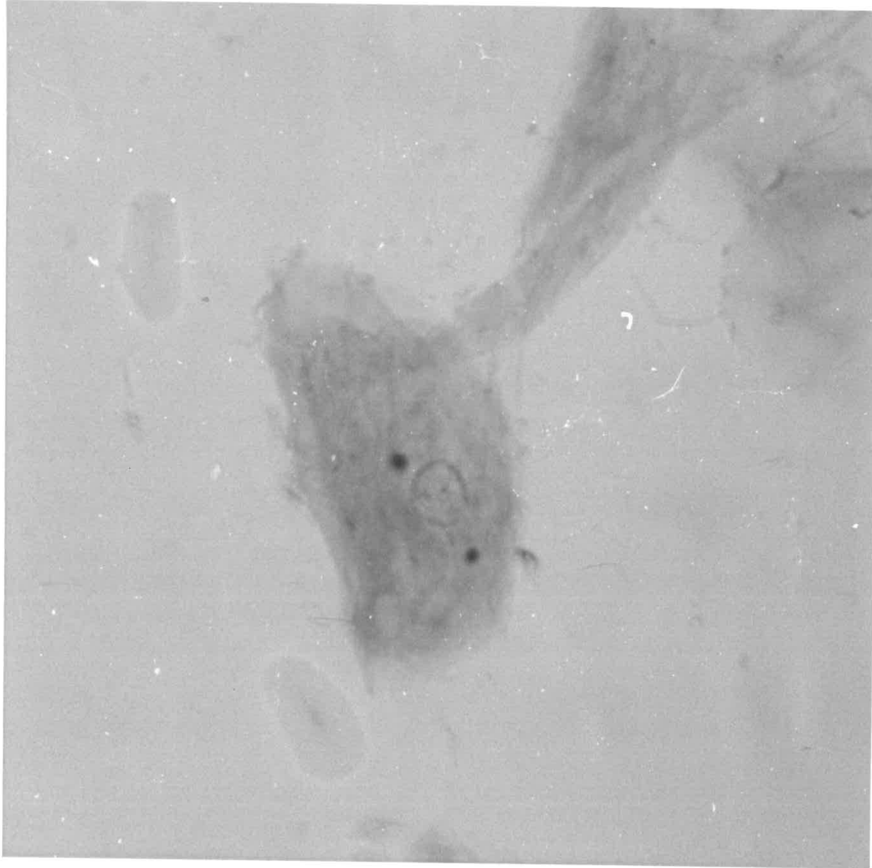

Fig. 16. Photomicrograph of an oral epithelial cell containing two cytoplasmic granules. Gram stain. X1000.

Discussion and Conclusions.

Further comment on the conclusions formed in Chapter I is now possible. The smears stained by the Papanicolaou method demonstrated that cytoplasmic granules occur in squamous epithelial cells from the normal mouths of both adults and infants, and from the vaginas of adult females. The buccal mucosa is normally non-keratinized or parakeratinized by histological definition, and the vaginal mucosa is also non-keratinized. Keratohyalin granules would thus not be expected to be found in such smears if it is accepted that they are associated only with completely keratinized squamous epithelial surfaces. However, the granules in the smears from non-keratinized areas could be divided again into two main types as in Chapter I. One of these types had similar properties to keratohyalin granules in cells from keratinized areas.

This first type of granule was similar to those staining blue in the haematoxylin and eosin stained sections described in Chapter I. They were stained deeply by the haematoxylin of the Papanicolaou stain but occasional ones stained red, indicating a probable loss of basophilic material. The red-staining with methyl green - pyronin, and the blue-staining with Azure A was

removed by digestion with ribonuclease, indicating the presence of RNA or RNP. A positive reaction occurred with the ferric-ferricyanide reduction test, and the Feulgen reaction was negative. The stains for pigments, and the PAS reaction were also negative.

These results are strong evidence for the suggestion that such granules are in fact the so-called keratohyalin granules. The only other possibilities, though unlikely, are that their appearance could be due to micro-organisms or staining artefacts.

The second type of granule was Feulgen positive, and much smaller than the first type. The smears left no doubt that such granules were the result of nuclear breakdown. The appearances in the smears prevented any confusion of these granules with bacteria.

In summary, the results of the examination of oral and vaginal smears confirmed some of the conclusions drawn in Chapter I.

1. Cytoplasmic granules with properties similar to those of keratohyalin granules do occur in epithelial cells from non-keratinized epithelium of the mouth and vagina.

2. The only other possible causes for the appearance of such granules could be

(i) Micro-organisms

(ii) Staining artefacts.

3. Small cytoplasmic granules also occur in squamous epithelial cells from the mouth and vagina. They contain DNA or DNP and are probably the result of nuclear disintegration.

CHAPTER III

COMPARISON OF THE INCIDENCE OF YEASTS WITH THE PRESENCE OF CYTOPLASMIC GRANULES IN ORAL SMEARS

Introduction.

The examination of sections and smears of oral epithelium reveals the presence of large numbers of bacteria in intimate relation to the epithelial cells, yet no pathology is evident. It is thought that a harmonious relationship exists between the oral microbial flora and the epithelium. This concept of a vital biological relationship between oral micro-organisms and epithelium was proposed by Bloomfield (1922). He found that the oral mucosa could be scrubbed until the integrity of the epithelium was threatened, without dislodging the organisms as detected by smear and culture. In the smears described in Chapter II, it appeared that the organisms were adherent to the surface of the epithelial cells. This view was also held by Montgomery (1951).

On the other hand, Thoma and Goldman (1960) suggest that oral epithelial cells are phagocytic, and that the micro-organisms seen in relationship to them in smears, are in fact <u>inside</u> the cell. Since histological and histochemical evidence depicts oral epithelium as a mechanically protective tissue, this phagocytic property

is difficult to accept.

The possibility that some cytoplasmic bodies in oral epithelial cells may be virus particles cannot be neglected. The pathological changes caused by virus infection in the mouth (particularly Herpes simplex infection) have been described by Cahn (1950), Cooke (1958), Silverman (1959), and Thoma and Goldman (1960). They include eosinophilic ballooning degeneration of epithelial cells, formation of multi-nucleated giant cells (so-called "Tzanc cells") and intra-cellular or intra-nuclear inclusion bodies (so-called "Lipschutz bodies") which are believed to be colonies of the virus enveloped in a matrix. No such changes were observed in the cells containing cytoplasmic bodies which were examined in the sections and smears of Chapters I and II. This is not unlikely as the specimens selected had no clinical evidence of virus infections. The only possibility remaining is that the cytoplasmic granules could be viruses in eclipse phase. Although this may be an acceptable explanation in adults, most of whom have a positive Herpes simplex antibody titre, it is not acceptable in infants who are unlikely to have come in contact with the Herpes virus without showing clinical signs of infection. However, many cytoplasmic bodies occur in the

epithelial cells from the infants' oral mucosa. Exclusion of viruses as cytoplasmic granules could only be made certain by tissue culture studies. These are beyond the resources of this study. It is considered, however, that the presence of viruses is unlikely in oral epithelial cells from clinically normal mucosa.

Bacteria are far too small to be confused with the larger cytoplasmic bodies in oral epithelial cells. However, yeast cells do conform in some respects, and therefore warrant investigation. Candida albicans was described by Burnett and Scherp (1962) as an oval, budding yeast two to four microns in diameter. Candida species are inhabitants of the normal mouth, intestinal tract, and vagina. Yeasts were not present in the edentulous mouths without artificial dentures examined by Lilienthal (1950), but occurred in mouths containing natural teeth; they reappeared in edentulous mouths after the insertion of artificial dentures. Thrush (infection with yeast organisms, usually Candida albicans) occurs mostly in poorly nourished infants and aged adults. As infants (up to six months of age) and old adults are often edentulous, they are two categories in which Lilienthal would least expect to find yeasts as a member of the oral flora. Children born of mothers having yeasts in the vagina have about 35 times greater chance of

developing oral thrush than those born of non-infected mothers (Woodruff and Hesseltine, 1938).

Although no bodies suggestive of yeast cells stained with the PAS reaction in the smears examined in Chapter II, the fact remains that cytoplasmic granules staining with the haematoxylin, and with a size range similar to that of yeast cells occurred in Papanicolaou-stained smears. An experiment to prove that such bodies were <u>not</u> yeast cells is described below.

Materials and Methods.

The subjects chosen for the study included five normal infants between 10 and 14 days old, five edentulous adults who were not wearing artificial dentures, and 10 normal adults with natural dentition. Squamous epithelial cells were obtained from each subject by scraping the buccal mucosa with a metal spatula. This material was spread evenly on an albuminized glass slide and fixed in 96 per cent ethyl alcohol for a minimum of 30 minutes. Two such smears were obtained from each subject and stained by different methods.

1. The Papanicolaou stain.

This allowed examination of the smears for the presence of cytoplasmic granules staining with haematoxylin.

2. The Gram stain.

This smear was examined by a microbiologist who expressed an opinion as to the presence of yeast cells in the smear.

A third smear was taken from each subject by wiping a sterile cotton swab over the buccal mucosa. The swab was then placed immediately in nutrient broth at pH 6.4 which contained 1000 units of penicillin and 500 units of

streptomycin per millilitre. The antibiotics prevent the growth of all organisms in the oral microflora except the yeasts. After incubation at 37°C for seven days, the broth was plated onto corn meal agar (pH6). These plates were incubated for a further three days and then examined for the presence of Candida colonies.

Results.

The results are shown in Table III.

In the opinion of the microbiologist, none of the Gram-stained smears contained yeast cells. All but five of the Papanicolaou-stained smears contained epithelial cells with cytoplasmic granules, staining with haematoxylin, in the size range between two microns and four microns in diameter. Three of the 20 subjects gave cultures of Candida albicans.

Table III

Comparison of the presence of Candida albicans (as detected by smear and culture) in oral smears, with the presence of cytoplasmic granules in oral epithelial cells in similar, Papanicolaou-stained smears.

Subject number	Age	Natural Dentition	Artificial Dentures	Gram + ve yeasts	Granules (Papanicolaou stain)	Culture
1	10 days	-	-	-	+	-
2	14 "	-	-	-	+	-
3	10 "	-	-	-	+	-
4	12 "	-	-	-	+	-
5	13 "	-	-	-	+	-
6	45 years	-	-	-	+	-
7	61 "	-	-	-	-	-
8	58 "	-	-	-	+	-
9	53 "	-	-	-	+	-
10	49 "	-	-	-	-	-
11	21 "	+	-	-	+	-
12	23 "	+	-	-	-	+
13	18 "	+	-	-	+	-
14	30 "	+	+	-	-	-
15	25 "	+	-	-	+	-
16	20 "	+	-	-	+	+
17	19 "	+	-	-	+	+
18	26 "	+	+	-	-	-
19	20 "	+	-	-	+	-
20	22 "	+	-	-	+	-

Discussion and Conclusions.

It is theoretically possible that the presence of only one viable yeast cell in a smear may result in a positive culture. One would therefore not necessarily expect large numbers of yeast cells to appear in a stained smear from an individual with a positive culture. This is borne out by the fact that no yeasts were identified in Gram-stained smears from the subjects with positive cultures. One would therefore never expect to see yeast cells in a smear from a subject with a negative culture. In fact, none of the Gram-stained smears (from individuals with either positive or negative cultures) contained yeast cells in the opinion of the microbiologist. However, in the Papanicolaou-stained smears, granules staining with haematoxylin and in the size range two to four microns in diameter, occurred in 15 of the 20 subjects. If these granules were yeast cells, one would expect the cultures from all of these subjects to be positive. In fact, only two of the 15 were positive.

(The remaining five Papanicolaou-stained smears contained granules staining with haematoxylin but less than two microns in diameter. Of these, one had a positive culture.)

These results indicate that the granules staining with the haematoxylin of the Papanicolaou stain are <u>not</u>

yeast cells. Furthermore, it may be that even when yeast cells are present as natural microbial flora on the buccal mucosa, they are probably in small numbers and are not readily identified in smears.

Viruses and bacteria have already been mentioned as being extremely unlikely to produce the appearance of cytoplasmic granules in normal oral squamous epithelial cells. Thus, the only remaining explanations of the nature of the granules are:-

1. Artefacts.

2. Intrinsic organelles of the epithelial cells, namely, keratohyalin granules.

CHAPTER IV

EXAMINATION OF SQUAMOUS EPITHELIAL CELLS BY PHASE CONTRAST MICROSCOPY

Introduction.

It has been established that the granules occurring in oral and vaginal squamous epithelial cells from non-keratinized mucosa are either keratohyalin granules or artefacts. A method which can test the latter possibility is phase contrast microscopy. This allows examination of cells under conditions closely approximating those "in vivo", as no fixation or staining is involved.

Materials and Methods.

The subjects chosen were the same ones as those used in Chapter III (see Table III), plus five women aged 27, 35, 38, 52 and 64 years. Buccal epithelial cells were obtained from the infants and adults described in Table III by scraping the buccal mucosa with a metal spatula as described previously. Vaginal smears were obtained from the five women described above using a wooden spatula.

The cells from each individual were then suspended in a drop of physiological saline solution (0.9 per cent) on a clean, flat glass slide and covered with a clean glass cover slip. The margins of the cover-slip were sealed to the slide with clear nail polish to prevent evaporation and drying of the specimen. Examination with the phase contrast microscope was performed immediately at magnifications up to approximately 900 diameters with the oil immersion objective.

Results.

Cytoplasmic granules occurred in cells from every subject. They were similar to the granules occurring in fixed stained specimens (fig. 17). The appearance of the epithelial cells varied. In each specimen, the appearances described below were noted.

1. Aggregation of material inside the nucleus (fig. 18).

2. Bulging of the nucleus with the nuclear membrane still intact (figs. 19, 20).

3. Constriction of the neck of the bulge to produce a bud containing the nuclear material. The nuclear membrane did not necessarily surround the bud (fig. 21).

4. A cytoplasmic granule in close proximity to the nucleus often had the appearance of having resulted from separation of the bud from the nucleus (fig. 22).

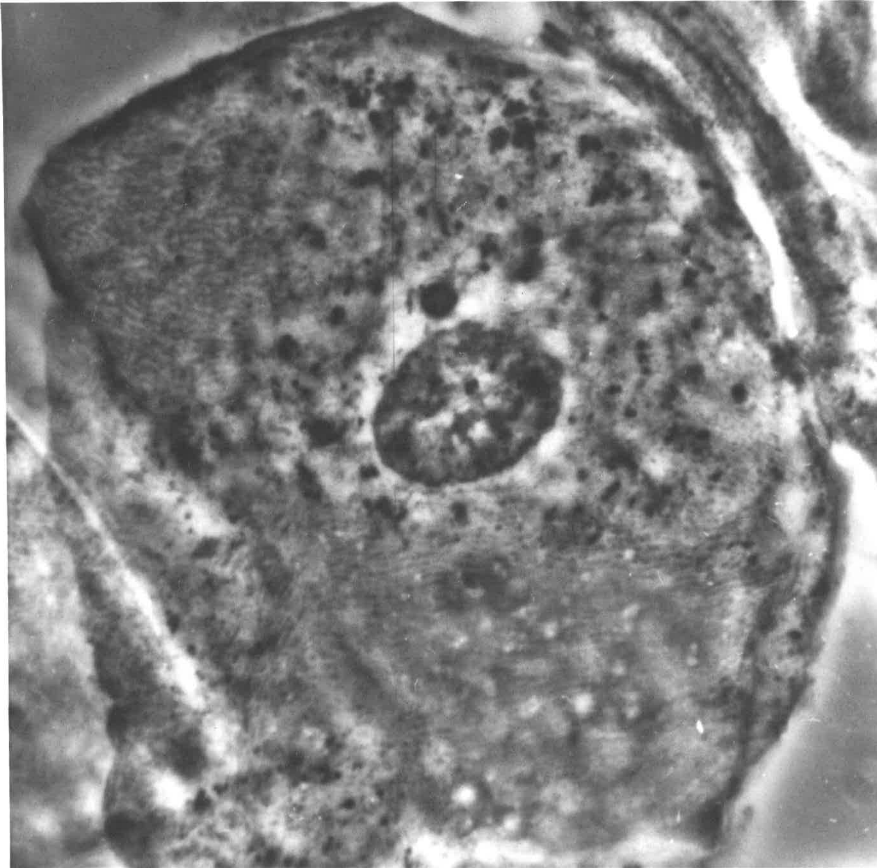

Fig. 17. Phase photomicrograph of an oral epithelial cell in a buccal scraping. There is one large cytoplasmic granule adjacent to the nucleus. X3500.

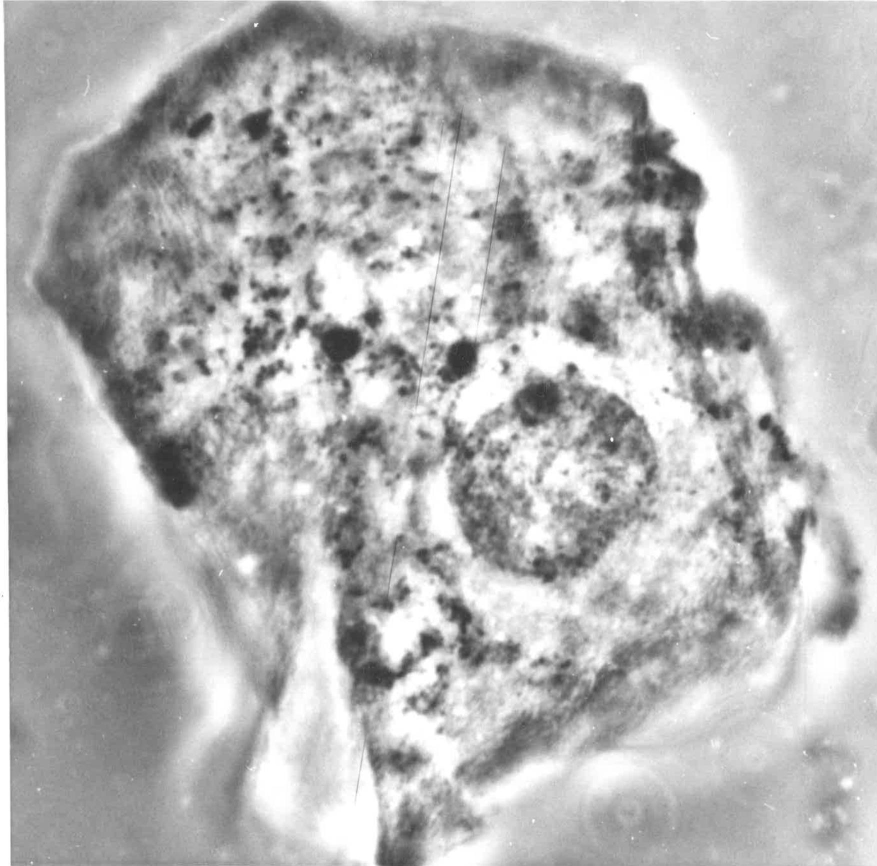

Fig. 18. Phase photomicrograph of an oral epithelial cell containing two large cytoplasmic granules. The nucleus appears to contain a mass of material of similar density to the granules. X3500.

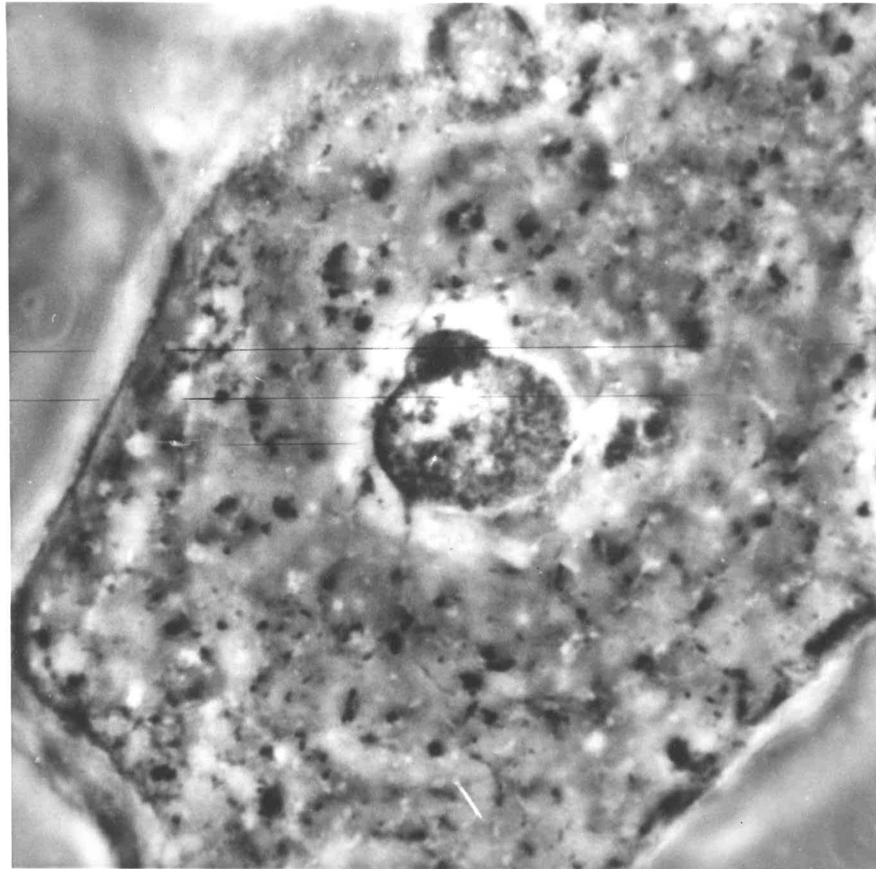

Fig. 19. Phase photomicrograph of an oral epithelial cell with a marked bulge on one side of the nucleus. X3500.

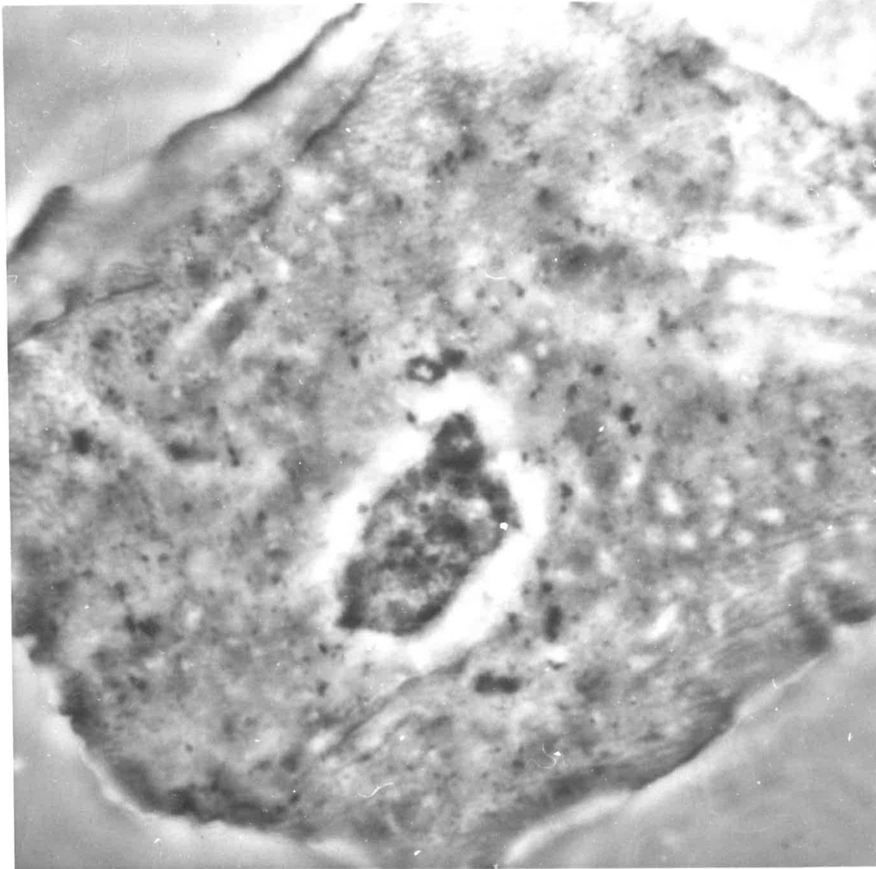

Fig. 20. Phase photomicrograph of an oral epithelial cell with a more pronounced bulge on one side of the nucleus. X3500.

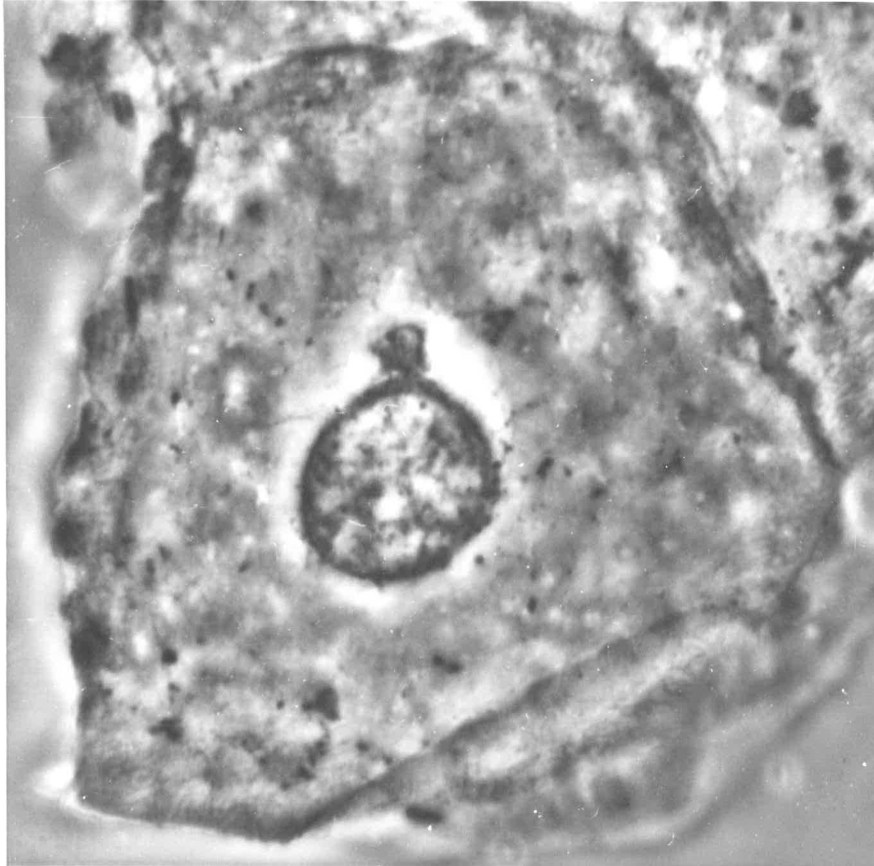

Fig. 21. Phase photomicrograph of an oral epithelial cell with the neck of the nuclear bulge constricted giving the appearance of a bud. X3500.

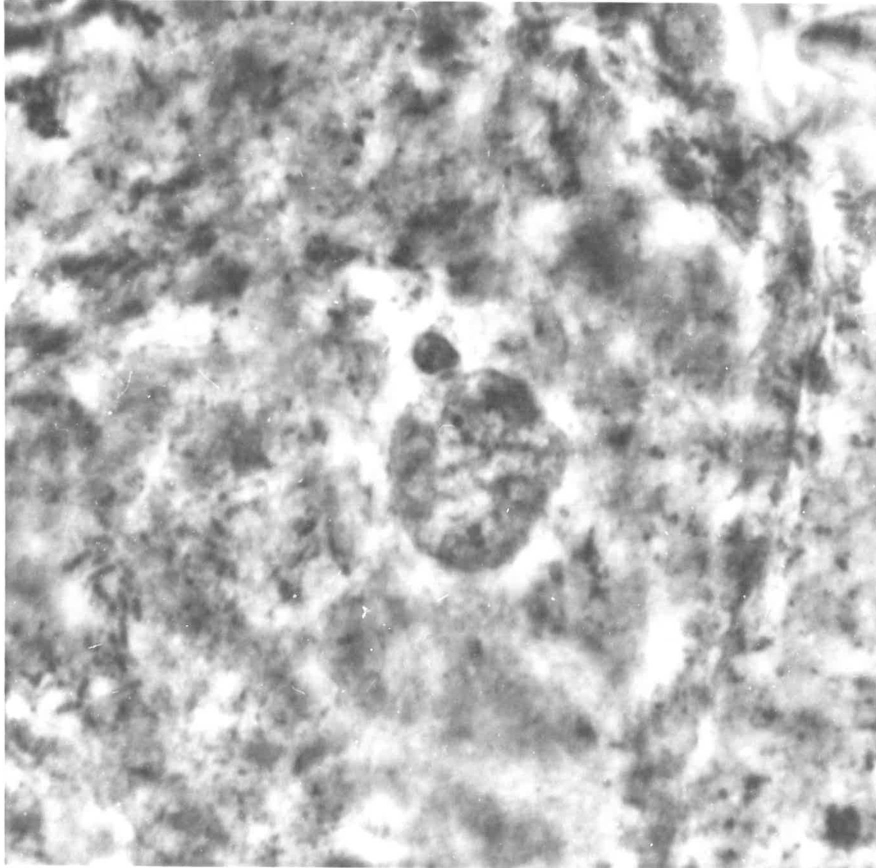

Fig. 22. Phase photomicrograph of an oral epithelial cell containing a cytoplasmic granule in close proximity to the nucleus. X3500.

Discussion and Conclusions.

The results of this investigation show that the cytoplasmic granules occurring in squamous epithelial cells from non-keratinized oral and vaginal mucosa are not artefacts. This establishes beyond doubt that they must in fact be keratohyalin granules.

The appearances of the cells provide evidence that the keratohyalin granules may arise from the nucleus of the cell in the sequence of events described in the results.

A number of changes associated with the formation of keratohyalin granules in oral epithelial cells from the buccal mucosa as seen by the electron microscope were described by Sognnaes and Albright (1958). These changes were:-

"1. Foldings of the nuclear membrane.

2. Nucleolar fragmentations and the formation of nucleolar satellites migrating to the nuclear surface.

3. The formation of cytoplasmic complexes in near proximity to the nuclei containing granules of various dimensions and mitochondrial fragments besides the high density keratohyalin granules."

The writers stated that they considered that the implications of the nucleolus, with known RNA activity, to be significant to the formation of keratohyalin granules

which contain RNP.

The suggestion that the keratohyalin granules of epidermis had a nuclear or nucleolar origin was made years before by Kollmann and Papin (1914) who believed them to be transformed nucleolar extrusions. A similar view was held by Ludford (1924) who believed that the nucleolus played a part in keratohyalin granule formation, but who also suggested that the Golgi apparatus, which disappears in the stratum granulosum of the epidermis was the origin of the granules. The Golgi apparatus was thought to be the origin of the keratohyalin granules by Parat (1928).

A change of or within mitochondria produced keratohyalin granules in the opinion of Sheldon and Zetterquist (1956), who described the accumulation of a dense substance within mitochondria believed to suffer from altered metabolism during vitamin A deficiency. However, they too noted that where the granules first appeared they were most often at the periphery of the nucleus. The concept of a mitochondrial origin of keratohyalin granules was supported by Favre (1950) and Meneffe (1957); the latter studied the electron microscopic appearance of the epidermis of embryo skulls during differe ntiation.

A number of possible origins were proposed by Martinotti (1914 a, b, 1915, 1921) who suggested that keratohyalin was formed by a process of "fibrillohexis" in epidermal fibrils, from the basophilic granules of the ground cytoplasm in stratum Malpighii, from the nucleus by karyolysis, and from the cell membrane. More recently, Matoltsy and Matoltsy (1962), in an electron microscopic study, stated that the development of epidermal keratohyalin granules begins in the cytoplasm when sub-microscopic corpuscles appear at the time the cells enter their course of differentiation, grow to various sizes independent of one another, and cease to grow when the cells are mature. This concept arose when Brody (1959), using electron microscopy, noticed similar fine granular material in both the cytoplasm and the keratohyalin granules; Matoltsy and Matoltsy extended the idea further to suggest that the granules originate at points in the cytoplasm, and grow by accretion of this material.

There is thus considerable support for the suggestion that the keratohyalin granules of the oral and vaginal epithelium arise from the nucleus. However, evidence suggesting the involvement of the mitochondria, the Golgi apparatus, the cell membrane, and the ground cytoplasm in the formation of keratohyalin granules of epidermis

prevent one from being dogmatic. It is possible that the components of keratohyalin granules may be variable and that the granules may arise by different means at different sites.

The final conclusions of this discussion are therefore:-

1. The cytoplasmic granules occurring in oral and vaginal squamous epithelial cells from non-keratinized mucosa are not artefacts or yeast cells, but are so-called keratohyalin granules.

2. The keratohyalin granules appear to arise from the nucleus of the epithelial cell.

CHAPTER V

THE INCIDENCE OF CYTOPLASMIC GRANULES IN BUCCAL EPITHELIAL CELLS

Introduction.

In 1940, Weinmann described the use of the oral smear and the Ernst-Gram stain to assess the degree of keratinization of the human oral mucosa. Other workers subsequently published similar papers dealing with different areas of the oral mucosa, and with the vaginal mucosa of the human female. Oral and vaginal smears stained by the Ernst-Gram method were compared by Ziskin, Kamen and Kittay (1942) who found "general uniformity in degree of keratinization between vaginal mucosa and alveolar gingivae". Using similar methods, Ziskin and Moulton (1948) concluded that slight hormonal changes could be more readily detected by vaginal smears than oral smears. The Shorr stain was used by Papic and Glickman (1950). They found no correlation between variations in gingival keratinization and specific phases of the menstrual cycle. Variations in buccal and vaginal smears stained by the Papanicolaou method were studied by Iuseum (1950); he claimed to demonstrate a correlation between changes occurring in both of them during the menstrual cycle of the human female. This led

to the conclusion that the maturity of squamous epithelial cells from the buccal and vaginal mucous membranes was influenced by oestrogen levels in the female. However, Museum suggested that local factors probably influence the keratinization of the oral mucosa to a greater extent than hormonal changes, particularly in the case of the gingival epithelium.

The colour of the cytoplasmic staining of oral squamous epithelial cells (as stained by the Papanicolaou stain) was used as a means of assessing degree of keratinization by Miller et al. (1951). They quoted Papanicolaou (1933) as stating, "Cornified cells take on an intense eosin colour and are sharply differentiated. On the other hand, cells derived from the deeper vaginal layers which are free from cornification are stained strongly blue". On this basis they assessed degree of keratinization by making differential counts of cells occurring in oral smears (stained by the Papanicolaou method), and dividing them into three categories:-

1. Yellow-staining cells.
2. Red-staining cells.
3. Blue-staining cells.

Yellow-staining cells were anuclear and indicated complete keratinization. Blue-staining cells were considered to be non-keratinized.

Examples of their differential counts (in increasing degrees of keratinization) are as follows:

Ventral surface of tongue: Red-staining cells, 21 per cent
(Standard deviation 11.1)
Blue-staining cells, 79 per cent
(Standard deviation 10.9)

Cheek: Red-staining cells, 73 per cent
(Standard deviation 10.5)
Blue-staining cells, 27 per cent
(Standard deviation 10.5)

Gingiva: Yellow-staining cells, 89.4 per cent
(Standard deviation 12.2)
Red-staining cells, 10.3 per cent
(Standard deviation 12.3)

The Papanicolaou stain, and a similar method of differential counting to assess degree of keratinization in different areas of the oral mucosa were used by Montgomery (1951). The counts were found to be significantly different (at the five per cent level) in smears from different areas; that is, each area of the mucosa studied had a characteristic cytologic pattern. The areas so differentiated were soft palate, cheek, vestible, tongue (anterior dorsum and posterior dorsum), and gingiva. Age, sex or menstrual cycle did not significantly influence these cellular patterns in

Montgomery's study. Papanicolaou stained smears from clinically normal buccal mucosa, hard palate, dorsal surface of tongue, gingiva, and floor of mouth, of both males and females in different age groups were studied by Silverman, Becks and Farber (1958). They concluded that a relationship appeared to exist between cytoplasmic cornification and local functional irritation. Degree of cornification was determined by cellular morphology in addition to cytoplasmic staining. Extensive clear cytoplasm, usually pink, yellow or orange staining, and a pyknotic nucleus or no nucleus at all, were the criteria for cellular maturity or cornification. Sandler, Stahl, Cahn, and Freund (1960) used a similar classification for cellular maturity of normal squamous epithelial cells in oral smears.

As stated earlier, Peters (1958) commented on the occurrence of cytoplasmic granules in oral squamous epithelial cells but could demonstrate no correlation between their incidence, and sex or menstrual cycle. Studies described in Chapters I to IV indicate that these granules can be regarded as being identical with the so-called keratohyalin granules of keratinized squamous epithelium. The smear technique, the Papanicolaou stain, and the method of differential counting to assess degree

of keratinization, are methods of investigating the relationship between the incidence of the granules and degree of keratinization. The following statements and predictions can be made to elucidate some aspects of this discussion.

1. The findings of Montgomery (1951) and Miller et al. (1951) concerning the differential counts made in smears from the buccal mucosa may be explained as follows.

They claimed that the more red-staining cells and the less blue-staining cells found in the smear, the higher the degree of keratinization. This can be explained firstly by the fact that the tougher keratinized surface limited penetration by the spatula used to scrape and collect the cells, and secondly by the fact that in such keratinized epithelium the red-staining superficial layers were thicker, so that relatively more red-staining cells would be collected, even if the spatula penetration had been equal to that obtained with less keratinized epithelium.

2. Histological appearances (Chapter I) suggest that more granules should be expected in cells from sub-surface epithelial layers (that is, blue-staining cells with the Papanicolaou stain) than in superficial cells (red-staining cells) and that there is no simple

relationship between degree of keratinization and incidence of keratohyalin granules. On occasions an anuclear keratinized surface occurred above an ill-defined and poorly developed granular layer, while parakeratinization occurred above a well-devloped granular layer.

3. As it would be expected that most granules would occur in blue-staining cells (Papanicolaou stain) and as a higher incidence of red-staining cells is taken to indicate a higher degree of keratinization, a comparison of granule incidence with degree of keratinization should logically be made by comparing the incidence of granules in blue-staining cells with the incidence of red-staining cells in the smear.

The following study was designed primarily to determine what relationship, if any, existed between the incidence of keratohyalin granules and degree of keratinization.

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Materials and Methods.

Squamous epithelial cells were obtained as described in Chapter II by scraping the buccal mucosa with a metal spatula. The cells were spread onto an albuminized glass slide, and fixed for a minimum of 30 minutes in 96 per cent ethyl alcohol. As all material was obtained from the buccal mucosa, the patients were chosen to ensure a wide range of degree of keratinization. Twelve of the patients were premature infants whose different ages, sexes and feeding methods ensured a variable oestrogen level. Details are given in Table IV. Eight were adults, three females and five males, of different ages. Details are given in Table V.

One smear was obtained from each individual. All smears were stained by the Papanicolaou method.

The stained smears were examined by the light microscope at a magnification of 450 diameters and the following counts were made.

1. One hundred epithelial cells were counted and divided into:-

(i) Red-staining cells.

(ii) Blue-staining cells.

This was repeated to make five counts in all, and mean percentages were then calculated.

2. (i) One hundred red-staining cells were counted and divided into

Table IV

Details of premature infants.

Patient number	Age (days)	Sex	Clinical state of buccal mucosa (N = normal) (J = jaundiced)
1	10	F	N
2	14	F	N
3	20	F	N
4	6	F	N
5	13	F	N
6	3	F	N
7	5	F	N
8	7	F	N
9	7	F	J
10	7	M	J
11	1	M	N
12	16	M	N

Table V

Details of adults.

Patient number	Age (years)	Sex	Clinical state of buccal mucosa (N = normal)
13	18	F	N
14	22	F	N
15	36	F	N
16	31	M	N
17	25	M	N
18	26	M	N
19	18	M	N
20	28	M	N

A. Cells containing cytoplasmic granules identifiable with those described in Chapter II.

B. Cells without cytoplasmic granules.

This was repeated to make five counts in all, and a mean percentage of the red-staining cells containing granules was calculated.

(ii) One hundred blue-staining cells were counted as above, and a mean percentage of blue-staining cells containing granules was calculated.

3. (i) One hundred of the red-staining cells containing granules were examined, and the number of granules in each cell was recorded to enable a mean number of granules per cell to be calculated.

(ii) One hundred blue-staining cells containing granules were examined as above, and a mean number of granules per cell obtained.

These data were then examined statistically. The methods used are described in Appendix IV.

1. Standard deviations were calculated for:-

(i) Mean percentage red-staining cells in each smear.

(ii) Mean percentage blue-staining cells in each smear.

(iii) Mean percentage of red-staining cells containing granules.

(iv) Mean percentage of blue-staining cells containing granules.

2. A chi-squared test was used to determine the significance of the difference between 2 (i) and 2 (ii) above.

3. The product of the mean percentage of red-staining cells containing granules and the mean number of granules per red-staining cell was calculated, thus giving the number of granules per hundred red-staining cells. A similar product was calculated for blue-staining cells. An analysis of variance was then carried out to determine the significance of the difference between the incidence of the granules in red-staining cells and their incidence in blue-staining cells, and the significance of the variations between individual patients.

4. The incidence of granules in blue-staining cells (represented by the mean number of granules per 100 blue-staining cells in smears from each patient) was plotted against the incidence of granules in red-staining cells (represented similarly) in the same smear. A correlation coefficient was calculated for the relationship between the incidence of granules in red-staining cells and the incidence of granules in blue-staining cells.

5. Differences between smears from infants and smears from adults were tested for significance by student "t" tests. The differences tested are listed below.

(i) The difference between the incidence of red-staining cells in infant smears and their incidence in adult smears.

(ii) Similarly for blue-staining cells.

(iii) The difference between the incidence of red-staining cells containing granules in infants' smears and their incidence in adult smears.

(iv) Similarly for blue-staining cells containing granules.

6. The incidence of granules in blue-staining cells (represented by the number of granules per 100 blue-staining cells in smears from each patient) was plotted against the incidence (percentage) of red-staining cells in the same smear.

Results.

The results are presented in Tables VI, VII, VIII, IX, X, XI and XII.

The incidence of red-staining and blue-staining cells in the smears is shown in Table VI.

Table VII compares the incidence of cells containing cytoplasmic granules in the red-staining and blue-staining categories, and indicates that blue-staining cells containing granules occur more frequently than red-staining cells containing granules in every smear.

The differences between the incidence of red-staining cells containing granules and the incidence of blue-staining cells containing granules are revealed to be significant (at the five per cent level or better) in all but two patients in Table VIII.

The mean number of granules per cell in red-staining cells containing granules and in blue-staining cells containing granules are shown in Table IX. There were more granules per cell in blue-staining cells than in red-staining cells in every patient. The mean number of granules per cell in both red-staining and blue-staining cells was higher in adults than in infants.

Table X shows the number of granules per hundred red-staining cells and per hundred blue-staining cells

Table VI

Incidence of red-staining and blue-staining cells in buccal smears.

	Patient number	Mean percentage of red-staining cells	Mean percentage of blue-staining cells	Common standard deviation
INFANTS	1	16.6	83.4	1.14
	2	5.2	94.8	0.84
	3	20.6	79.4	1.84
	4	56.4	43.6	2.88
	5	34.8	65.2	1.72
	6	74.6	25.4	1.67
	7	17.0	83.0	1.87
	8	13.4	86.6	2.30
	9	45.8	54.2	1.48
	10	58.0	42.0	1.58
	11	50.2	49.8	1.50
	12	29.8	70.2	2.55
	Mean	35.1	64.9	21.7
ADULTS	13	94.0	6.0	2.24
	14	52.8	47.2	2.49
	15	65.4	34.6	1.82
	16	52.2	47.8	2.39
	17	73.8	26.2	1.67
	18	75.6	24.4	1.84
	19	75.2	24.8	1.92
	20	63.0	37.0	2.45
	Mean	69.0	31.0	13.75

Table VII

Incidence of cells containing cytoplasmic granules in buccal smears.

	Patient number	Mean percentage of red-staining cells containing granules	Standard deviation	Mean percentage of blue-staining cells containing granules	Standard deviation
INFANTS	1	44.8	2.39	86.2	1.48
	2	33.0	2.12	50.2	2.39
	3	37.8	1.88	66.0	2.34
	4	53.8	1.48	67.0	1.87
	5	46.8	2.28	56.2	1.64
	6	17.8	2.24	42.2	1.48
	7	15.6	1.14	49.8	2.88
	8	41.4	1.52	88.6	3.05
	9	74.2	1.92	94.2	2.59
	10	40.0	1.87	65.4	1.14
	11	20.6	1.82	67.6	2.61
	12	46.0	1.58	97.2	1.26
	Mean	39.3	16.46	69.2	18.43
ADULTS	13	34.6	1.79	47.2	2.14
	14	5.2	1.76	26.6	1.18
	15	10.6	1.26	52.4	1.84
	16	38.2	1.22	49.4	2.65
	17	49.4	1.52	65.4	1.97
	18	19.6	2.07	26.8	2.19
	19	34.4	2.39	45.0	2.59
	20	9.8	1.05	16.4	1.26
	Mean	25.2	16.98	41.2	20.39

Table VIII

Values of chi-squared (χ^2) and probability (n=1) for the difference between the mean percentage of red-staining cells containing granules, and the mean percentage of blue-staining cells containing granules in each patient.

Patient number	Chi-squared (χ^2)	Probability (n=1)
1	144.08	0.001
2	11.802	0.001
3	30.12	0.001
4	7.882	0.01
5	3.589	0.10
6	24.41	0.001
7	46.78	0.001
8	220.54	0.001
9	73.227	0.001
10	28.503	0.001
11	100.85	0.001
12	963.28	0.001
13	5.68	0.02
14	23.787	0.001
15	70.29	0.001
16	5.017	0.05
17	11.277	0.001
18	2.6321	0.20
19	4.54	0.05
20	4.2128	0.05

Table IX

Incidence of cytoplasmic granules in cells containing granules, occurring in buccal smears.

	Patient number	Mean number of granules per cell in red-staining cells containing granules	Mean number of granules per cell in blue-staining cells containing granules
INFANTS	1	2.53	3.41
	2	1.71	2.86
	3	4.05	5.22
	4	2.90	3.39
	5	2.18	3.01
	6	1.18	1.59
	7	2.24	3.39
	8	2.51	3.41
	9	4.09	5.58
	10	3.05	4.77
	11	1.89	4.46
	12	3.76	5.02
	Mean	2.67 $\pm$ 0.9307	3.82 $\pm$ 1.166
ADULTS	13	2.19	3.86
	14	2.12	3.68
	15	7.71	9.83
	16	5.30	6.94
	17	5.83	9.30
	18	1.79	4.32
	19	2.58	4.36
	20	3.04	4.17
	Mean	3.82 $\pm$ 2.177	5.81 $\pm$ 2.534

Table X

Incidence of cytoplasmic granules in all cells in buccal smears.

	Patient number	Number of granules per 100 red-staining cells.	Number of granules per 100 blue-staining cells.
INFANTS	1	113	294
	2	56	143
	3	149	344
	4	156	227
	5	102	169
	6	21	67
	7	35	169
	8	104	302
	9	303	526
	10	122	312
	11	39	301
	12	173	488
	Mean	115	279
ADULTS	13	76	182
	14	11	98
	15	82	515
	16	202	343
	17	288	608
	18	35	116
	19	89	196
	20	30	69
	Mean	101	266

and again demonstrates the fact that granules occur more frequently in blue-staining cells than in red-staining cells.

The correlation coefficient for the relationship between the incidence of granules in red-staining cells and the incidence of granules in blue-staining cells (as shown in Table X) was 0.808. Therefore there is a high degree of concomitant variation in the incidence of granules in the two staining categories (red-staining cells and blue-staining cells) as the correlation coefficient differs from 1.0 significantly at the one per cent level. This relationship is demonstrated in graph form in figure 23.

The incidence of granules in infants is only slightly greater than that for adults in Table X.

The analysis of variance shown in Table XI (from the data in Table X) indicates that the difference between the incidence of granules in red-staining cells and that in blue-staining cells in all the smears examined is significant at the one per cent level. It is also demonstrated that significant variations (at the one per cent level) in the incidence of granules occur between individual patients.

The significance of differences between adult smears and infant smears is shown in Table XII. Adult smears

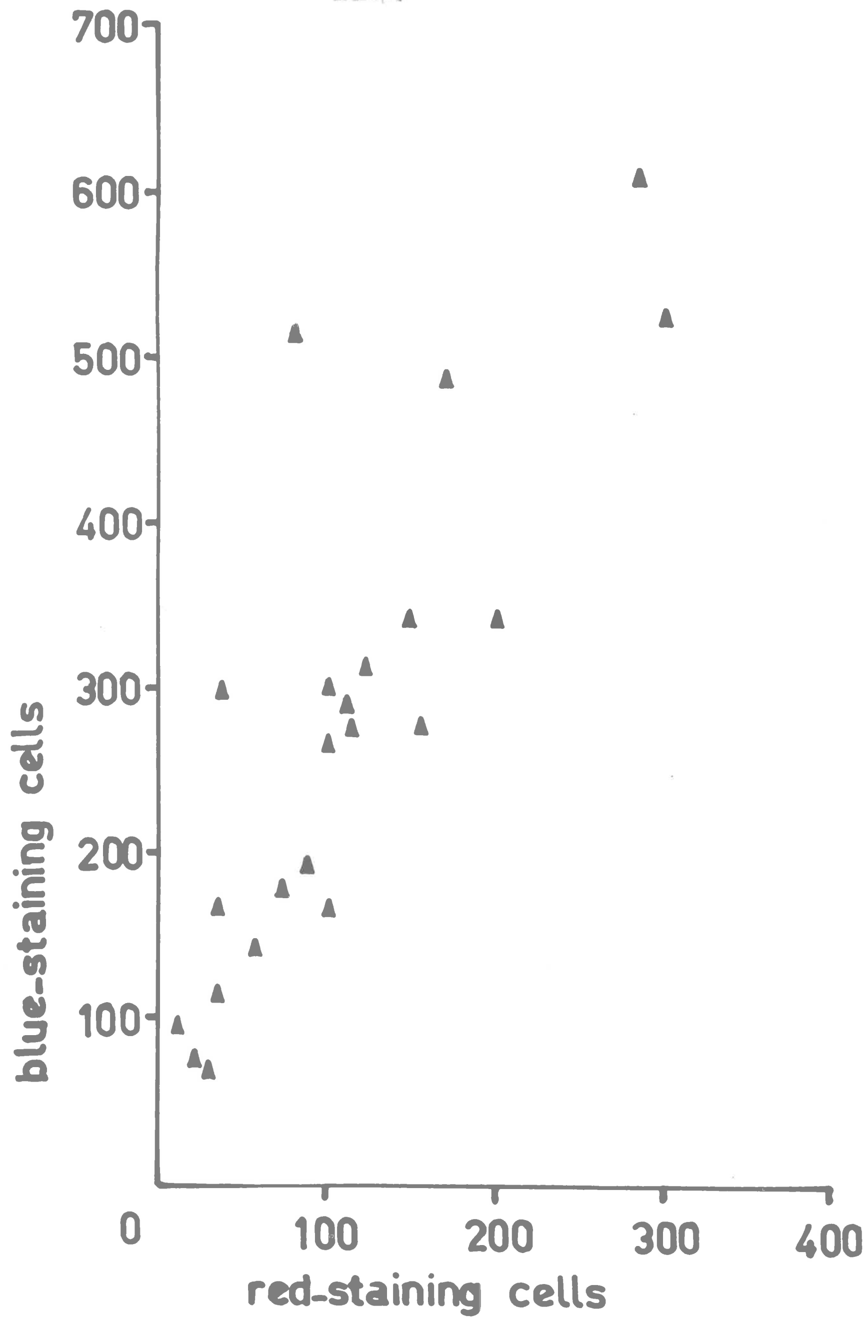

Fig. 23. Graph showing the relationship between the incidence of cytoplasmic granules in blue-staining cells and red-staining cells in Papanicolaou-stained buccal smears.

Table XI

Analysis of variance between the incidence of granules in red-staining cells and blue-staining cells, and between the incidence of granules in specimens from individual patients (data from Table X).

Both variance ratios are significant at the one per cent level.

Variation due to:-	Degrees of freedom	Sum of squares	Mean sum of squares	Variance ratios
Between cells	1	271,422.20	271,422.2	50.04
Between patients	19	514,626.90	27,085.6	4.99
Error	19	103,060.30	5,424.2	
Total	39	889,109.40		

Table XII

Differences between infant smears and adult smears.

	Infants	Adults	Difference	Level of significance of difference
Mean percentage of red-staining cells per smear	35.1	69.0	33.9	0.1%
Mean percentage of blue-staining cells per smear	64.9	31.0	33.9	0.1%
Mean percentage of red-staining cells containing granules	39.3	25.2	14.1	5%
Mean percentage of blue-staining cells containing granules	69.2	41.2	28.0	0.1%

contain more red-staining smears than infant smears but infant smears contain a higher percentage of cells containing granules than adults smears. These differences are significant at the five per cent level or better.

Figure 24 clearly depicts the lack of correlation between the incidence of granules in blue-staining cells and the incidence of red-staining cells in the same smear.

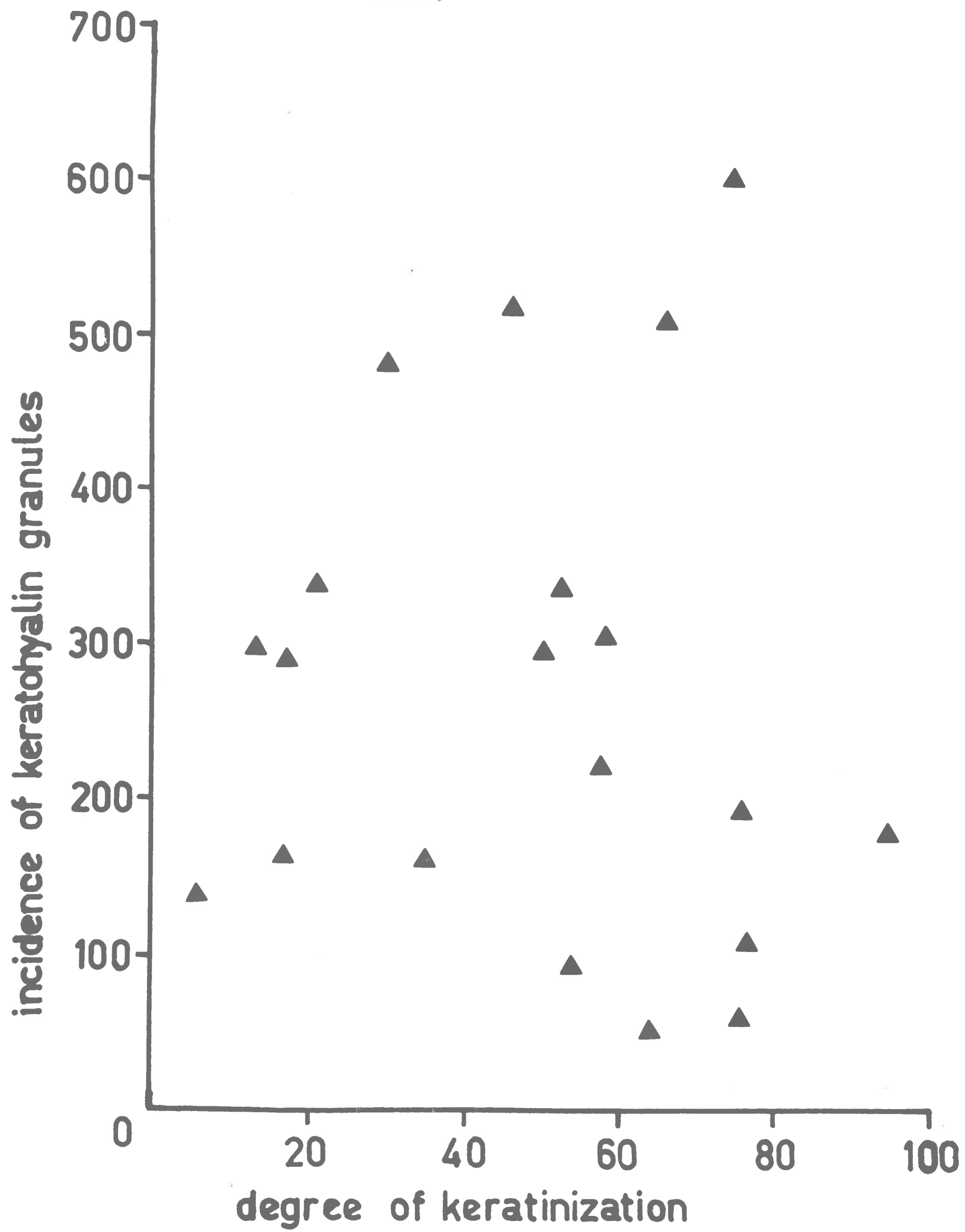

Fig. 24. Graph showing the lack of correlation between incidence of keratohyalin granules and degree of keratinization in buccal epithelium.

Discussion and Conclusions.

Smears from adults contained, on the average, twice as high a percentage of red-staining cells as smears from infants (Table VI). As there is no doubt that more functional masticatory trauma exists in the adult mouth, and if in fact the percentage of red-staining cells is a valid indication of degree of keratinization, this observation supports the view of Silverman et al. (1958) that there is a direct relationship between local functional irritation and degree of cornification of the oral epithelium.

Greater variations in percentages of red and blue-staining cells occurred in smears from infants than in those from adults. This may be due to ante-natal variations in oestrogen levels in the foetal circulation, combined with post-natal variations in the milk received by each infant. Cornification of the infant vaginal mucosa was stated by Osborn (1953) to be due to the action of the mother's oestrone; higher oestrone levels induced higher degrees of cornification of the infants' vaginal epithelium which persisted for up to four weeks after birth. This persistence of effect long after the oestrone is removed from the infants' circulation is readily explained if we accept the idea that keratinization is initiated in the basal layer of

the epithelium. It would then take some weeks for a cell in the basal layer at birth to reach the surface of the epithelium, resulting in a more keratinized surface long after the direct action of the oestrogen had ceased.

Both blue-staining and red-staining cells in infant smears contained granules more frequently than similar cell populations in adult smears (Table VII). However, less granules in each infant cell (Table IX) resulted in adults and infants having a similar overall incidence of granules in their smears (Table X). However, Table VI shows that the degree of keratinization in adults (as assessed by the incidence of red-staining cells) is twice that of infants, and there is no correlation between degree of keratinization and incidence of keratohyalin granules in sub-surface (blue-staining) cells of the buccal epithelium (fig. 24).

The fact that granules occurred more frequently in blue-staining cells than red-staining cells confirmed the impression gained from observation on sections (Chapter I). There can be no doubt that a change occurs in the granules as the epithelial cells proceed from the intermediate layers to the surface. This change involves the loss of RNA or RNP which is probably involved in the synthesis of the final product of the cell. Furthermore, the high correlation between the incidence of granules in

blue-staining cells and their incidence in red-staining cells (fig. 23.) suggests that the relative reduction of granule incidence which accompanies the change in colour of cytoplasmic staining may be at a uniform level.

The following conclusions can be drawn from this discussion.

1. The buccal mucosa of adults possessed a higher degree of keratinization (as assessed by the incidence of red-staining cells in Papanicolaou-stained smears) than that of infants.

This is probably due to the adult mucosa being subjected to more functional irritation than infant mucosa.

2. Variations in the degree of keratinization of infants' buccal mucosa (as assessed above) could be due to variations in oestrogen levels both ante-natally and post-natally.

3. There is no correlation between the degree of keratinization and the incidence of keratohyalin granules in the buccal mucosa of infants and adults.

4. Keratohyalin granules occur more frequently in the subsurface epithelial cells than in the cells of the superficial layers.

GENERAL DISCUSSION AND CONCLUSIONS

The origin, nature and function of the keratohyalin granules of stratified squamous epithelium have long been a source of speculation. Although recent work has clarified this subject to some degree, a variety of opinions are expressed in histological textbooks.

In describing the keratohyalin granules of epidermis, Maximow and Bloom (1957) state that their origin is not clearly established. However, they imply indirectly that there may be a nuclear origin of keratohyalin granules when they say "with the gradual increase in size and number of granules, the nucleus disintegrates and becomes pale." Of the oral epithelium they state that "in man, under physiologic conditions, it does not undergo cornification", but that it may contain granules of keratohyalin.

Similarly vague reference to the keratohyalin granules of epidermis is made by Ham (1953) who considers that they are involved in some phase of the process by which soft keratin is formed. Montagna (1962) in surveying the literature concerning the nature, origin and function of the keratohyalin granules of epidermis, arrives at some more definite conclusions. He states, "Keratohyalin granules seem to be more numerous in sites of slow

keratinization than in those of rapid keratinization. The lack of either sulfhydryl or disulphide groups in these granules, however, eliminates almost entirely the possibility that they play a primary, direct role in keratinization." An almost identical statement is made in "Bailey's Text-book of Histology" (1958); "Since the keratohyalin granules lack sulfhydryl groups characteristic of tonofibrils, and disulfide groups characteristic of keratin, it seems doubtful that they have any direct role in the process of keratinization." While echoing the opinion that the granules are not directly concerned with keratinization, Lever (1961) appears to contradict completely Montagna's statement concerning the relationship of granule incidence to rate of keratinization when he says, "The thickness of the granular layer varies from one to three cells, and as a rule, stands in direct relation to the degree of keratinization. The layer is thickest in areas where keratinization is most active. In areas of imperfect keratinization (parakeratosis), the granular layer is usually absent". Furthermore he limits his remarks about the oral epithelium to, "The mucous membranes of the mouth normally possess no granular cells and no horny layer. There the epithelial cells in their migration from the basal layer to the surface, first become vacuolated, then

shrink and finally desquamate." Lever's statement is in turn contradicted by that of Sognnaes (1954) writing in Greep's "Histology". He reports that in epithelium of mucosal surfaces "granules of the refractive horny substance, keratohyalin, are said to occur in the outer cells, even in the oesophagus." Orban's Oral Histology and Embryology (1962) allows that keratohyalin granules do occur in oral epithelium, but offers no opinion beyond the well-known fact that they "are basophil and stain blue in haematoxylin and eosin preparations," which has has been accepted since Waldeyer (1882) named such cytoplasmic bodies "keratohyalin granules." A "Synopsis of Oral Histology" (Bhaskar, 1962) contains a comment almost as antiquated; "These granules are believed to be the precursors of keratin."

Recent studies with the electron microscope have done much to clear the confusion evident in the foregoing review of the literature. Although Zelickson and Hartmann (1962) in an electron microscopic study of the epithelium from the inside of the lower lip of adult white males state that keratohyalin granules are absent, Sognnaes and Albright (1958) on the other hand, describe keratohyalin granules in cells from adjacent buccal epithelium. The diversity of opinion concerning the origin of the granules has been discussed previously

(Chapter IV).

Finally, the ultimate purpose and destination of the granules has been exhaustively investigated by Brody (1959, 1960). He proposes that the keratohyalin granules and cytoplasmic fibrils (of keratin) are equally involved in the formation of the final horny component of epidermis. However he states that the granules do not undergo a fibrillar transformation, but appear to become dissociated and mixed with the fibrous content of keratinizing cells, their ultimate fate thus being a dispersion into the interfibrillary spaces. This concept was supported by Charles (1959) who also considered that the keratohyalin granules appeared as a precipitate adjacent to tonofibrils.

From this discussion a number of points of controversy arise. Firstly, there is some doubt as to whether keratohyalin granules occur in the epithelium lining the oral mucosa and other mucous surfaces, although the preponderance of opinion suggests that they are indeed present.

Secondly, the origin of the granules has stimulated many hypotheses over a number of years (see Chapter IV); this work has involved the use of both the light microscope and the electron microscope.

Finally it has been suggested by Lever (1961) that the incidence of keratohyalin, granules in epidermis bears a direct relationship to the degree of keratinization, whereas Montagna (1962) considered keratohyalin granules to be fewer in areas of rapid keratinization and more numerous in areas of slow keratinization.

The present investigation produced facts which may clear the controversy concerning these three points.

1. Keratohyalin granules were invariably present in sections of the oral epithelium from the lips, cheeks, tongue and hard palate of adults and from the buccal mucosa of foetuses. They were also present in smears from the buccal mucosa of both adults and infants of both sexes and in vaginal smears from adult women. Histochemical evidence, along with phase contrast microscopy and the microbiological work, left no doubt that the granules present in the smears were in fact the so-called keratohyalin granules and were not pigments, microorganisms or artefacts of any kind.

2. From the observations made with the phase contrast microscope, it appears that the keratohyalin granules of buccal and vaginal epithelial cells arise from the nucleus of the cell and possibly from the nucleolus.

3. In the buccal epithelium of adults and infants the degree of keratinization bears no relationship to the

incidence of keratohyalin granules. If the keratohyalin granules were directly involved in the keratinization process one would expect the opposite, a correlation between granule incidence and degree of keratinization. This evidence thus supports the suggestion of Brody (1959, 1960) that the granules are not directly involved in the process of keratinization, but ultimately become an interfibrillary component of the keratinized squame. It would not then be inconceivable for the amount of interfibrillary substance to vary independently, with no relation to the amount of fibrillar keratin.

APPENDIX I

<u>Ribonuclease digestion technique.</u>

Three specimens were used and the pre-staining treatment was different for each.

(i) Incubation at 37°C for one hour in a $\frac{1}{10,000}$ solution of ribonuclease in glass distilled water.

(ii) Incubation at 37°C for one hour in glass distilled water.

(iii) No incubation.

APPENDIX II

Azure A stain.

The formula used was as follows:

Azure A, 125 mgm.

Citric acid 0.1 M, 30 ml.

Disodium phosphate 0.2 M, 20 ml.

Staining method

(i) Smears brought to water.

(ii) Stained with Azure A for 10 minutes.

(iii) Washed in running water for five minutes.

(iv) Dehydrated with butyl alcohol.

Once again three specimens were used, and were treated in exactly the same manner as those stained with methyl green - pyronin.

(i) Incubation at 37°C for one hour in a $\frac{1}{10,000}$ solution of ribonuclease in glass distilled water.

(ii) Incubation at 37°C for one hour in glass distilled water.

(iii) No incubation.

APPENDIX III

Benzidine stain for haemoglobin.

Benzidine solution.

Dissolve 0.2 mgm. of benzidine and a 5 mm. crystal of sodium nitro-prusside in 15 ml. of methyl alcohol. Add four drops of glacial acetic acid and shake.

Ozonic ether.

H_2O_2, 10 volumes . . 50 ml.

Methyl alcohol . . .100 ml.

Ether . . . . 50 ml.

Permanganate solution.

The solution used is similar to that used for the silver reticulin method, diluted in the ratio 1:4 with distilled water. It is bleached with oxalic acid.

Method: (i) Any fixative.

(ii) Bring to water, remove any mercury and rinse with methyl alcohol.

(iii) Stain the inverted slide on a tile for five to ten minutes with the benzidine solution.

(iv) Wash off with ozonic ether.

(v) Leave on the rack with fresh ozonic ether for five to ten minutes.

(vi) Wash off with tap water and examine. If there is a precipitate remove it with weak permanganate.

(vii) Wash in water for ten or fifteen minutes.

(viii) Counterstain with neutral red.

(ix) Dehydrate, clear, and mount.

Haemoglobin stains dark blue.

APPENDIX IV

<u>Examples of statistical methods.</u>

1. <u>Standard deviations.</u>

The standard deviations were calculated as in the following example.

<u>Patient 3.</u>

1. (i) Percentage of red-staining cells.

(total = n) Count number	Red cells	(x) Differences from 20	(f) Number of observations	($fx = \Sigma x$) Contribution to sum	($fx^2 = \Sigma x^2$)
1	21	+1	1	+1	1
2	20	0	1	0	0
3	23	+3	1	+3	9
4	18	-2	1	-2	4
5	21	+1	1	+1	1
				+3	15

$$\text{Mean} = 20 + \frac{\Sigma x}{n}$$

$$= 20 + 3/5$$

$$= \underline{20.6}$$

$$\text{Standard deviation} = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n - 1}}$$

$$= \sqrt{\frac{15 - \frac{(+3)^2}{5}}{4}}$$

$$= \sqrt{3.4}$$

$$= \underline{1.84}$$

2. Chi-squared test.

The value of chi-squared was calculated using the following method.

Example:-

Patient 3.

Cell colour	Red	Blue
Percentage containing granules	37.8	66.0
Percentage without granules	62.2	34.0

$$\text{Chi-squared } (\chi^2) = \Sigma \frac{(f - f_e)^2}{f_e}$$

$$= \frac{(37.8 - 66.0)^2}{66.0} + \frac{(62.2 - 34.0)^2}{34.0}$$

$$= \underline{30.12}$$

3. <u>Analysis of variance.</u>

This test involved the combined results of all 20 patients as shown below.

(data from Table X)

Patient number	Number of granules per 100 cells: Red-staining cells	Blue-staining cells	Totals
1	113 ($x_{1.1.}$)	294 ($x_{1.2.}$)	407 (X_1)
2	56	143	199
3	149	344	493
4	156	227	383
5	102	169	271
6	21	67	88
7	35	169	204
8	104	302	406
9	3,030	526	829
10	122	312	434
11	39	301	340
12	173	488	661
13	76	182	258
14	11	98	109
15	82	515	597
16	202	343	545
17	288	608	896
18	35	116	151
19	89	196	285
20	30 ($x_{20.1}$)	69 ($x_{20.2}$)	99 (X_{20})
Totals	2,186 (X_r)	5,469 (X_b)	7,655 Grand Total (G.T.)

A. Correction Factor (C.F.) $= \frac{G.T.^2}{40}$

$$= \frac{7655^2}{40} = 1,465,005.6$$

B. Sums of squares (S.S.)

(i) Between cells S.S. $= \frac{X_r^2 + X_b^2}{20} - C.F.$

$= \frac{2,186^2 + 5,469^2}{20} - C.F.$

$= 1,734,427.8 - 1,465,005.6$

$= \underline{271,422.2}$

(ii) Between patients S.S. $= \frac{X_1^2 + X_2^2 \ldots\ldots + X_{20}^2}{2} - C.F.$

$= 1,979,632.50 - 1,465,005.6$

$= \underline{514,626.9}$

(iii) Total S.S. $= x_{1.1.}^2 + x_{1.2.}^2 + x_{2.1.}^2 \ldots\ldots x_{20.2}^2 - C.F.$

$= 2,354,115.00 - 1,465,005.60$

$= \underline{889,109.40}$

(iv) Error S.S. = Total S.S. - (Cells S.S. + Patients S.S.)

$= \underline{103,060.30}$

C. Mean sums of squares (M.S.S.)

$$\text{Mean sum of squares} = \frac{\text{Sum of squares}}{\text{Degree of freedom}}$$

So (i) Between cells M.S.S. $= \frac{271,422.20}{1}$

$= 271,422.20$

(ii) Between patients M.S.S. $= \frac{514,626.90}{19}$

$= 27,085.6$

(iii) Error M.S.S. $= \frac{103,060.30}{19}$

$= 5,424.2$

D. Variance ratio (V.R.)

(i) Between cells V.R. $= \frac{\text{M.S.S. between cells}}{\text{M.S.S. error}}$

$= \frac{271,422.2}{5,424.2}$

$= 50.04$

(ii) Between patients V.R. $= \frac{\text{M.S.S. between patients}}{\text{M.S.S. error}}$

$= \frac{27,085.6}{5,424.2}$

$= 4.99$

These results are contained in Table XI.

4. Correlation coefficient.

The correlation coefficient was obtained from the same data as used for the analysis of variance.

(data from Table X)

$$\text{Correlation coefficient } (r) = \frac{\Sigma(x_1 - \bar{x}_1)(x_2 - \bar{x}_2)}{\sqrt{(\Sigma(x_1 - \bar{x}_1)^2 . \Sigma(x_2 - \bar{x}_2)^2}}$$

$$= \underline{0.808}$$

5. Student "t" tests.

The following is an example of the "t" tests.

(data from Table VI)

	Mean percentage of red-staining cells	Standard deviation
Infants	35.1 (x_i)	21.7 (S_i)
Adults	69.9 (x_a)	13.75 (S_a)

(n = number of patients.
Thus n_i = number of infants (12)
n_a = number of adults (8))

$$t = \frac{x_a - x_i}{\sqrt{\frac{S_a^2}{n_a} + \frac{S_i^2}{n_i}}}$$

$$= \frac{69.0 - 35.1}{\sqrt{\frac{(13.75)^2}{8} + \frac{(21.7)^2}{12}}}$$

$$= \underline{3.907}$$

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ADDENDUM

The initial objectives of this thesis were outlined in the Introduction. To briefly reiterate, it was decided to attempt to characterize histochemically the cytoplasmic granules occurring in non-keratinized stratified squamous epithelium to see if they could be identified as so-called "keratohyalin granules". Further studies were directed with the hope that knowledge of the origin and role of the granules in the normal cell could be extended. The relationship between keratohyalin granule incidence and degree of keratinization was thought to be an important field for investigation in this respect.

It may be appropriate at this time to review some of the findings described in the text. Firstly, a review of the literature revealed that it has been generally accepted that keratohyalin granules occur only in epithelium which could be classified histologically as "keratinized" according to the definition outlined in the Introduction. In consequence most histological text books state that the non-keratinized areas of oral epithelium contain no keratohyalin granules. It must be remembered however that because epithelium is classed as non-keratinized this does not necessarily mean that its cells are entirely devoid of keratin. In fact a disadvantage of histological classification of epithelium into three types, keratinized, parakeratinized and non-keratinized is that it suggests that these three types are distinct entities, whereas the truth is that there is a gradual transition from one type to another. Not infrequently the histological appearances of stratified squamous

epithelium prevent easy classification. This point is illustrated by figures 4 and 5 (pp. 36 and 37). The results of examination of histological sections of oral epithelium from a variety of sites revealed that granules with the characteristic staining reactions of keratohyalin granules were present in all types of epithelium, including that conforming to the definition of non-keratinized epithelium. The appearances could be interpreted to mean that no constant relationship existed between degree of keratinization and incidence of keratohyalin granules.

Histochemical studies were extended to smears of normal buccal epithelium which can be normally classed as non-keratinized; this resulted in confirmation of the histo-chemical findings of the work using histological sections. The possibility that artefacts or micro-organisms could account for the granules was eliminated by the results described in Chapters III and IV. From the appearances of oral and vaginal cells under phase-contrast microscopy it seemed that the keratohyalin granules originated from the nucleus by an active "budding" process.

The statistical analysis in Chapter V provided further evidence that degree of keratinization and incidence of keratohyalin granules could vary independently in normal buccal epithelium.

Additional specimens of oral epithelium have since been obtained and evaluated in order to elucidate some of the findings described in the text. The results are described below.

1. <u>Histological examination of normal and inflamed oral mucosa.</u>

In the preliminary studies described in Chapter I, the epithelial specimens used for histological study were from hyperplastic lesions. Consequently it was thought advisable to conduct a further study to compare epithelium from normal mucosa and that of inflamed areas so that the conclusions concerning the occurrence of keratohyalin granules in histological sections of oral epithelium could have broader application. (Although the smear technique allows more accurate assessment of granule incidence, the area of oral mucosa most commonly found to be inflamed is the gingival interdental papilla from which adequate smears are impossible to obtain. Histological methods were therefore used in this study.)

Specimens of oral mucosa from various sites were excised from patients undergoing oral surgery, fixed in 10 per cent formol saline, processed, sectioned, stained with haematoxylin and eosin, and examined microscopically. The details of the specimens are given in tables XIII and XIV.

Granules identifiable with those described previously in the text as keratohyalin granules were present in the epithelium of every specimen regardless of the degree of keratinization as determined by histological criteria outlined in the Introduction. In specimens with inflamed sub-epithelial tissue but intact epithelium the general incidence and appearance of the granules seemed to be no different from that of normal tissue.

Thus the appearance and occurrence of keratohyalin granules have been found to be similar in histological sections of normal epithelium, epithelium from hyperplastic lesions (Chapter I) and epithelium from areas of inflammation.

Table XIII

Details of specimens of normal oral mucosa

Age (years)	Sex	Site	Epithelial type
59	M	Buccal sulcus	Non-keratinized
69	M	Mylohyoid region	Parakeratinized
39	F	Buccal sulcus	Non-keratinized
65	F	Gingival margin	Parakeratinized
46	M	Gingival margin	Keratinized
30	M	Buccal sulcus	Non-keratinized
15	F	Hard Palate	Parakeratinized
37	F	Median frenum	Non-keratinized
31	F	Buccal sulcus	Non-keratinized
63	F	Labial mucosa	Non-keratinized

Table XIV

Details of specimens of inflamed oral mucosa

Age (years)	Sex	Site	Epithelial type
84	F	Gingival margin	Parakeratinized
13	M	Gingival papilla	Non-keratinized
74	M	Gingival papilla	Non-keratinized
63	F	Gingival papilla	Parakeratinized
40	F	Gingival papilla	Non-keratinized
55	F	Gingival margin	Parakeratinized
15	M	Gingival papilla	Non-keratinized
13	M	Gingival margin	Non-keratinized
39	F	Gingival margin	Non-keratinized
50	M	Gingival papilla	Parakeratinized

2. <u>The relationship between the incidence of cytoplasmic granules and degree of keratinization.</u>

The most important conclusion of Chapter V, namely that there is no correlation between the degree of keratinization and the incidence of keratohyalin granules in normal buccal epithelium, depends upon the validity of using cytoplasmic colour of Papanicolaou-stained cells as an indication of degree of keratinization. To test the efficacy of the method used in Chapter V, a buccal smear was taken from each of the twenty adults with clinically normal buccal mucosa. Smears were fixed while wet in 96% ethyl alcohol for 20 minutes, as were those described previously in the text, then all stained simultaneously with haematoxylin and eosin, mounted, and examined microscopically at magnifications up to 400 diameters. The cells were divided into two groups depending upon the characteristics of their nuclei, which were classified as either pyknotic or non-pyknotic. The criteria for pyknosis were as follows:

1. Marked reduction in size of nucleus.
2. Dense staining with haematoxylin to produce a uniformly "black" appearance with no clearly defined nuclear membrane or chromatin particles.

The criteria for a non-pyknotic classification were:

1. No marked reduction from normal size.
2. Normal staining with clearly defined nuclear membrane and chromatin particles.

Those few nuclei with equivocal characteristics were not counted. Anuclear cells, which were also exceedingly rare, were placed in the pyknotic group. A higher percentage of pyknotic nuclei was interpreted as being indicative of a higher degree of keratinization, and a lower percentage of pyknotic nuclei was taken to represent a lower degree of keratinization.

Five separate counts of 100 cells each were made on each smear and a mean percentage of pyknotic nuclei was calculated for each specimen.

Cytoplasmic granules identifiable with the so-called keratohyalin granules, and identical with those described as such in Chapters II and IV, and those counted in Chapter V, were present in all smears. The number of the granules occurring in 100 cells with non-pyknotic nuclei was recorded for each smear. In rare instances granules which appeared to be the result of nuclear disintegration with dissolution of the nuclear membrane were seen. These granules were thus not keratohyalin granules and were not included in the counts.

The results of the counts are shown in Table XV. The incidence of granules was plotted against the percentage of pyknotic nuclei representing degree of keratinization in the same smear. The resulting graph is shown in figure 25. (cf. figure 24, p.118). Statistical analysis of these data indicates that the results are significant at the five per cent level. These results clearly show that, using this method, there is no correlation between degree of keratinization and incidence of keratohyalin granules in the buccal epithelium and thus agree with the conclusion reached in Chapter V.

Table XV

Incidence of pyknotic nuclei and cytoplasmic granules in normal buccal epithelial cells

Age (years)	Sex	Mean percentage of cells with pyknotic nuclei	Number of granules per 100 cells with non-pyknotic nuclei
35	M	33.4	113
36	M	16.6	19
24	F	20.0	249
27	M	42.0	357
20	M	28.6	18
22	M	37.8	214
21	M	28.8	286
34	M	25.2	453
24	M	22.6	454
21	F	37.8	159
24	F	39.8	63
24	F	11.0	223
34	M	15.6	133
25	M	29.6	252
30	M	11.6	48
50	F	7.0	47
53	F	1.6	38
43	F	25.8	567
20	M	30.2	436
21	M	21.4	215

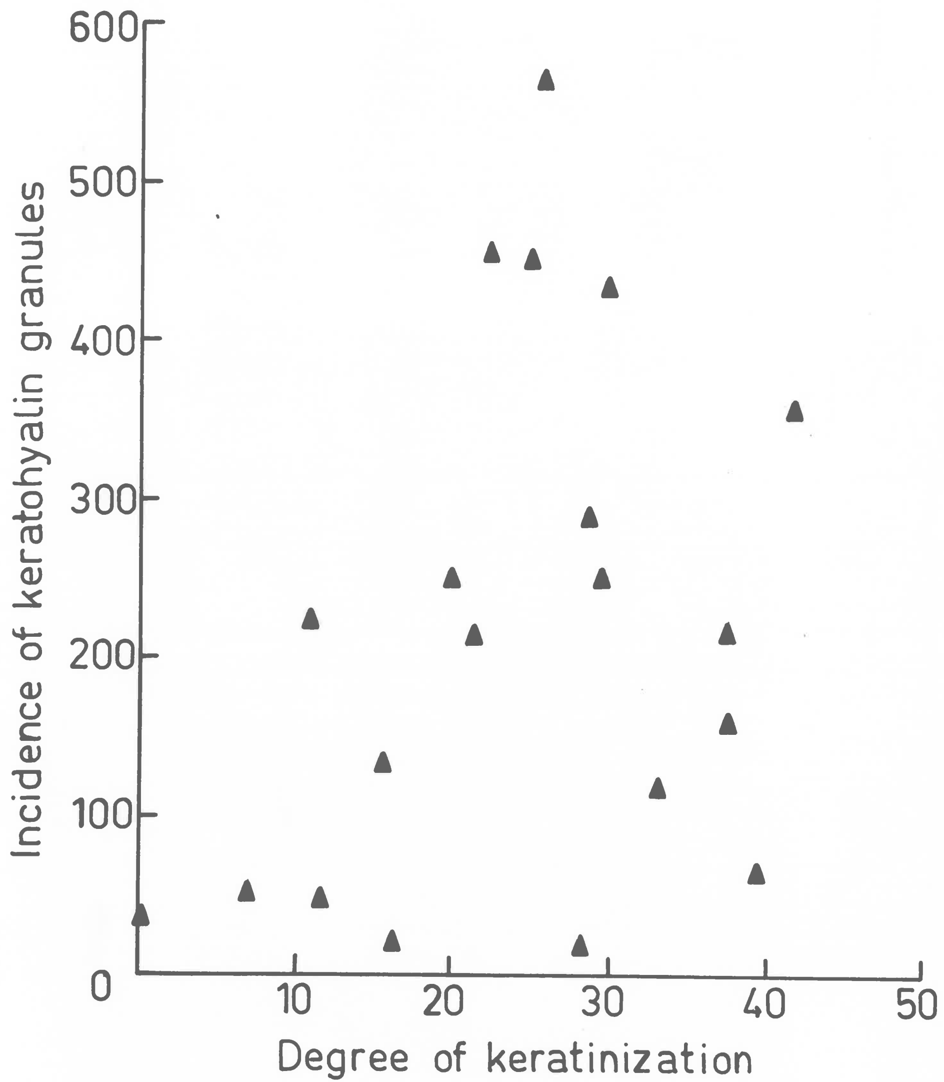

Fig. 25. Graph showing the lack of correlation between incidence of keratohyalin granules and degree of keratinization in buccal epithelium.

3. Irradiated specimens of oral epithelium.

The effects of X-irradiation on oral epithelial cells were described by Peters (1958) and recorded previously in the text. The main finding concerned the enormous increase in the number of granules identifiable with those described as keratohyalin granules in Chapters II and IV.

In order to relate Peters' findings to those of this thesis, buccal smears of otherwise normal irradiated epithelium were obtained from five patients undergoing treatment for facial or oral malignancies at the Radiotherapy Department of the Royal Adelaide Hospital. Radiation doses ranged from 2000 to 3000 rads, and treatment had begun between three and six weeks previously and had not been concluded for more than one week. Smears were obtained, fixed, stained and examined as described previously in this Addendum.

The findings of Peters were completely confirmed. Granules were so numerous in most cells as to prevent accurate counting, but approximate assessments gave the number of granules per 100 cells with non-pyknotic nuclei to be between 5000 and 7000. This represents a ten-fold increase over the highest incidence of the granules recorded in any of the twenty normal smears.

On the other hand, the incidence of pyknotic nuclei in the irradiated cells did not increase. Nuclear abnormalities were quite frequently observed and were often bizarre, but percentages of pyknotic nuclei varied between 14 per cent and 31 per cent, i.e. within normal limits as defined by table XV. The incidence of completely degenerate nuclei did not appear to be increased and thus could not be associated with the increase in granule numbers.

Discussion.

The facts to emerge from these additional studies are mainly confirmatory of those elicited by the work described in the text. In particular it can now be said that so-called "keratohyalin granules" occur in oral epithelium covering normal, inflamed, and hyperplastic tissue and that the incidence of the granules in normal buccal epithelium is not related to the degree of keratinization as assessed by two different methods. Smears from X-irradiated epithelium have provided supportive evidence for a number of suggestions concerning the origin and role of the granules in the epithelial cell. This evidence can now be included in a brief speculative review of epithelial cell function.

As mentioned previously in the Introduction a number of workers have found that cytoplasmic organelles including mitochondria, Golgi apparatus and rough-surfaced endoplasmic reticulum are abundant in basal epithelial cells but disappear in cells of more superficial layers. Enzymes have been demonstrated in virtually all layers of the epithelium. Some of these, in the deeper cell layers are doubtless involved in the mitotic process, and other energy requiring processes such as protein synthesis, while others, although probably synthesized in cells of the deeper layers, persist in more superficial cells. It is almost certain that these enzymes are contained in membrane bound vesicles (lysosomes) and they may be indirect evidence of further organisation of cell components in the superficial epithelial layers.

The accumulated morphological and biochemical evidence in the literature can only be interpreted to mean that the major energy-requiring syntheses occur in

the deeper cell layers. Keratin synthesis must surely be among these processes. On the other hand, the appearance of keratohyalin granules occurs in more superficial cell layers as do the accepted manifestations of keratinization, namely nuclear morphological changes and cytoplasmic staining differences. It seems then that the chain of biochemical events initiated in the basal layer of the epithelium continues to varying degrees, its ultimate being the production of an anuclear, fully-keratinized squame. The fact that no quantitative relationship can be demonstrated between keratohyalin granule incidence and degree of keratinization suggests that perhaps no qualitative relationship exists. In the light of the evidence that granule production seems to involve nuclear membrane activity and thereby phospholipid metabolism, it is not unlikely that this should be unrelated to the synthesis of proteins such as keratin. It is interesting to note that Elston (1963) described nuclear formations similar to the appearances depicted in figures 19 to 22 (pp. 87 to 90) in human bone marrow, skin and fascia lata cells in vitro, and in oral mucosa cells in vivo. Furthermore, Fogh, Biedler, and Denues (1961) found that X-irradiation of human amion cells in vitro produced budding and break-off of nuclear material. In view of the evidence presented in this Addendum, along with that of Peters (1958) it seems that X-irradiation may stimulate similar nuclear budding and thus increase keratohyalin granule incidence in oral epithelial cells. As the irradiation is designed to disrupt nucleic acid synthesis and hence mitosis in cancer cells, it is highly likely that it should at least in part reduce the level of synthesis of proteins such as keratin. One would thus

expect the finding that the increase in number of keratohyalin granules would be unaccompanied by an increase in degree of keratinization.

It is also interesting that Peters (1958) noticed that an increase in granule incidence did not reach a peak until 10 days after the beginning of radiotherapy which suggests that the initial effect may be in the deeper cell layers, only to become obvious after the cells have progressed to more superficial strata. Two alternative explanations of this phenomenon suggest themselves. Firstly, although unlikely, it may be that irradiation results in direct stimulation of granule production in the deep cell layers but that the granules only become stainable when they reach the more superficial layers. Secondly, it may be that irradiation produces a change in the nucleus which results in increased granule production when the cell reaches the superficial strata.

As granule production and the nuclear changes in superficial cells may be dependent on variations in membrane stability, interesting future studies could come from the use of known membrane "stabilizers" and "labilizers" such as cortisone and vitamin A in combination with irradiation. Such studies would necessarily be conducted with suitable experimental animals and could possibly determine where the initiation of keratohyalin granule production occurs.

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