AN ELECTRON MICROSCOPIC STUDY
OF NORMAL AND MIGRATING
EPITHELIUM IN THE PALATE
OF THE RAT.

Thesis submitted to the University of Sydney
as a requirement for admission to the
degree of Doctor of Philosophy.

John Richard Gibbins, M.D.S.
1964.
PREFACE

The work reported in this thesis was carried out to provide a basis for further studies into the development of oral carcinoma. At the same time it was hoped that it would provide some information on the biology of oral epithelium.

A knowledge of the fine structure of normal epithelium and of the changes occurring in epithelial cells following injury was considered to be an essential pre-requisite to any attempt to interpret the morphological alterations which might occur in palatal epithelium during attempts to produce carcinoma experimentally. In addition, the relative ease with which carcinoma can be induced in traumatised epithelium when compared with the difficulties of tumor induction in normal epithelium indicated that the effect of trauma on oral epithelium would have to be more thoroughly investigated.

Although a great deal of work has been done on superficial wound healing in humans and various animals, epithelium, especially stratified squamous epithelium, has received much less attention than the components of the
connective tissue. Little information is available on either the processes involved in the epithelial response or the mechanisms underlying them.

All the experimental work reported in this thesis is original and was carried out in the Department of Pathology, University of Sydney, while I was a research fellow of the New South Wales State Cancer Council. The observations and results are based on electron micrographs taken by myself and are set out in Chapters 2, 3 and 4. The magnifications after the captions referring to the electron micrographs are only approximate. The final chapter is a general discussion in which an attempt is made to interpret the results and observations reported in the earlier chapters.

I wish to thank my supervisor, Professor F.R. Magarey, for his encouragement and assistance during the period of my candidature and for obtaining and ensuring financial support. I am indebted to Associate Professor D.A. Cameron for training in the techniques of electron microscopy and for continual guidance and assistance. I wish to thank Professor B.T. Mayes for the use of the Philips EM100B in
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CHAPTER 1
MATERIALS AND METHODS

EXPERIMENTAL MATERIAL.

Material for study was taken from the ante-molar palate (Kutuzov and Sicher, 1952) of adult Wistar albino rats weighing approximately 250 gms. All the animals used in the standardised wound series were males. Other material (normal and after injury with iris scissors) was from both males and females. The diet of all animals was the stock diet with water ad lib.

NORMAL.

Collection of Specimens. Normal tissue was obtained under ether anaesthesia. The incisive papilla and antemolar rugae were excised and transferred immediately to ice-cold osmium tetroxide fixative (Caulfield, 1957). The second and third antemolar rugae were then separated and trimmed in the coronal plane into strips approximately 1 mm. wide. The trimming was carried out in a pool of fixative on a piece of polythene over ice. The small pieces of tissue were then processed according to one of the schedules
described below.

WOUND HEALING. The tissue examined in the studies of wound healing was taken from the bud of epithelium that arose from the margin of the wound nearest the crest of the ruga.

Investigations with the electron microscope were mainly concerned with the cells at the leading edge of the bud. This area was located in thick sections (1/2-1μ) and the block accurately trimmed so that the tip of the advancing bud was in the centre of the ultra-thin sections.

Experimental Procedure and Collection of Specimens.

Part I. The animals were anaesthetised with ether and the crest of the second antemolar ruga was removed with fine iris scissors. The animals were then allowed to recover and returned to their cages for 3, 6, 9 or 12 hours, during which time they were allowed food and water ad lib. At the end of the respective periods they were re-anaesthetised with ether. The injured rugae were removed in toto and transferred immediately to ice-cold 1% osmium tetroxide in Caulfield's (1957) buffer in which they were
trimmed in the coronal plane into 1 mm. wide strips. The blocks of tissue were embedded in methacrylate. This technique of wounding gave some useful information but was too crude to provide a uniform series of experimental wounds. Therefore, after the first introductory study was completed it was discontinued in favour of the following method.

Part II. The animals were rapidly anaesthetised with ether and given an intraperitoneal injection of Nembutal (0.3 ml. of an 0.6% solution in 10% alcohol for each 100 gm. body weight) and Atropine (0.06 ml. of an 0.0065% solution in 10% alcohol/100 gm.) as premedication. They were then transferred to a specially constructed operating table and anaesthesia maintained with ether and oxygen. The mouth was propped open with a combination mouth-gag and tongue depressor and the antemolar area of the palate well illuminated.

With the aid of a dissecting microscope an incision approximately \( \frac{1}{2} \) mm. wide and \( 1 \frac{1}{2} - 2 \) mm. deep was made with a specially constructed two-bladed scalpel in the anterior
surface of the 2nd antemolar palatal ruga. The strip of epithelial and connective tissue between these incisions was then carefully removed under the dissecting microscope with the tip of a No.11 Bard-Parker blade. The animals were then allowed to recover and returned to their cages for the experimental period (6, 12, 18 or 24 hours) during which they were allowed food and water ad lib. At the end of the experimental period the animals were anaesthetised with ether and given an intraperitoneal injection of Nembutal (approximately 1 ml./100 gms.). The injured rugae were then removed in toto and transferred to 1% or 2% osmium tetroxide in Caulfield's (1957) or Millonig's (1962) buffer over ice. They were trimmed immediately in the coronal plane with stainless steel blades into strips approximately 1 mm. wide and fixed in the cold for 1½-2 hours. The blocks were then processed according to one of the schedules below.

PROCESSING SCHEDULES

LIGHT MICROSCOPY. Blocks for examination by light microscopy only were fixed overnight in Bouin's fixative at
room temperature. They were then briefly washed with distilled water, dehydrated with alcohol (70%, 90%, absolute - 1 hour each), cleared in xylol and embedded in paraffin. Sections 5u thick were mounted and stained with haematoxylin and eosin by standard methods.

ELECTRON MICROSCOPY.

Fixation. Blocks for electron microscopy were fixed in solutions of osmium tetroxide prepared in the following manner.

Veronal Acetate Buffer.

1.94 gms. sodium acetate in 400 mls. of
2.94 gms. sodium veronal distilled water
pH of solution adjusted to 7.4 with N/10 HCl

Phosphate Buffer. (Millonig, 1962)

Solution A 2.26% sodium dihydrogen phosphate
Solution B 2.52% sodium hydroxide
Solution C 5.4% glucose
Solution D 41.5 ml. solution A + 8.5 ml. solution B
Final pH 7.3 - 7.4

(All pH measurements were made on a Radiometer pH meter 22 with a calomel electrode).

Osmium. 0.1 gm. vials of osmium tetroxide were mechanically cleaned in running water and gently scored
around the middle. They were then placed in concentrated nitric acid in 125 c.c. bottles and left overnight. The acid was poured off and the bottles thoroughly rinsed with distilled water.

The final osmolarity of the fixative was adjusted by the addition of 0.045 mg. of sucrose/ml. for the veronal buffer or 1 ml. of solution C to 9 mls. of solution D for the phosphate buffer. The necessary amount of buffer was added to make a final concentration of 1% or 2% osmium tetroxide. The bottle was capped and the vials fractured by sharp blows on a tightly rolled towel. When the crystals of osmium tetroxide had dissolved the solution was transferred to small glass tubes, sealed with plastic caps and chilled prior to use.

Dehydration was performed with either alcohol or acetone after a brief washing in buffer. Acetone was used for methacrylate embedding and alcohol for epoxy resin embedding. The procedure was the same in each case.

- 70% solution 15 minutes
- 90% solution 15 minutes
- absolute 2 x 30 minutes
Occasionally blocks were left overnight in dehydrating solution but preferably the blocks were carried through to the embedding stage as quickly as possible.

**Embedding.**

*Methacrylate.* Commercial methacrylate monomer was cleared of polymerisation inhibitors by adding one volume of 25% of sodium hydroxide to three volumes of methacrylate. This was then placed in a separating funnel and shaken vigorously. After standing, the brown sodium hydroxide solution collected at the bottom of the funnel and was run off. The monomer was washed by shaking with fresh water which was run off and the procedure with the sodium hydroxide repeated until the washing water remained clear. The washing was then repeated until the sodium hydroxide was removed completely.

The monomer was stored in a refrigerator in a tightly capped bottle containing anhydrous sodium sulphate.

The embedding mixture was prepared by mixing 1 part of methyl methacrylate monomer with 4 parts of butyl methacrylate monomer. Benzoyl peroxide (0.2–0.3%) was added
as a catalyst to the mixture of monomers.

The dehydrated blocks were placed in progressively increasing concentrations of the methacrylate monomer to remove the dehydrating solutions.

<table>
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<tr>
<th>Monomer</th>
<th>Acetone</th>
<th>Time</th>
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<tr>
<td>50%</td>
<td>50%</td>
<td>30 minutes</td>
</tr>
<tr>
<td>75%</td>
<td>25%</td>
<td>30 minutes</td>
</tr>
<tr>
<td>100%</td>
<td></td>
<td>1 hour</td>
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</tbody>
</table>

During this period a quantity of monomer was prepolymerised in a test tube in a water bath at 70-80°C. until the viscosity had increased to the extent that the mixture only just flowed out of the tube at room temperature.

Flat embedding moulds were prepared by punching \( \frac{1}{4} \)" holes in a 1/16" sheet of polythene and sticking the polythene to a flat glass surface. The prepolymerised methacrylate was placed in the wells and the blocks orientated with a needle. The surface of the wells was covered over with cellophane, another flat piece of glass applied, and the blocks cured in an oven at 60°C.

After curing the moulds were chilled, the polythene was removed and the blocks, now adherent to the glass, were removed by a sharp blow parallel to the surface.
Epoxy Resin.

During the course of these experiments epoxy resins gained in favour as embedding media for electron microscopy. Early attempts with this material were carried out with the procedure recommended by Glauert and Glauert (1958). However the results were extremely variable and the technique was discontinued until the publication of Luft (1961) on improvements in epoxy resin embedding. Application of Luft's methods, especially the use of propylene oxide, produced reasonable results but the procedure was still unreliable.

The most constant problem appeared to be irregular infiltration of the blocks. When sectioned at right angles to the surface, epithelium quite often cut satisfactorily but the underlying connective tissue usually could not be cut. On the odd occasions that sections were obtained they slowly disintegrated on the surface of the bath and dispersed under the influence of xylol vapour.

Since this problem did not arise after methacrylate embedding and had not been reported by other investigators,
I thought that it may be due to some technical difficulty in the handling of the resin. As a result an appraisal was made of the epoxy resins as embedding media for biological material.

Lee and Neville (1957) in their book on the theory and application of epoxy resins in industrial practice point out that anhydride curing of epoxy resins is relatively critical. In order to obtain the required physical and chemical properties the ratio of epoxy base and anhydride hardener must be determined in the light of the curing cycle employed.

The theoretical optimum ratio between the base and hardener reactive groups is 1. However, in industrial practice the ratio is reduced to 0.85 to compensate for competing reactions in the resin itself due to the unavoidable presence of reactive impurities.

Little accurate information is available on the reaction of osmium tetroxide with biological tissue (Reimersma, 1963) but it would appear that the reaction with the epithelium and the connective tissue of the rat
 palate is not identical.

After fixation in osmium tetroxide the epithelium is much more intensely stained than the underlying connective tissue. After prolonged washing this difference in staining is increased and if the washing is carried out in a hypotonic solution then the dense connective tissue undergoes a definite swelling while the epithelium retains its original size. These observations led me to conclude that the osmium tetroxide was not "fixing" the collagen in the connective tissue beneath the epithelium.

Since it is known that epoxy groups react with hydroxyl and amine groups I thought that the large amount of collagen in the dense connective tissue was affecting the curing of the resin. It seemed to me that the reactive groups of the collagen fibrils could be removing epoxy molecules from the embedding mixture which resulted in a disturbance of the correct hardener/base ratio in the immediate vicinity of the fibres. If this were so it could be compensated for by diffusion while the resin was thin in consistency, but as the viscosity of the resin
increased the diffusion of the molecules through the dense connective tissue would become progressively more difficult. The eventual result of this process would be that the dense collagen would become embedded in a resin which at best was improperly cured and at worst consisted virtually of a mass of liquid hardener. If the resin-connective tissue mass was sufficiently mechanically stable to be cut, flattening with xylol would dissolve the residual resin and disperse the connective tissue into the water of the cutting-bath. This is what happened in practice.

As a result two approaches to overcome this problem were attempted.

Fixation of Collagen by Aldehyde Fixatives. Formaldehyde and glutaraldehyde were used to post-fix the connective tissue. 10% formaldehyde with 2.5% potassium dichromate overnight at room temperature were found to be the most effective. However the fine structural morphology of the cells, especially phospholipid membranes was altered by these procedures.

Alteration of the Hardener/Base Ratio. Alteration of
the hardener/base ratio in an attempt to provide an excess of base molecules in the connective tissue was therefore investigated. However it was realised that it would be necessary to strike a compromise that would also provide acceptable cutting properties in the other tissues embedded in the resin.

As a result a range of hardener/base ratios were examined from 1 through to 0.2 in 0.2 steps, and it was found that the optimum ratio lay between 0.6 and 0.8. A ratio of 0.7 was finally chosen.

An example of the method used to determine the ratio is as follows, using information obtained from Lee and Neville (1957).

Epoxide equivalent of Araldite 502 (D)CY230 = 250
Molecular weight of DDSA (HY964, Ciba) = 266

Assuming that monoester formation is the most frequent reaction at the curing temperatures used for biological material, then, 266 gms. DDSA contain 1 gram. mol. of carboxyl. Therefore assuming that 0.7 is the optimum ratio, 250 gms. 502 would require 0.7 x 266 gms. DDSA 25 gms. " " " 18.6 gms. DDSA
(Epoxide equivalent is the weight of resin in grams that contains one gram chemical equivalent of epoxy. In resins with linear chains and no side branching the epoxy equivalent is normally half the average molecular weight of the resin).

Embedding Schedule for Araldite D(502) CY230 Ciba.

After dehydration in alcohol the blocks were taken through 1% phosphotungstic acid in absolute alcohol - 1 hour absolute alcohol 15 min.
1% uranyl nitrate in absolute alcohol 1 hour re-distilled absolute alcohol 2 x 30 min.
propylene oxide 2 x 15 min.

During this part of the processing the epoxy resin mixture was prepared,

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<th>Base</th>
<th>Araldite 502</th>
<th>25.0 gms.</th>
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<tr>
<td>Hardener</td>
<td>DDSA</td>
<td>18.6 gms.</td>
</tr>
<tr>
<td>Accelerator</td>
<td>DMP 30</td>
<td>0.5 gms.</td>
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and mixed with a mechanical rotary mixer for 30 minutes.

The excess propylene oxide was poured off and only sufficient left to cover the blocks. The epoxy resin mixture was poured in and the tubes placed on a rotator and left overnight.

Next morning the resin mixture was replaced and the tubes transferred to a vacuum oven and left all day at
35°C.

In the evening the tissues were transferred to flat polythene embedding wells, orientated, labelled and placed in an oven at 45°C. overnight. The next morning the orientation of the blocks was checked and they were transferred to an oven at 60°C. and left for 2 days. After cooling the blocks were readily removed from the wells by flexing the sheet of polythene.

SECTIONING.

Sections (silver to gold) were cut on Porter-Blum, Huxley or LKB microtomes, using glass or diamond knives, flattened with xylol vapours and picked up on 100 or 200 mesh copper grids that had previously been covered with a layer of Formvar. Grids for methacrylate sections were carboned and grids for Araldite sections were used uncarboned.

Thick sections \( \frac{1}{2} \) to 1μ were cut on Porter-Blum or Huxley microtomes and examined either with phase contrast or stained with alkaline toluidine blue. They were used to localise the area of the block to be examined in the electron microscope. After selection of the area the
blocks were trimmed with razor blades under a dissecting microscope.

**STAINING.**

Staining for examination in the electron microscope was carried out by 3 methods.

(a) Methacrylate sections were stained with lead hydroxide prepared by the method of Watson (1958).

(b) Araldite sections were stained in the block by alcoholic solutions of phosphotungstic acid and uranyl nitrate.

(c) In addition, some araldite sections were stained prior to examination in the electron microscope with sodiumplumbite prepared by the method of Karnovsky, M.J. (1961).

**EXAMINATION AND PHOTOGRAPHY.**

Light microscope sections were photographed with a Zeiss Photomicroscope on panchromatic film.

Electron microscope sections were examined in either a Philips 100B or Siemens Elmiskop I.

Electron micrographs were taken in the Philips EM 100B on 35mm Agfa Agape film and developed with Rodinal
developer (1:30, 8 mins., 68°C.)

In the Siemens Elmiskop I micrographs were taken with Ilford Microneg Pan 35mm film, Ilford N50 plates or Kodak Statfile 70mm film. The 35mm film was developed in Rodinal, the N50 plates in ID36, and the 70mm in Kodak D11. All exposures were standardised with a photomultiplier exposure meter reading off the final screen.

Final prints were made on Ilford B3IL or B2IL.

**ACID PHOSPHATASE LOCALISATION.**

The first attempts to localise acid phosphatase were carried out with tissue fixed in formaldehyde and incubated in Gomori's Medium using the methods recommended by Holt and Hicks (1961). The results obtained with this method were encouraging, but the preservation of fine structure was poor. It was impossible to differentiate cytoplasmic organelles.

Following the publication of Sabatini et al (1963) a changeover to glutaraldehyde was made. This fixative produced a much better preservation of fine structure and cytoplasmic organelles could be clearly recognised. However some difficulty was experienced in obtaining preservation without distortion of cellular outline,
apparently due to hypertonicity of the fixative.

The method finally adopted was as follows:

**FIXATION.** 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.3 - 7.4.

(The fixative was buffered with cacodylate buffer to avoid the possibility of excess phosphate ions that may be left in the block after fixation in phosphate buffers).

At 18 or 24 hours after injury the wounded rugae were removed *in toto* and transferred to ice-cold fixative, trimmed into thin slices (approximately \( \frac{1}{2} \) mm) and fixed in the cold for 1\(\frac{1}{2}\)-2 hours. They were then washed with cacodylate buffer, transferred into 0.1M tris-maleate buffer at pH 7.3 - 7.4, containing 0.2M sucrose and left there till required. This period in tris-maleate buffer varied but was never less than overnight. When left for longer periods, the buffer was changed frequently.

The blocks were removed from the buffer and transferred to the incubating medium at room temperature. The incubating media were prepared immediately before use and were based on the modified Gomori medium recommended by Barka and Anderson (1962).
**Stock Solutions.**

1.25% sodium b-glycerophosphate (pH adjusted to 6.2 with 1N HCl).

0.2M tris-maleate buffer pH 6.2
2.9 gm. maleic acid
3.04 gm. tris (hydroxy methyl) amino methane in
50 ml. of distilled water. Add 43 mls. of 0.5N
sodium hydroxide and make up to 125 mls. with
distilled water.

0.2% lead nitrate in distilled water (CO₂ free).
Filtered immediately before use.

**Incubating Medium.**

10 ml. tris-maleate buffer (0.2M, pH 6.2)
10 ml. distilled water
10 ml. sodium b-glycerophosphate

Add 20 ml. of lead nitrate by drops with continuous
stirring. The resulting medium is clear and will produce
minimal nuclear staining artifacts.

**CONTROLS** were prepared as follows:

(i) Incubating medium + NaF to make a final
concentration of 0.01M.

(ii) Incubating medium - after immersion of the
blocks for 10 mins. in 5% trichloracetic acid.

(iii) Incubating medium with the substrate (sodium
b-glycerophosphate) replaced by an equal amount of
distilled water.
INCUBATION. The blocks were left in the incubating media for 30 mins. at room temperature and transferred to a water bath at 37°C. for another 30-45 minutes.

POST-FIXATION. At the end of the incubating period the blocks were quickly washed in veronal-acetate buffer at pH 7.3 - 7.4 and transferred to 1% or 2% osmium tetroxide in veronal-acetate buffer. (N.B. Phosphate buffer cannot be used to post-fix since there is still sufficient lead in the blocks to produce a diffuse precipitate by reacting with the phosphate ions in the buffer).

DEHYDRATION AND EMBEDDING was carried out according to the Araldite schedule described above except that staining with phosphotungstic acid and uranyl nitrate in the final dehydrating alcohols was omitted.

INCUBATION OF FROZEN SECTIONS. The majority of the experiments were carried out with incubated blocks. This was found to be satisfactory since it produced the minimum disruption of fine structure. Adequate penetration of incubating media occurred because of the proximity of the migrating bud to the surface of the block. In addition, trimming of the specimen blocks often produced
artifact splitting along the interface between the superficial edge of the bud and the wound coagulum, with the result that the incubating medium could come into direct contact with the outer surface of the bud.

However some blocks were treated by methods similar to those recommended for liver by Holt and Hicks (1961).

The rugae were removed, placed in cold fixative and cut into thirds. After fixing and washing the blocks were infiltrated with 0.1M tris-maleate buffer (pH 7.3) with 0.2M sucrose. They were placed on a piece of aluminium foil on the end of a length of cotton and immersed in liquid propane in a large test tube surrounded by liquid air in a Dewar flask. After removal from the propane the blocks were transferred rapidly to the pre-frozen stage of a freezing microtome and 50u frozen sections cut. These were incubated and processed by the same methods as used for the blocks.

EXAMINATION IN ELECTRON MICROSCOPE. Thin sections were examined in the electron microscope without staining or after short (1-2 mins.) stained with lead prepared by the method of Karnovsky, M.J. (1961).
PHAGOCYTOSIS OF ELECTRON OPAQUE COLLOIDS.

The first experiments to determine the presence or absence of phagocytic ability by the migrating epithelial cells were carried out with colloidal solutions of silver and saccharated iron oxide.

COLLOIDAL SILVER was prepared by a modification of the method for Luppo-Cramer's Nuclear Solution (Weiser, 1933).

- 10 ccs. of 10% dextrin
- 10 ccs. of 10% sodium hydroxide
- 75 ccs. of 10% silver nitrate

mixed and left standing. A very concentrated silver solution (approximately 20 mgm./ml.) was produced with a shelf life of approximately 1 month.

Prior to use the excess sodium hydroxide was removed by dialysis against distilled water for 24 hours, and any residual sodium hydroxide neutralised by adjusting the solution to pH 7.2 with N/10 HCl. The solution was then centrifuged at 4,000 r.p.m. in an MSE centrifuge. The resulting solution contained a high concentration of silver particles in the 60-80 μm size range.

This preparation was extremely well tolerated by the animals after intravenous injection. 0.5 ml. was injected into the dorsal penile vein immediately before wounding.
and 0.5 ml. immediately after wounding. At the end of the experimental period (18 or 24 hours) the wounds were processed by one of the schedules described above.

**SACCHARATED IRON OXIDE.** A commercial preparation (Imferon, Bengers Laboratories) with a concentration of 250 mgm./ml. was used. Little success was obtained with this material, however, despite its small particle size and low toxicity. It was very rapidly removed by the reticulo-endothelial system and a high peripheral concentration could not be maintained without repeated injections. Direct intra-carotid injection was attempted but the technique could not be standardised and was not without risk in small animals.

**THOROTRAST.** A commercial preparation of thorium dioxide (24-26% stabilised with dextrin) supplied by Testagar Corp. was finally selected as the colloidal marker of choice. However the high toxicity of this material necessitated a modification to the experimental technique used with the other less toxic materials.

In order to have a high circulating concentration of marker immediately after the wounding the reticulo-
endothelial system was "blockaded". The animals were anaesthetised with ether and 0.5 ml. of non-flocculating carbon (Pelikan C11/1431a) was injected into the dorsal penile vein. This carbon had previously been immersed in boiling water bath for 10 minutes. The animals were then allowed to recover and 2 hours later were re-anaesthetised and given an injection by the same route of 0.5 ml. of Thorotrust containing 0.013 mgm. of atropine. (The atropine was used to counteract the sudden cardiac arrest and respiratory obstruction by mucus secretion characteristic of overdoses of Thorotrust and which frequently resulted in the death of the animal.). During the injection the animal was also given 100% oxygen to breathe. This technique resulted in 100% survival in the 24 animals used for the experiments.

Within a 5 minute period after the injection of the Thorotrust a standard wound was made in the anterior surface of the second antemolar ruga. The animals were allowed to recover and returned to their cages for the experimental period (18 or 24 hours).

At the end of the experimental period the animals were
re-anaesthetised and the injured rugae were fixed in 2% osmium tetroxide in Millonig’s buffer (1962) and processed according to the Araldite schedule described above.
CHAPTER 2

NORMAL PALATAL EPITHELIUM

INTRODUCTION

Oral epithelium does not appear to have been extensively studied with the electron microscope and the majority of the studies carried out so far have been confined to human tissue (Sognnaes and Albright, 1956, 1958, Sognnaes et al., 1956, Albright, 1960, Zelickson and Hartman, 1962, 1963).

The epithelium covering the antemolar rugae of the rat palate was selected as being best suited to the type of experimental investigation planned. However, rodent oral mucosa only appears to have been examined by Rhodin and Reith (1962) and they were mainly concerned with lingual epithelium. As a result it was necessary to study the fine structure of normal epithelium from the antemolar palate in order to have a firm basis for accurate interpretation of any alteration in fine structure that might be found after experimental intervention.

OBSERVATIONS.

LIGHT MICROSCOPE. The second antemolar ruga of the rat
FIGURE 1. Light micrograph of the second antemolar ruga of the rat palate. A stratified squamous epithelium covers a triangular shaped mass of dense connective tissue. The thickness of the epithelium is relatively constant over the whole of the ruga and rete pegs are not highly developed. The external surface is uniformly covered with a thin cornified layer. The anterior surface of the ruga is the slightly convex right hand side of the triangle. H & E. x 60.

FIGURE 2. The prominent oval nuclei in the basal and lower spinous layers tend to lie with their long axis at right angles to the external surface. Cell borders can be clearly distinguished in the spinous and granular layers. The cytoplasm of the cells in the granular layer is almost completely obliterated by a densely staining material and the junction with the cornified layer is sharp. H & E. x 325.

FIGURE 3. Under polarised light the cornified layer is strongly birefringent. Orientated birefringence is also present in the other cell layers but is not as prominent as in the cornified layer. In the lower spinous and basal layers the pattern of birefringence is at right angles to the mucosal surface whereas in the upper spinous and granular layers it is parallel to the surface. Crossed prisms. x 225.
palate consists of a pyramid of dense connective tissue supporting a well developed stratified squamous epithelium (Fig.1). The characteristics of this epithelium are relatively uniform over the whole surface of each ruga. Its deep edge is irregular and is sharply demarcated from the underlying connective tissue except at the apex of the ruga where fingers of basal cells may extend into a cellular region of the connective tissue. The basal and lower spinous layers cannot easily be distinguished from each other because the cells at the junctions are closely packed and cell boundaries are not clear (Fig.2). The oval nuclei that stand out prominently in the cytoplasm have their long axes at right angles to the surface.

The cell borders become distinct in the spinous layer where the cells and their nuclei are elongated parallel to the surface. The spinous layer is approximately 5 to 8 cells thick. Its thickness cannot be accurately determined since the regions of transformation from the basal cells below and into the granular layer above are not sharply demarcated.

The cells in the granular layer are thinner and more
elongated than the cells in the spinous layer beneath them. The nuclei appear smaller and quite often cannot be seen in the cells immediately below the cornified layer. The layer is distinguished by the presence of very fine, darkly stained granules in the cytoplasm of the cells. The thickness of this layer is approximately 3 to 4 diameters but once again the transformation of spinous cells into granular cells does not give a clear line of demarcation. The transformation into the cornified layer above, however, is sharply defined.

In the cornified layer the cells have lost the densely staining granules that had accumulated earlier. Cell borders are not clear but the cells appear to be more compressed and elongated than those in the granular layer beneath. The number of cells in this layer cannot be determined from light micrographs of sections stained with haematoxylin and eosin but this layer is approximately one third of the thickness of the whole epithelium. The cornified layer is not separated from the granular layer by a stratum lucidum.

When examined in polarised light the cornified layer is strongly bi-refringent. The intensity of bi-refringence
FIGURE 4. The lower border of the basal cells is irregularly folded and is separated from the underlying connective tissue by an amorphous, electron dense basement membrane (b) which is itself separated from the plasma membrane by a less dense space of approximately the same width. Tonofilaments run vertically through the cytoplasm and some of them are inserted into the incomplete desmosomes (d) along the plasma membranes apposed to the basement membrane. The collagen fibrils (c) between the folds are irregularly intertwined and are associated with very fine filaments. Deeper in the connective tissue the arrangement of the collagen fibrils is more regular and the fine filaments are much less frequent. Araldite. PTA, uranyl and lead stain. x 16,000.

FIGURE 5. The structure of the desmosomes is clearly visible. Tonofilaments (f) are inserted into dense plaques (a) that appear to be thickenings of the plasma membranes of the apposed cells. These plaques are uniformly separated by a dense material in which can be seen three dense lines that run parallel to the dense plaques. Araldite. PTA, uranyl and lead stain. x 80,000.
is much less in the layers beneath the cornified layer. In the granular and upper spinous layers the pattern of bi-refringence is parallel to the surface whereas in the lower spinous and basal layers it is at right angles to the surface (Fig. 3).

**ELECTRON MICROSCOPE.**

**Basement Membrane.** The plasma membrane of the cells of the basal layer is separated from the underlying connective tissue by a thin layer (300-600A approximately) of electron dense amorphous material (Fig. 4b). This layer (the basement membrane) follows exactly the intricate corrugations and undulations of the plasma membrane of the basal cells and is separated from them by a less electron dense space approximately 300A wide. The collagen fibrils immediately beneath this membrane are randomly orientated, especially between the deeper folds of the irregularly corrugated plasma membrane of the basal cells. In these sites there are also numerous thin fibrils (200A approx.), without clearly defined periodic banding, arranged in a complicated meshwork amongst the larger collagen fibrils (Fig. 4c).
FIGURE 6. An oblique section through the lower spinous and basal layers. The plasma membranes of adjacent cells are closely apposed and run parallel courses along most of their length but are randomly separated to form irregularly shaped small intercellular spaces(a). The impression is gained that these intercellular spaces form an anastomosing network. Infiltrating cells (w) which distend the spaces and distort the adjacent epithelial cells are occasionally found. The plasma membranes of the infiltrating and the epithelial cells are closely approximated but no desmosome-like attachment areas are present between them. Araldite. PTA, uranyl and lead stain. x 16,000.
Along a line approximately level with the lowest extension of the cytoplasmic processes of the basal cells into the connective tissue, the collagen fibrils are arranged in a more orderly fashion and tend to be collected into bundles. The bundles, however, do not appear to have any constant arrangement relative to each other. The very fine fibrils are absent from this region.

**Plasma Membranes.** In the basal and lower spinous layers the plasma membranes after fixation in OsO₄ have a fine structure identical to the plasma membranes of other cells. Along the lower border of the basal cells the membrane is thrown into large irregular folds. Along the lateral and superficial margins of the basal cells and all around the lower spinous cells the membrane is irregularly corrugated and the membranes of adjacent cells tend to run an almost parallel course along portions of their length. As a result the cells interdigitate with one another (Figs. 6 and 7). The other portions of the membranes are separated to form irregularly shaped spaces (Fig. 6a). The individual spaces are not large but in oblique sections of this region they give the appearance of forming intercellular channels. Occasionally other cells
are found wedged in between the epithelial cells which are somewhat distorted as a result (Fig. 6w). The characteristics of the cell membranes are similar throughout the spinous and granular layers. In the more superficial layers intercellular membrane spaces are infrequent and infiltrating cells are not present.

In the cornified layer, however, the plasma membranes are greatly thickened and cannot be easily distinguished from the adjacent cytoplasm (Figs. 10m and 11m). The cells are unevenly separated by long, narrow spaces which occasionally are partly filled by a dense granular material (Figs. 10i and 11i), but the spaces often appear empty.

**Desmosomes.** The plasma membranes of adjacent epithelial cells are united by desmosomes that are scattered along them. These structures consist of zones of the plasma membrane that appear more electron dense than other zones along the cell surface. When cut in cross section and examined at higher magnification these regions of the cell membrane are seen to be separated from the neighbouring cell membrane by a space of uniform width in which are 3 parallel lines against a less dense background.
material. The plasma membranes in these regions are thickened and appear as plaques into which some of the cytoplasmic fibrillar material is fused (Fig. 5).

This structural arrangement is not found along the lower border of the basal cells, in the cornified layer and along the interface between the cornified and granular layers. Scattered along the plasma membranes of the lower borders of the basal cells are incomplete desmosomes (Fig. 4d). The intracellular fibres, the plaque and one extracellular dense line can be seen but the extracellular dense background material is absent.

In the cornified layer the whole structure is different. The intracytoplasmic plaques and fibrillar material can no longer be seen since the cell membrane has the same appearance along its whole length. The intercellular background material is denser than in the lower layers and gradations in density rather than distinct lines are present. This background material is now a dense plate separated from adjacent cells by a definite zone that appears to be empty (Figs. 10d and 11d). The collections of dense material occasionally present between the cells of this layer are continuous with the
FIGURE 7. Basal cell. Mitochondria (M) are the most prominent cytoplasmic organelles. Their shape and the arrangement of their cristae (c), which arise by infolding of the inner membrane, are irregular. Golgi vesicles (g) are the major component of the endoplasmic reticulum. Rough surfaced endoplasmic reticulum (e) is rare but particles identical in appearance to RNP particles (r) are scattered throughout the cytoplasm. Tonofilaments (f) are present in the cell as bundles that run parallel to the long axis. Side branches from these bundles are inserted into desmosomes (d) that also tend to lie parallel to the long axis of the cell. The nucleus (N) is surrounded by a double layered membrane (h) that is reflected back at nuclear pores (p). Nucleoli (n) appear as aggregates of particulate components. Araldite. Lead stain. x 60,000.
altered intercellular attachment areas (Fig. 11b).

The number of desmosomes is greater and their orientation changes nearer the surface. Along the lateral and upper borders of the basal cells they are orientated at right angles to the epithelial surface. In the cells of the upper spinous and lower granular layers they lie at an angle to the surface and the increase in their number gives the cell membrane a "saw-tooth" appearance. In the upper granular and the cornified layers they are orientated parallel to the surface and at right angles to those in the basal cells.

Cytoplasm. In the basal and lower spinous layers the cytoplasm is a narrow band around the nucleus and is widest at the apex and base of the cells. The small number of organelles are mainly at the poles of the nuclei. Mitochondria are the most prominent organelles and may be round to elongated but their outline is often irregular (Fig. 7M). They are composed of an outer double membrane and contain irregularly arranged cristae that arise from infoldings of the inner layer of the outer membrane (Fig. 7c). The numbers of mitochondria decrease as the surface is approached and they are absent from the cornified layer.
FIGURES 8 and 9. In the cytoplasm of the cells of the granular layer an extremely electron dense granular material (g) is intermingled with the tonofilaments (f) which appear to be randomly orientated. The size of these accumulations of granular material varies over a wide range. A few mitochondria (M) are present in this layer but they are small and tend to be rounded up. The majority of the desmosomes (d) lie parallel to the long axis of the cell, which is now parallel to the surface of the epithelium, i.e., they are at right angles to the orientation of the majority of the desmosomes in the basal cells. Araldite. Lead stain. x 40,000.
The endoplasmic reticulum is poorly developed. Smooth surfaced membranes arranged in the pattern of the Golgi apparatus are clearly recognisable in the basal and suprabasal cells (Fig.7g). Rough surfaced membranes (Fig.7e) are rare but particles 150-200Å are scattered throughout the cytoplasm (Fig.7r). In the upper spinous layers these particles are more densely packed.

A fine filamentous material constitutes the major component of the cytoplasm in all layers except the cornified layer. In the basal layer these filaments are approximately 50Å in diameter and arranged into bundles that run in the long axis of the cell (Fig.7f). The ends of the bundles or side branches from them are inserted into the desmosomes (Fig.7d). In the more superficial cells of the spinous layer the orientation of the filaments is much more random. There is also an increase in the number and compactness of the filament bundles and individual filaments cannot always be recognised easily.

In the cells of the granular layer another component in the form of extremely electron dense masses (Figs.8g and 9g) are intermingled with the bundles of filaments.
FIGURES 10 and 11. In the cornified layer the cytoplasm is devoid of recognisable organelles and is filled with an electron dense material. Areas of slightly greater density are scattered throughout the cells. The plasma membranes (m) are diffusely thickened along their entire length and appear to merge into the dense material in the cytoplasm of the cells. The membranes of adjacent cells are much more widely separated than in the cells of the lower layers and the intercellular space is often filled with a dense granular material (i) that is continuous with the altered desmosomes (d). The desmosomes in the cornified layer have a different fine structure from the desmosomes in the lower layers. The dense plaques cannot be distinguished from the thickened plasma membranes and the triple line structure in the dense material between the cells is not present. Instead, the plasma membranes appear to be separated from the material between the cells by less dense lines. Fig. 10, Araldite. Fig. 11, methacrylate. x 60,000.
These masses differ in size and are usually adjacent and closely applied to the bundles of filaments. On their free sides there are accumulations of the small particulate component of the cytoplasm.

The cells in the cornified layer are completely different from any of the cells in the other layers. The alteration in the cell membrane and its specialised structures has already been mentioned. The cell consists of a dense granular material in which are dispersed irregular areas of slightly greater electron density. None of the cytoplasmic components described in the outer layers are recognisable. The fine structure of these cells is not organised into any constant pattern (Figs. 10 and 11).

**Nuclei.** In the basal and lower spinous cells the nuclei are the most prominent structures (Fig. 2). In the more superficial cells the ratio of nuclear size to cytoplasmic area is lower. In the cornified layer the nucleus has completely disappeared.

The nucleus is surrounded by a double membrane (Fig. 7h). The space between the layers of this membrane varies over
a very small range and pores are present (Fig. 7p). A particulate component similar to that of the cytoplasm is scattered throughout the nucleus but is concentrated around the periphery. Nucleoli are prominent and consist of linear aggregations of the particulate component (Fig. 7n). Mitotic figures have been seen occasionally.

**DISCUSSION.**

The fine structure of the stratified squamous epithelium covering the antemolar ruga of the rat is very similar to the fine structure that has been described for the epidermis of man and various other animals (Selby, 1955, Porter, 1956, Odland, 1958, Brody, 1959a, b, 1960).

**BASEMENT MEMBRANE.** This structure has been found to exist at the interface between mature epithelium and connective tissue in all normal epithelial tissues examined so far with the electron microscope (Mercer, 1961). Some confusion exists as to whether the structure seen with the electron microscope is equivalent to the basement membrane of light microscopists. Salpeter and Singer (1959) in their studies of amphibian epidermis considered that the two structures were different and to avoid
confusion suggested the term "adepidermal membrane". They subsequently altered the term to "adepithelial membrane" to include the structures present at other connective tissue - epithelial interfaces (Salpeter and Singer, 1960). Selby (1955) had earlier expressed similar views and in her study of epidermis coined the term "dermal membrane".

The other point of view was expressed by Otterson et al. (1953). They used the term basement membrane when they were obviously aware of the use of the same term by histologists. Gersh and Catchpole (1960) considered that the two membranes were identical. They explained the apparent contradiction of a membrane revealed by the electron microscope with dimensions below the level of resolution of the light microscope by regarding the membrane visible in the electron microscope as slightly corrugated. Thick sections (2-10u) examined in the light microscope would then show a membrane that, in fact, is 10 times thinner.

The confusion is added to by electron microscopists studying mammalian tissues, as they continue to use the
term basement membrane and the other terms suggested do not appear to have received general acceptance.

The composition and function of the basement membrane is not known but it has been shown to be involved in the maintenance of electrical potentials between epithelium and connective tissues (Otterson et al., 1953). It has also been ascribed roles influencing absorption and excretion from epithelium (Salpeter and Singer, 1959) and, during development, as a substratum for the crystallisation of collagen fibrils (Mercer, 1961). Some support for this last view has come from the work of Hay and Revel (1963). They found that tritiated thymidine injected into amphibia with 12 day regenerating limbs was located in both fibroblasts and epithelial cells after 30 mins. and that the label was concentrated at the epidermal-lamellar border after two hours. The juxta-epidermal band of collagen labelled after 4 hours became displaced inwards during the succeeding 4 to 7 days.

**DESMOSONES.** This type of intercellular attachment has been found between epithelial cells in specialised tissues as well as stratified squamous epithelium. In the
epithelia of stomach, intestine, gall bladder, uterus, oviduct, liver, pancreas, parotid and thyroid, the ducts of the last 3 glands and the tubules of the nephron, the desmosome forms a junctional complex with other specialisations of the cell membrane (Farquhar and Palade, 1963).

Unfortunately little is known at present about the desmosome except for the details of its fine structure. The life cycle, the rate of formation, the factors controlling formation, the permanence and the constancy or otherwise of its position on plasma membranes are questions still unanswered. At present it is thought that it is a relatively permanent structure (Mercer, 1961) and evidence has accumulated that suggests that it is important in the maintenance of intercellular attachment (Fawcett, 1958).

The presence of a totally different fine structure in the cornified cells indicated that the desmosome underwent a transformation during the passage from the granular to the cornified layer. This alteration appeared to be part of the process of cell maturation. The development of an extensive intercellular cement did not reach the
degree found in hair follicles (Birbeck and Mercer, 1957) or even that found in skin (Brody, 1959) but appeared to be confined to the areas of the modified desmosomes.

**CYTOPLASMIC PARTICLES.** The particulate material 150-200A in size scattered throughout the cytoplasm of the cells of the non-cornified layers, resembled the particulate material shown by Palade and Seikevitz (1956) to be RNP. In the cells that they examined the granules were attached to membranes that constituted the elements of the rough surfaced endoplasmic reticulum. Since this description electron microscopists have referred to particles attached to membranes as RNP particles. Similar particles, when found free in the cytoplasm, unassociated with a membrane, have not been shown to be RNP except in the case of embryonic chick liver. In that site the particles became attached to membranes at a later stage of development (Duck-Chong et al., 1964). Brody (1960) disputed that the particles in epidermal cells of the upper spinous and granular layers were RNP.

Without a correlated biochemical and electron microscopic study it is impossible to be sure that the particles
found in the cytoplasm of epithelial cells of the rat palate are RNP. However with this proviso in mind they will subsequently be referred to as RNP particles.

**CYTOPLASMIC FILAMENTS.** The filaments found in the basal spinous and granular layers correspond to the filamentous material in epidermal cells and their distribution is almost identical (Brody, 1959, 1960, Mercer, 1961).

**GRANULES IN THE GRANULAR LAYER.** These granules also have a morphological appearance almost identical to the granular material found in skin and designated kerato-hyaline (Brody, 1959, 1960).

**THE CORNIFIED LAYER.** The morphology of the cells in this layer was similar but not identical to that found in epidermis. The fine structure described by Brody (1960) in the cornified layer of the skin has not yet been shown in the rat palate but this may have been because of technical difficulties. However the strong bi-refringence in the cornified layer when examined in polarised light indicated that orientated fibres were definitely present in this layer. The contents of the cells were certainly not uniform as in the amorphous keratin of the hair cuticle
(Birbeck and Mercer, 1957) and some areas had patterns that suggested a fibrillar arrangement.

KERATINISATION IN THE RAT PALATE. The keratins are a group of proteins derived from ectodermal tissues and are usually classified on the basis of either structure or chemical composition (Lundgren and Ward, 1963). Since the major proportion of information on keratin has been obtained from highly keratinised structures such as wool, hair, etc., that are amenable to chemical and X-ray analysis, the resulting definitions can only be applied to related tissues by inference. A classification of keratinised tissues more useful to investigators interested in morphology has been advanced by Giroud and Leblond (1951). The classification of hard and soft keratin that they devised has been widely accepted (but not without some reservations) and has recently been re-assessed by Matoltsy (1962) in the light of current research. Matoltsy concluded that keratinised tissues fall into 3 basic groups; amorphous keratin (hair cuticle, Birbeck and Mercer, 1957) and fibrous keratin formed from either fibrous precursors alone (hair cortex, Birbeck and Mercer, 1957, Rogers, 1959) or
fibrous precursors and granules (skin, Brody, 1959, 1960).

One form of keratinisation therefore can be recognised by the formation of fibrous precursors in the basal and spinous layers followed by the elaboration of a granular material that becomes intermingled with these fibrils. This combination finally becomes consolidated and converted into an organised system in the cornified layer. This is the pattern found in the palate of the rat except that the final product in the cornified layer does not appear to have the level or organisation found in other keratinised structures.

In addition, Cancellaro et al., (1961) and Barrnett and Sognnaes (1962) have shown that the distribution of sulphydryls and disulphides in oral mucous membranes and skin is similar and Eastoe (1963) found that the amino acid composition of the human palatal epithelium conformed to a pattern recognisable in both epidermis and nail but that a greater variation was present than in the proteins from these other sites.

As a result I conclude that morphological observations of the stratified squamous epithelium covering the ante-
molar rugae of the rat palate allow this tissue to be classified as keratinised but that the stage of cellular maturation reached is not the same as in other keratinised tissue.

**SUMMARY.**

The fine structure of the epithelium covering the antemolar rugae of the rat palate is similar to that found in human, rat and guinea pig skin.

The basal cells are separated from the underlying connective tissue by a thin "basement membrane" and the cells of all layers are interconnected by desmosomes.

The major cytoplasmic component of the basal spinous and granular layers is a thin filamentous material approximately 70A in diameter that is arranged into bundles. The ends and side branches from some of these bundles are inserted into desmosomes. In the granular layer an extremely electron dense material becomes irregularly inter-mingled with the fibre bundles and is morphologically identical to the keratoxyline granules found in skin. Endoplasmic reticulum is not highly developed. Rough surfaced endoplasmic reticulum is rare and the cytoplasm
contains large numbers of "free" particles considered to be RNP.

Mitochondria are relatively frequent in the basal and lower spinous layers but are much less prominent in the granular layers.

In the cornified layer all the cytoplasmic constituents have disappeared and the cells are compressed and filled with a relatively uniform electron dense material that contains irregularly scattered areas of increased density. The plasma membranes and desmosomes have also undergone a distinct change in their fine structure.

The fine structure of these cells indicated that this epithelium may be classified as keratinised stratified squamous epithelium.
CHAPTER 3

THE MORPHOLOGY OF MIGRATING EPITHELium DURING THE EARLY STAGES OF WOUND HEALING

INTRODUCTION

Although many authors have investigated the response of epithelial tissues to mechanical injury few have made more than a passing reference to the changes that take place in the first 24 hours (Arey, 1936, Johnson and McMinn, 1960, Washburn, 1960). The relative neglect of this period is surprising when it is realised "that most epithelia appear to be capable of beginning their migration across a defect within 24 hours" (Johnson and McMinn, 1960). In addition it appears that the electron microscope has not been used to investigate the morphological changes that take place in migrating epithelium in mammals although it has been used to some extent in the study of the role of epithelium in regeneration in amphibia (Weiss and Ferris, 1956, Singer and Salpeter, 1961). I consider the effect of this early period on subsequent wound healing to be important and its study forms the basis of this thesis. The events that occur illustrate dramatically
FIGURE 12. Light micrograph of a paraffin section of tissue removed 12 hours after injury with fine iris scissors. A definite bud of epithelium (a) is present and the cells of the spinous layer are orientated in the direction of movement of the bud. Necrotic epithelium (n) above the bud extends to the wound margin which is on the left hand side of the picture. H & E. x 325.

FIGURE 13. Light micrograph of a paraffin section of a six hour scissor wound. The wound margin is on the left of the picture. In this instance the necrotic epithelium (n) has been partially displaced by the migrating cells (a). A layer of cells containing flattened hyperchromatic nuclei forms the boundary between the necrotic and migrating epithelial cells. H & E. x 325.

FIGURE 14. Phase contrast micrograph of a nine hour migrating bud after fixation in osmium tetroxide. Two types of granules are evident in the cells. One type (u) is large, dense and closely applied to the nucleus. The other type (v) is much smaller and less dense and not as obvious as the former type. The wound edge is on the left hand side of the picture. Methacrylate. x 1,600.

(Note. The tissue in these illustrations was taken from wounds exhibiting the minimal amount of traumatic necrosis of the marginal epithelium.)
the structural changes that are coincident with the functional response of the epithelial cells to trauma.

**OBSERVATIONS.**

**PART I. REMOVAL OF CREST OF THE ANTEMOLAR RUGA WITH IRIS FINE SCISSORS.** The trauma resulting from the use of this method was extremely variable in extent and could not be reproduced in an experimental series. The technique was useful as an introduction to the study of wound healing but was abandoned subsequently.

**Light Microscope.** The operation crushes the marginal epithelium for a variable distance from the cut edge of the wound. This crushing produces cellular necrosis recognisable after 3 hours in sections stained with haematoxylin and eosin by the pyknosis of the nuclei and the loss of the basophilic staining of the cytoplasm (Figs. 12n and 13n). By 6 hours the epithelium has begun to migrate under and past the necrotic cells (Fig. 13). During the migration the nuclei of the cells in the basal and lower spinous layers retain their oval or rounded profiles but the nuclei of the cells comprising the leading edge become elongated in the direction of movement (Fig. 12).
The nuclei of the cells forming the surface layer of this migrating bud are thin and hyperchromatic. After 9 hours the migration of the epithelium has produced a definite bud some 4 to 6 cells thick and 10 to 12 cells long (Fig. 14). This has increased slightly in length by 12 hours (Fig. 12).

When the tissues are fixed in osmium tetroxide and examined with the phase contrast microscope the cytoplasm of the surviving cells does not show the uniform appearance seen in the formalin fixed material. Dense, osmiophilic bodies that are visible at 3 hours have, by 9 hours, increased in number and are found in the cells of the migrating bud and also in a large number of the cells of the basal and spinous layers of the adjacent epithelium (Fig. 14). These dense bodies can be divided into at least 2 types. One type is extremely dense and perinuclear in distribution, obscuring the nuclear membrane (Fig. 14u). The other is much less dense, smaller and less obvious, and is not closely applied to the nucleus (Fig. 14v).

**Electron Microscope.** The observations of the fine structure of the regenerating cells made with the electron microscope support and extend those made with the light microscope.
FIGURE 15. Electron micrograph of necrotic cells in the lower spinous layers at the margin of a wound three hours after injury with fine iris scissors. The nuclei (n) have almost entirely disappeared and the cytoplasm is completely disorganised. Vesicular structures (v) are randomly dispersed throughout the cells. Cell membranes and desmosomes can still be recognised and the general outline of the cells appear not to be affected. Methacrylate.  x 14,000.

FIGURE 16. A higher power view of a desmosome and adjacent cytoplasm from an area similar to that in Fig. 15. The desmosome, plasma membranes and tonofilaments do not appear to be altered. RNP particles have completely disappeared. Methacrylate. Lead stain.  x 50,000.

FIGURE 17. A higher power view of a basal cell in the necrotic marginal epithelium from a 3 hour wound. The cytoplasm of the cell (c) can hardly be recognised since it is completely devoid of RNP particles and other organelles and only the compressed tonofilaments remain. The basement membrane (b) is still present as is also the remains of an incomplete desmosome (d). Methacrylate. Lead stain.  x 50,000.
At 3 hours the cells that had been killed by the wounding retain their outline but much of their fine-structure is lost or disrupted (Fig.15). The plasma membranes, tonofilaments and desmosomes are still intact (Fig.16) and vesicular structures are present (Fig.15v). The nuclear membrane and the RNP-like particles found in the cytoplasm of normal epithelium have completely disappeared. Little of the nuclear chromatin remains in the basal cells but in the more superficial cells it is often in large clumps (Fig.15n). The basement membrane (Fig.17b) beneath these necrotic cells (Fig.17c) remains closely applied to their corrugated lower borders.

At 6 hours the cell membranes, tonofilaments and desmosomes are no longer clearly discernable in the necrotic cells (Figs.18n and 20n). They have lost their attachment to the underlying connective tissue (Fig.18c) but the basement membrane remains and retains its integrity and complicated shape (Figs.18b, 19b and 20b). The surviving epithelial cells have begun to migrate after 6 hours (Fig.21). The intercellular spaces become distended (Fig.21s) and minute pseudopodia are evident along the free
FIGURE 18. The necrotic epithelial cells (n) have disintegrated further after 6 hours have elapsed since the injury and they have begun to separate from the dermis (c). After separation the dermal collagen fibrils remain covered with the basement membrane (b) that previously had formed the boundary between the two tissues. Methacrylate. Lead stain. x 15,000.

FIGURE 19. A higher power view to show the basement membrane (b) covering the collagen fibrils (c) after the necrotic cells have separated. Methacrylate. Lead stain. x 50,000.

FIGURE 20. A higher power view near the point of detachment of the necrotic cells. The basement membrane (b) has no obvious connection with the necrotic cytoplasm (n) and the plasma membranes have disappeared. Various vesicular structures (v) are still present. Methacrylate. Lead stain. x 50,000.
FIGURE 21. Electron micrograph 6 hours after injury shows pseudopodia (p) of migrating epithelial cells extended out along the residual basement membrane (b). The pseudopodia have displaced the remains of the necrotic epithelial cells (n). Cytoplasmic debris is present in the widened intercellular spaces (s) between the pseudopodia. Methacrylate. Lead stain. x 15,000.

FIGURE 22. A basal cell from the migrating epithelial bud of a 12 hour wound. A large juxtanuclear uniform dense body (u) is a prominent feature. There are vesiculated dense bodies (v) and some rough surfaced endoplasmic reticulum (e) in the cytoplasm. The arrangement of the tonofilaments (f) is irregular. Methacrylate. Lead stain. x 17,000.
edges of the cells (Fig. 21p). The cell margins have also
become extremely convoluted and the orientation of the
desmosomes does not follow any recognisable pattern (Fig.
21d).

At 9 hours the forward progression of the cells of the
epithelial bud is more obvious, and by 12 hours elements
of rough surfaced endoplasmic reticulum have appeared in
the cytoplasm (Fig.22e). The cytoplasm of these migrating
cells has a more complex appearance than that in normal
epithelial cells. The tonofilaments have lost their
normal orientation and are randomly disposed in the
cytoplasm (Fig.22f).

Cytoplasmic inclusions that have not previously been
seen in normal epithelial cells are found in some of the
migrating cells at all stages examined. These inclusions
can be divided into 2 general categories on the basis of
their fine structure. One type is relatively large,
irregular in outline, uniformly dense and usually close to
the nucleus. They are not surrounded by a membrane (Fig.
22u). The second type is found at random in the cytoplasm
and is always membrane bound (Figs.23,24 and 25v). The
FIGURE 23. A vesiculated dense body from an apparently viable cell 3 hours after injury. One component of the organelle is an electron dense granular material which appears to be a condensation of particles similar to those in the surrounding cytoplasm. In the upper portion of the dense body is what appears to be the "ghost" of a mitochondrion. Two irregularly shaped vesicular areas are evident. The whole structure is surrounded by an electron dense membrane. Methacrylate. Lead stain. x 50,000.

FIGURE 24. Another vesiculated dense body from an apparently viable cell 3 hours after injury. In this instance the resemblance of the components in the organelle to mitochondria outside it is clearer. A small uniform dense body (u) is nearby. Methacrylate. Lead stain. x 50,000.

FIGURE 25. The cytoplasm of the cell containing the vesiculated dense body (v) illustrated in Fig. 23. The general appearance of this cell is quite different from the appearance of the necrotic cell (n) nearer the cut edge of the wound. The cytoplasm appears to be somewhat swollen but RNP particles are prominent and profiles of rough surfaced ER (e) are present. This cell has apparently survived the trauma which has killed those at the margin of the wound. Methacrylate. Lead stain. x 17,000.
details of their internal structure are not constant, but an overall pattern is present. Prominent within them are masses of material with a granular texture and an electron density similar to the RNP-like particles in the surrounding cytoplasm. They contain vesicular structures of various sizes and shapes. The remaining areas are filled with material of uneven density and in the 3 hour material contain rounded bodies resembling mitochondria in both size and shape. In order to simplify further discussion, dense bodies of the first type will be referred to as uniform dense bodies and those of the second type as vesiculated dense bodies.

During the whole 12 hour period the migrating cells retain the structural components characteristic of cornifying cells (tonofibrils and desmosomes) but their arrangement is often irregular.

PART II. REMOVAL OF A STRIP OF EPITHELIUM 0.5mm. WIDE FROM THE ANTERIOR SURFACE OF THE SECOND ANTEMOLAR RUGA. The careful removal of a strip of epithelium 0.5mm. wide produces much less trauma to the wound margins than the previous method and results in a more uniform series of
experimental wounds. Necrosis often occurs only in the cells immediately adjacent to the cut edge although it sometimes extends for 2 to 3 cell diameters into the epithelium. This is in marked contrast to the previous method which often produces necrosis that extends 20 to 30 cell diameters from the cut edge.

**Light Microscope.** When examined with the dissecting microscope immediately after injury the walls of the wound are perpendicular and the floor is wide and irregular. In sections examined 6 hours after the injury however, the walls of the wound are inclined obliquely and the base is v-shaped (Fig. 26). The wound space is filled with dense fibrin meshwork which contains an irregularly distributed cellular population consisting of red blood cells and migrating white blood cells. The surface of the wound is composed mainly of white blood cells intermingled with bacteria and debris.

By 6 hours the epithelial cells have begun to migrate and have displaced the necrotic epithelial cells. A definite bud 4 to 6 cells thick has formed by 12 hours (Fig. 27). At this stage the migrating bud moves parallel
FIGURE 26. Light micrograph of a wound 6 hours after injury with parallel mounted scalpel blades. The walls of the wound are obliquely inclined and the base is "v" shaped. H & E. x 80.

FIGURE 27. The bud formed 12 hours after injury. The necrotic cells have been displaced and the bud has started to move down the inclined walls of connective tissue. The wound space is filled with fibrin and contains migrating white blood cells. The surface of the wound is composed almost entirely of white blood cells many of which are necrotic. H & E. x 210.

FIGURE 28. In this wound the bud formed 18 hours after the injury is not much longer than the 12 hour bud. No mitoses are present in the bud or the adjacent epithelium but the cells have moved a distance at least equivalent to the length of the band of necrotic epithelium (n). H & E. x 210.

FIGURE 29. A wound 24 hours after injury with parallel mounted scalpel blades showing the definite increase in the length of the epithelial bud. In this instance the cells have migrated down the inclined connective tissue walls. H & E. x 210.
with the inclined walls of the wound. Its base appears to be moving directly over the dense connective tissue while its leading edge is in contact with the fibrin and other debris in the wound. After 18 hours the migration has continued further along the wound wall (Fig.28) and quite often this is the only difference observed from the 12 hour stage. However on some occasions the bud is very irregular in outline and migrated directly into the wound. Under these circumstances it is heavily infiltrated with debris from the wound and many of the cells are irregularly arranged and partly separated by wide spaces. By 24 hours there appears to be an increase in migratory activity because the buds are often twice as long as those seen after 18 hours. The position and shape of the bud are not constant and appear to depend on the previous stages of migration. It is usually 3 to 4 cell diameters thick, and extends down the sides of the wound (Fig.29). The end of the bud is often fan-shaped and directed into the wound coagulum. Occasionally the wound is completely sealed over as a result of direct migration through the clot. 

**Electron Microscope.** This description will be mainly
FIGURE 30. The leading edge of a bud formed 12 hours after injury. The free plasma membranes of the cells are heaped up into many irregularly shaped folds (p) with outlines that vary from long, thin finger-like projections to squat, button-like protuberances that are devoid of cytoplasmic organelles. Part of the lower border of one of the cells is in contact with the basement membrane (b). The plasma membrane in this site conforms to the shape of the basement membrane. A desmosome can be seen at d and the cytoplasm contains many "free" RNP particles. Methacrylate. Lead stain. x 20,000.

FIGURE 31. The lower border of a cell from a bud 12 hours after injury. No basement membrane is present and the cell is covered with short, irregularly shaped protuberances (p) that contain RNP particles. The intercellular spaces (s) are enlarged and contain many irregular extensions of the plasma membranes of adjacent cells. The cells are attached to each other by desmosomes (d). Methacrylate. Lead stain. x 20,000.
concerned with the 12, 18 and 24 hour periods since the essential features of the preceding period were similar to those described in Part I of this chapter.

**Basement Membranes.**

This membrane is usually absent beneath the cells at the leading edge after the first 6 hours (Figs. 31, 33, 45 and 47). When present the edge of the cell in contact with it follows it very closely (Figs. 30 and 32) except where it undergoes sudden directional changes. Even then the cells often have cytoplasmic extensions partially filling the corrugations (Figs. 43 and 44).

**Plasma Membranes.**

The most characteristic and constant feature of the migrating cells is the appearance of their free surfaces at the advancing edge of the bud and bordering the distended intercellular spaces. The plasma membrane is heaped up in cytoplasmic projections that vary from finger-like extensions of different lengths to bulbous button-like protuberances (Figs. 30p and 34p). The cytoplasm in these projections is similar to that in the rest of the cell but contains no organelles.

The shape of the plasma membrane on the deep surface
FIGURE 32. The lower border of basal cells some distance from the leading edge in a wound 18 hours after injury. The basement membrane (b) is separated from the plasma membrane by a less electron dense space of relatively uniform width. Nearby, in the cytoplasm of the cells, small vesicles are frequent (v). Methacrylate. Lead stain. x 40,000.

FIGURE 33. The lower border of a basal cell nearer the leading edge in the same bud as in Fig. 32. The basement membrane is not present and the plasma membrane is unevenly corrugated. Some of the collagen fibrils (c) beneath the cell appear to be in contact with the plasma membrane. Mitochondria (m) are irregular in outline and the tonofilaments (f) are elongated in the direction of migration. Methacrylate. Lead stain. x 40,000.
of the bud varies with its sub-stratum. When a basement membrane or other orientated sub-stratum is absent the plasma membrane is covered with cytoplasmic protuberances (Figs. 31, 33, 45 and 47). However these protuberances differ from the projections at the other free margins of the cells in that they often contain RNP particles.

The plasma membranes still have specialised attachment areas (desmosomes) scattered along their length (Fig. 30d, 31d, 39d and 40d). The orientation of the desmosomes does not follow the pattern present in the cells of the lower layers of normal epithelium but instead is quite random. The plasma membranes of the epithelial cells and infiltrating white blood cells are often close together but there are no specialised attachment areas (Figs. 39 and 40).

Cytoplasm.

Mitochondria. The mitochondria in the cells of the migrating bud retain their characteristic structure of an outer double membrane and transverse cristae (Figs. 33m, 45m and 47m). They are unevenly scattered throughout the cell cytoplasm in contrast to the perinuclear distribution and polar aggregations found in normal epithelium.
Endoplasmic Reticulum. At 12 hours the cytoplasm of most of the migrating cells still contains a high density of free RNP particles such as seen in normal cells (Figs. 30 and 31). As the migration continues there is a gradual increase in the rough surfaced endoplasmic reticulum (Figs. 34e, 48e and 51e) but the degree of development or organisation never reaches that found in some other cells, e.g., the parenchymal cells of pancreas, liver (Fig. 37e) or salivary gland. The rough surfaced sacs thus formed may be flattened, elongated ribbons (Figs. 48e and 51e) or have swollen rotund outlines (Fig. 34e). They all contain a fine granular material more dense than the surrounding cytoplasm. Communication between rough surfaced endoplasmic reticulum and the extracellular space has not been observed. The smooth surfaced endoplasmic reticulum is more obvious than in normal cells and is often seen in different parts of the same cell (Fig. 51g). Vacuoles and microvesicles are more frequent especially near the plasma membrane at the free edges of the cell (Figs. 32v, 34v and 45). The vacuoles and micro-vesicles appear to be empty but occasionally contain material similar to that in the
FIGURE 34. A cell from the leading edge of a bud 18 hours after injury. The irregular surface membrane (p) is associated with strings of vesicles (v) of various sizes and shapes. The most prominent constituents of the cytoplasm are vesiculated dense bodies. The variety in the size and shape of these structures is evident. Rough surfaced endoplasmic reticulum (e) is more extensive than in normal cells. Methacrylate. Lead stain. x 20,000.

FIGURE 35. This cell from the superficial surface of the migrating bud near the leading edge 18 hours after injury also contains vesiculated dense bodies that vary in size, shape and content over a wide range. The plasma membrane is relatively smooth in this instance, probably due to the relatively compact nature of the wound coagulum adjacent to it. The vesicle (v) contains components similar to those present in the coagulum outside the cell. The deeper surface of the plasma membrane of the cell is attached to the adjacent cell by desmosomes (d). Methacrylate. Lead stain. x 20,000.
wound space (Figs. 35v, 42v and 46v), especially when the cells are close to the leading edge of the bud.

Other Cytoplasmic Structures. After 12 hours the dense bodies become more obvious in the cytoplasm of the cells in the bud (Figs. 34 and 35). These dense bodies fall into the general classification of uniform (Fig. 39u) and vesiculated (Fig. 40v, 48-51) dense bodies described in the first part of this chapter. They may be found anywhere in the cytoplasm but most frequently become aggregated at the poles of the nucleus (Figs. 34 and 35).

Desmosomes and tonofilaments are still present in the cells of the bud. Often, in cells that are widely separated by distended intercellular spaces or infiltrating cells, the desmosomes appear to be the only means of intercellular attachment. The tonofilaments are irregularly distributed in the cytoplasm but in none of the cells examined are they completely absent. The orientation of the tonofilaments is also altered. The bundles tend to lie parallel to the direction of migration, i.e., at right angles to their orientation in normal basal and lower spinous cells (Figs. 38f, 39f, 41f and 47f).
FIGURE 36. A dense granular material (g) is occasionally found in the cytoplasm of migrating cells 24 hours after injury. The individual particles of this granular material are distinct from RNP particles (r) since they are much larger. An infiltrating polymorph (w) occupies a distended intercellular space. Araldite. Lead stain. \( \times 20,000 \).

FIGURE 37. The granular material (g) present in liver and recognised as glycogen has a basic particle component similar to the granular material in the epithelial cells in Fig. 36. The individual particles however, tend to be aggregated into larger clumps than in the epithelial cells. Rough surfaced ER (e) is well developed in this cell. Araldite. PTA, uranyl and lead stain. \( \times 20,000 \).
In the material examined, cornification is never complete in the migrating bud even though stratification is often evident behind the fan-shaped leading edge (Fig. 52). Keratohyaline granules are not found and the cells on the surface appear to undergo a process of necrosis and condensation without becoming cornified (Fig. 53).

Glycogen. Occasionally, small accumulations of a granular material (approximately 300A) are found in the cytoplasm of some of the migrating cells (Fig. 36g). They stain intensely with lead and appear similar to the particles forming the granular material present in liver cells and recognised there as glycogen (Fig. 37g).

Nuclei.

The nuclei of the cells in the migrating bud do not appear to be significantly altered from normal. Their outline is quite often irregular (Fig. 39). Nucleoli are prominent and are usually connected to the peripheral chromatin (Fig. 47).

During the first 24 hours of wound healing mitoses are absent from the cells of the migrating bud. In all the blocks examined only 1 mitosis was seen in the bud and
FIGURE 38. The epithelial cells in an 18 hour migrating bud separated to varying degrees by distended spaces which contain an unevenly dispersed fine granular material. Irregular cytoplasmic projections arise from the cells bordering the spaces. Migrating white cells (w) partially or completely fill many of the spaces. Tonofilaments (f) in the cytoplasm of the epithelial cells tend to be arranged parallel to the direction of migration. Araldite. PTA, uranyl and lead stain. x 30,000.
this was a 24 hour specimen.

Distended Intercellular Spaces.

The cells in the migrating bud are separated to varying degrees by distended intercellular spaces. In the light microscope these spaces often give the appearance of being an artifact due to poor fixation. However in the electron microscope, they are seen to contain an unevenly dispersed granular material into which extend cytoplasmic projections similar to those on the free margins of the cells at the leading edge (Fig. 38). Occasionally fibrin strands or fragments of red cells are also present and frequently polymorphs or macrophages partially or completely fill the spaces (Figs. 39 and 40).

At the leading edge of the bud, especially in the fan-shaped buds formed after 24 hours, the cells become extremely elongated and are often separated by wide spaces containing various combinations of the components found in the wound (Figs. 41 and 42). These spaces are often so large and the epithelial cells so elongated that an individual epithelial cell in any one section may appear to be separate from the rest of the bud (Fig. 41). However, in these instances continuity can be shown in serial sections.
FIGURE 39. A migrating white blood cell (w) infiltrated between the migrating epithelial cells 18 hours after injury. The epithelial cells contain irregularly shaped mitochondria (m), uniform dense bodies (u) and the tonofilaments (f) are orientated parallel to the direction of migration. The epithelial cells are interconnected by desmosomes (d). Similar structures do not occur along the interface between the epithelial cells and the white cells despite the close approximation of their plasma membranes. Araldite, PTA, uranyl and lead stain. x 30,000.
FIGURE 40. An area similar to Fig. 39 but nearer to the leading edge of the bud. A collection of polymorphs is wedged into an enlarged intercellular space. Vesiculated dense bodies (v) and a small uniform dense body (u) are present in the cytoplasm of an adjacent epithelial cell which is connected to a neighbouring epithelial cell by a desmosome (d). No specialised intercellular attachments of the desmosome type can be seen between the epithelial and white blood cells. Araldite, PTA, uranyl and lead stain. x 30,000.
FIGURE 41. An elongated epithelial cell from a 24 hour bud. The plasma membranes are in contact with migrating white blood cells. Fibrin (c), red blood cells and other debris from the wound coagulum are nearby. The plasma membrane (p) is extremely irregular where it is in contact with the coagulum and relatively smooth where it is in contact with the white blood cells. Tonofilaments (f) are arranged parallel with the long axis of the cell. Araldite, PTA, uranyl and lead stain. x 20,000.

FIGURE 42. Another group of cells from the leading edge of a 24 hour bud. A mass of wound coagulum (c) is partially enclosed in a widened intercellular space and vesicles (v) in the cytoplasm of adjacent cells contain material with an electron density similar to that in the coagulum. Araldite, PTA, uranyl and lead stain. x 20,000.
FIGURE 43. A basement membrane (b) is present ahead of the migrating epithelial bud in this wound 18 hours after injury. An extremely tenuous extension of epithelial cell cytoplasm (a) is closely applied to the crests of the corrugations in the basement membrane. Shorter processes reach partly into the depressions between the corrugations. A group of bacteria is present in one of the depressions. Araldite. Lead stain. x 20,000.

FIGURE 44. A similar region in another 18 hour bud in which the epithelial cell has extended a process of cytoplasm (a) that partially fills a deep crevice in the basement membrane (b). Methacrylate. Lead stain. x 20,000.
FIGURE 45. The lower margin of a migrating bud 18 hours after injury. A basement membrane is absent. The plasma membrane is irregular and the cytoplasm contains many small vesicles (v). Beneath the cells the wound coagulum is intermingled with collagen fibrils in the wound wall. (The dense granular material in the wound coagulum is an artifact of lead staining). Some irregularly shaped mitochondria (m) can be seen in an adjacent cell. Araldite. PTA, uranyl and lead stain. x 30,000.
AN ELECTRON MICROSCOPIC STUDY OF NORMAL AND MIGRATING EPITHELIUM IN THE PALATE OF THE RAT.

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SUMMARY

Covering the antemolar rugae of the rat palate is a stratified squamous epithelium which the electron microscope shows to be undergoing a process of soft keratinisation. There is a gradual increase in the number of tonofilaments in the cells as they approach the surface. The tonofilaments become associated with keratohyaline granules before the cells undergo the transformation into the cornified layer. In the cornified layer, the fine structure described in skin has not been demonstrated. In all layers the cells are interconnected by desmosomes. In the cornified layer the desmosomes have a fine structure different from those in the lower layers.

Mechanical injury involving the epithelium and underlying connective tissue results in migration of the epithelium into the wound space. During the first 24 hours the migratory activity is not associated with mitotic activity but some of the epithelial cells that move into the wound develop cytoplasmic organelles and undergo other alterations in their morphology that appear to be associated with migration. The organelles are described as vesiculated and uniform dense bodies. They appear at the same time as the cells exhibit increased activity. This activity is
evident as an increase in the quantity of rough surfaced endoplasmic reticulum and development of convolutions in the plasma membranes both at the free margins and bordering the distended intercellular spaces.

The vesiculated dense bodies react positively for acid phosphatase after incubation in a modified Gomori medium and therefore are presumed to be related to the cytoplasmic organelles described as lysosomes.

These organelles are shown to be a morphological expression of the development, in the migrating epithelial cells, of a phagocytic ability. This is conclusively demonstrated by the presence of intracellular concentrations of electron opaque colloidal markers that had been intravenously injected immediately before wounding.

In conclusion, an interpretation of the phenomena observed in epithelial cells during the early phases of wound healing based on the observations and results reported in this thesis is presented.
FIGURE 46. The wound coagulum into which the epithelial cells of an 18 hour bud are migrating. It consists of a complex mixture, the major component of which is an electron dense material containing a randomly arranged fibrillar material. Migrating white blood cells, bacteria and debris from necrotic cells (n) are mixed with the coagulum. The vacuole (v) in one of the migrating epithelial cells contains material similar to the cell debris in the wound coagulum. Araldite. PTA, uranyl and lead stain. x 30,000.
FIGURE 47. This epithelial bud from an 18 hour wound is migrating into an area of the wound coagulum containing a large amount of cell debris (n). The plasma membranes on the sides of the cells adjacent to this debris are thrown into many irregularly shaped folds (p). At the bottom of the micrograph the portion of the plasma membranes in contact with the connective tissue of the wound wall is uneven and is devoid of a basement membrane. The nucleus (N) does not appear to be different from the nuclei in normal epithelial cells. The nucleolus is prominent and is continuous with the peripheral chromatin at one point. The cytoplasm contains mitochondria (m) and a vesiculated dense body (v). The tonofilaments (f) tend to be arranged parallel to the direction of migration. Araldite. PTA, uranyl and lead stain. x 30,000.
FIGURE 48. Vesiculated dense bodies from an 18 hour bud. They are composed mainly of an electron dense granular material and are surrounded by a definite membrane. Rounded, less electron dense areas differ both in size and number but are most frequent around the periphery. Some profiles of rough surfaced ER (e) are nearby. Methacrylate. Lead stain. x 40,000.

FIGURE 49. Another pair of vesiculated dense bodies that illustrate some of their variety in shape, size and content. Methacrylate. Lead stain. x 40,000.
FIGURE 50. Variety in the appearance of the vesiculated dense bodies in an 18 hour bud. The features which distinguish these structures from other cytoplasmic organelles (limiting membrane and heterogeneous contents) can still be recognised despite this variety. Methacrylate. Lead stain. x 40,000.

FIGURE 51. This group of vesiculated dense bodies from an 18 hour bud are closely associated with elements of the Golgi apparatus (g) and rough surfaced ER (e). Methacrylate. Lead stain. x 40,000.
FIGURE 52. Away from the leading edge of a bud 18 hours after injury. Some stratification is present and the majority of the intercellular spaces are not greatly enlarged. The surface of the bud is not keratinised. The tonofilaments in the cytoplasm of the cells tend to be elongated in the direction of migration. Araldite, PTA, uranyl and lead stain. x 30,000.
FIGURE 53. Further from the leading edge of
the same bud as in Fig. 52. Stratification
is more in evidence but normal keratinisation
is not occurring. No granular layer is
present and the surface layer consists of
a single cell which is not keratinised but
appears to be necrotic. The dense granular
material (g) is probably a small accumulation
of glycogen granules. Araldite. PTA, uranyl
and lead stain.  x 30,000.
DISCUSSION.

A large proportion of the experimental work on wound healing has been done with amphibia and much of this work has been correlated with wound healing in mammals. However it is unwise to assume that similarity in a few aspects allows speculations and generalisations to cover the whole field of repair following injury. Unless definite correlations can be demonstrated in the specific aspect under discussion this tendency to generalisation, although helpful in formulating concepts, can sometimes cloud over important details and discourage their thorough investigation.

The skin of amphibia has been used by many investigators for the study of superficial healing because it is a simple tissue (Weiss, 1959). In this study the anterior maxillary palatal epithelium of the rat was chosen as a subject for examining wound healing since it is a stratified squamous epithelial tissue structurally similar to mammalian skin but uncomplicated by hair follicles, accessory glands or other specialised structures, and exposed to a relatively constant environment.
NECROSIS AT THE WOUND EDGE. The problem of cell death, especially its recognition by morphological signs, has received much less attention than the subject deserves. Majno et al. (1960) found that up to the time of their study there had been no integrated attempt to define the morphological and biochemical events characteristic of the early stages of cell death. In their critical study of the recognition of cell death they concluded that the time of death is best defined as the time at which a cell reaches the "point of no return"; a point determined by the lack of ability of a cell to survive a given injury. This injury was achieved in their experiments by interruption of the circulation of a piece of rat liver.

The loss of cytoplasmic basophilia and the nuclear pyknosis that I saw with the light microscope in the cells adjacent to the cut edge 3 hours after wounding was one of the first recognisable changes and corresponded with the disappearance of the cytoplasmic RNP particles and the clumping of the nuclear chromatin as seen with the electron microscope. Mitochondria also have disappeared at 3 hours although in cells in other sites they may exist
for long periods following death (up to 10 hours, Hibbs and Black, 1963). This difference may be related to the cause of the epithelial cell death, i.e., direct trauma. The presence of the plasma membranes, virtually intact for the first 3 hours, and the vesicular structures in the cytoplasm are evidence for the slower destruction of some lipoprotein membranes. However these begin to disintegrate after 6 hours. The persistence of desmosomes and of tonofilaments also indicates the resistant nature of these structures since they often remained when the cell membranes were disintegrating.

Although these changes were not uniform throughout the necrotic area they show that the loss of the apparatus for protein production is one of the earliest signs of epithelial cell death after mechanical injury.

THE BASEMENT MEMBRANE. The persistence of the basement membrane after the necrotic cells have become detached is of interest. As discussed in Chapter 2 the derivation and function of this membrane are unknown but it is clear that in the conditions present in a healing wound the membrane can exist in the absence of an epithelium—
connective tissue interface. When the epithelium begins to migrate it moves over this membrane and the plasma membranes apparently become adapted to its irregularities and convolutions.

Its absence beneath the cells at the leading edge of the migrating bud after the first 12 hours corresponded to its absence during the early stages of limb bud regeneration in amphibia (Salpeter and Singer, 1960). Salpeter and Singer (1960) and Singer and Salpeter (1961) thought that chemical contribution by the epithelium to the underlying tissues might be hindered by a basement membrane. Hence they considered that its absence during regeneration would be advantageous and might help to explain the influence that the epithelium of the apical cap exerts on the reconstitution of the amputated limb. This hypothesis is inadequate because physiologically important small molecules pass through it to normal epithelia; e.g., epithelium subject to hormonal influence. In the kidney glomeruli, large molecules can also pass through it (Farquhar and Palade, 1960) and during inflammation the basement membrane surrounding blood
vessels can allow the passage of cells without permanent loss of integrity (Florey and Grant, 1961). As was stated earlier the significance of this membrane awaits further investigation and speculations on the reasons for its absence must rely on the elucidation of the reasons for its presence.

The apparently empty space 300-400A wide that was always seen between the basement membrane and the plasma membrane of the basal cells in favourably sectioned material must also have some significance. When the migrating bud was moving over densely packed fibrin in wound coagulum this uniform space was maintained (Fig.69). This indicates that the plasma membrane of the migrating cells may be covered by a material that cannot be easily seen with the electron microscope. This material may be determining the contact relations of the cell since it is forming the true interface between the electron dense components that are more readily demonstrated.

MIGRATION. In the healing of wounds the restoration of structural integrity is entirely dependent on cellular migration. Locomotion, orientated locomotion in particular,
is one of the basic properties of living cells and although it has been studied for many years the means by which this locomotion is achieved is still a mystery (Wollfahrt-Bottermann, 1964).

Epithelium is the first tissue to respond in the healing of superficial wounds (Johnson and McMinn, 1960) and has a profound effect on subsequent healing (Gillman and Penn, 1956). The cell layers that are involved in migration are not accurately known. All the living cell layers in human epithelium were considered to be potentially migratory by Hartwell (1929, 1955) but he thought that the majority of the cells were derived from the middle and uppermost layers of the adjacent "normal" epithelium. Micro-wounds in human skin explants in tissue culture healed by migration of the basal and spinous layers in the opinion of Matoltsy (1955). In my study it was impossible without specific marking to determine which layers were primarily involved in migration. However the apparent continuity between the cells of the bud and the cells of the lower spinous and basal layers did favour the view that these are the layers concerned in the migratory response
Figs. 27-29). The epithelium at the margins of wounds does not become detached and mobilised as it does in amphibia (Lash, 1955), but retains its attachment to the underlying dermis except beneath the migrating bud.

The Method of Migration. The means by which the epithelial cells move across the wound is also not clear. However work by Weiss and Matoltsy (1959) has shown that cellular migration after wounding is independent of cellular proliferation. They showed that wounds made in embryonic chick skin and cornea prior to the tenth day of incubation failed to close even though cellular proliferation occurred at the wound margin. If this same wound were left till after the tenth day or if a wound were made after the tenth day migration took place in both. Wounds made prior to the tenth day and transferred to tissue culture also healed rapidly by migration. This experiment adds support to the observations made by earlier workers that migration across the wound is independent of mitosis (Arey, 1936). In the rat palate mitosis was only observed on one occasion in a 24 hour migrating bud, some distance from the leading edge.
Hartwell (1955) described the movement of the cells as amoeboid. Weiss (1961) also described the migration as a "sort of amoeboid motion" whereas in an earlier paper (Weiss, 1959), referring to the observations of Lash (1955) he thought that amoeboid motion was not the explanation. The description of the mode of migration as amoeboid does little to elucidate the problem since this mechanism itself is not understood. Two theories of amoeboid motion currently receiving the most attention are those supported by Goldacre (1961) and Allen (1961a and b).

Goldacre favoured the theory that the cell membrane provided the motive force by active contraction at the rear and that membrane flow took place at the surface from front to rear. Wolpert and O'Neill (1962) tested the membrane theory of amoeboid movement by labelling the cell surface with fluorescent antibody. They concluded that rapid formation of the surface at the advancing end or contraction and re-entry at the tail did not satisfactorily explain the uniform fluorescence of the membrane that they observed.

Allen, on the other hand considered that the motive force was provided by contraction in the cell cytoplasm at
the front of the cell with the formation of a "fountain zone". This fountain zone produced protoplasmic streaming with the endoplasm moving forward through a zone of contraction where it was everted to form a collar of ectoplasm that moved backward through the cell. Bell and Jeon (1963) did not think that there was sufficient evidence to support any comprehensive theory of locomotion and considered that moving cytoplasm itself was not able to change the shape of the cell. This shape they thought to be a consequence of the state of the surface membrane and to be dependent upon the properties of adhesion and change in electric charge exhibited by the membrane.

Application of these theories to migrating epithelial cells can only be speculative. Nevertheless if current thinking on the permanence of the epithelial desmosome is correct then it would appear that the theories relying on the dissolution and reformation of the plasma membrane would not explain epithelial migration.

Ambrose (1961) examined the problem of cell locomotion by using interference and "surface-contact" microscopy to study the movements of mammalian and avian fibrocytes in
tissue culture. He concluded that undulations of the cell membrane provided the mechanism for locomotion when cells were moving over a solid substrate. These undulations were due to random contraction of hypothetical cytoplasmic contractile fibrils lying parallel to the surface which became orientated by tension or cytoplasmic flow.

The means by which a moving cell activates contractile molecules is unknown but Weber (1955) has shown that it was dependent on a reaction between the contractile fibrillar proteins and ATP resulting in the formation of ADP. He showed that both fibroblasts and muscle fibres made similar use of ATP, but that contractile proteins were known only to exist in muscle.

The presence of cytoplasmic fibrils (tonofilaments) in the migrating epithelial cells and their orientation parallel to the direction of migration may have some significance in the light of Ambrose’s theory of locomotion. The similarity between the protein fibrils of epidermis and muscle was shown by Astbury (1950). He described keratin as not only extensible but as "also potentially contractile". The one major chemical difference between
myosin and keratin in the view of Astbury (1947) was the lower level of SH groups in mature keratin. Therefore it may not be unreasonable to suppose that the immature keratin fibrils in the cells of the lower spinous and basal layers and in the cells of the migrating bud are also "potentially contractile". This potentiality may be turned to advantage by the epithelial cells during wound healing. The only necessities apparently, would be for the cell to provide the mechanism to change the physico-chemical environment in a controlled manner and to produce an adequate supply of ATP.

DIRECTION OF MIGRATION. Weiss has probably contributed most to the understanding of the directional control of migration during wound healing. He contended that the direction of movement depended on a phenomenon that he described as "contact guidance" (Weiss, 1958). A great deal of the evidence for this theory was based mainly on experiments with culture cells seeded on to various substrata so that the movement of the cells was confined to movement over the substratum.

In healing wounds in the rat palate the epithelium
moved through and not over the wound coagulum. The fibrin network illustrated so well by the work of Hawn and Porter (1947) was not only beneath the migrating epithelial cells but also directly in front of them. The epithelial cells as a result had literally to "blaze a trail" to reach the migratory bud from the other edge of the wound. Clark and Clark (1953) observed similar phenomena in the dissolution of the blood clot ahead of the migrating epithelial cells in rabbit ear chambers.

Orientation of cell movement by contact inhibition of random, non-specific membrane movements except along an orientated substratum (Weiss, 1961) does not explain the ability of the cells to select the fibres that they follow relative to the fibres that they destroy. Weiss's theory of the directional control of cell movement, therefore, while explaining movement in a system of 2 planes does not appear to be adequate for the migrating epithelial cells in the 3 planes present in the wound space.

MEMBRANE ACTIVITY. The "activity" of the plasma membranes of the cells at the leading edge of the bud was evident in the variety of profiles that were seen in fixed preparations.
The irregularity in the outlines of these membranes, the uniform and vesiculated dense bodies and the endoplasmic reticulum often became so well developed that leading edge cells infiltrating through the wound coagulum could be distinguished accurately from other migrating macrophages only by their tonofilaments and desmosomes.

The relationship of this membrane "activity" to amoeboid movement of the cells has already been discussed. Its association with the appearance of intracellular vacuoles and vesicles prompts speculation about the possibility of its involvement in active transport across the cell membrane (Bennett, 1956). Small vesicles are found in the cytoplasm adjacent to the regions of membrane "activity" and are often seen fused with the plasma membrane of the cell. It is impossible to tell whether these fused vesicles are moving into or out of the cell but it is not unreasonable to assume that passage in both directions is taking place.

**CYTOPLASM OF THE MIGRATING CELLS.**

*Endoplasmic Reticulum.* An increase in the quantity of endoplasmic reticulum similar to that in the migrating
epithelial cells of the rat palate has been observed during the early stages of amphibian regeneration by Singer and Salpeter (1961). They thought that the epithelial cells could produce and secrete proteins that would influence the underlying blastema. However the protein may be produced for use by the epithelial cells themselves (See Chapter 5).

Dense Bodies. The terms uniform and vesiculated dense bodies have been adopted in an attempt to clarify the great variety in the morphological appearances revealed by these structures.

The uniform dense bodies had some of the morphological appearances generally considered as indicative of intracytoplasmic lipid, as distinct from absorbed lipid (Palay and Karlin, 1959; Ashworth et al, 1960). However oil red O staining of formalin fixed frozen sections failed to show conclusively intracellular lipid. The true nature of these particles, therefore, is not known.

The vesiculated dense bodies have been more thoroughly investigated in this study. They are morphologically similar to the cytoplasmic organelles described by Novikoff and Essner (1960) in liver as lysosomes.
The importance of lysosomes in cell physiology has become evident in the large volume of literature that has accumulated since their original description as a biochemical entity by DeDuve et al. (1955), and their recognition (Novikoff et al., 1956) as the peri-biliary dense bodies already known to electron microscopists. DeDuve (1959) from his experimental evidence described them as having functions in intracellular digestion and engulfing processes and in physiological and pathological autolysis. Further experimental evidence and criticisms proffered over the subsequent years have further clarified and consolidated the concept of lysosomes and many of the details of the applicability of this concept have been worked out (deReuck and Cameron, 1963). The definition of a cytoplasmic organelle as a lysosome has also become more precise during this period. In 1960 Novikoff considered "that most bodies described by electron microscopists as 'microbodies', 'cytosomes' and 'large granules' are lysosomes - cytoplasmic organelles delimited by 'single' outer membranes and possessing high levels of acid phosphatase". In 1963, however, Novikoff expressed the view
that the term "lysosome" would be best confined to "pure lysosomes", and that the organelles that receive acid hydrolases from the pure lysosomes, be described by their specific names.

The vesiculated dense bodies that arose in the cytoplasm of some of the migrating epithelial cells were similar to "derivative lysosomes", i.e., organelles that are heterogeneous in size, shape and content but which are always surrounded by a unit membrane and are obviously distinct from other cytoplasmic organelles (Novikoff, 1961). As a result it became necessary to determine the relationship of these organelles to lysosomes and their significance and role in the migrating cells.

At first it seemed that they were involved in the autolysis of injured epithelial cells. This possibility cannot be entirely ruled out but the vesiculated dense bodies were found at the 3 hour stage in cells that retained an otherwise normal appearance. The fine structure of these dense bodies closely resembled the organelles found in injured cells in other sites: i.e., they contained structures that appeared to be remnants of other cytoplasmic
components, mainly mitochondria and RNP particles (Ashford and Porter, 1962, Hruban et al., 1963). On the other hand the type of vesiculated dense body most frequently found in the migrating cells at later stages contained a uniformly granular electron dense component almost exclusively. The possible significance of these differences will be discussed later.

The presence of the vesiculated dense bodies in migrating cells at all the stages examined and in cells that showed evidence of increased rather than decreased cellular activity did not favour the view that they were involved in cell death at this site. The possibility of their role in phagocytosis was therefore considered and subsequently was tested experimentally. Their significance in this regard will be discussed further in the subsequent chapters of this thesis.

Glycogen. The small collections of granular material stained heavily with lead that were occasionally present in a few of the cells of the migrating bud are interpreted as glycogen (Revel et al., 1960). It was not possible to confirm this histochemically because of the difficulties of
adequately preserving and detecting small quantities of glycogen (Trott, 1961). The presence of glycogen in healing wounds after 24 hours has been described many times (Bradfield, 1951, Washburn and Blocker, 1954, Argyris, 1956) but the reasons for its appearance, and its subsequent disappearance after the conclusion of epithelial healing, are unknown. Washburn (1960) related its presence to the absence of keratinisation in migrating epithelium and its disappearance to the onset of keratinisation. This association does little to explain the phenomenon. Its possible significance in the light of the experiments reported in this thesis will be discussed in Chapter 5.

TONOMETRICAL, DESMOSES AND KERATOHYALINE GRANULES.

The presence of tonofilaments and desmosomes in the migrating epithelial cells showed that the development of the other specialised structures in the cytoplasm of these cells was not associated with a concurrent loss of the characteristic structural components of cornifying stratified squamous epithelium. It is a matter for conjecture, however, whether their formation was taking place during the migratory phase. The cells that formed the bud appeared to arise from
the basal and spinous layers of the adjacent epithelium by a process of migration. Mitotic activity was not apparent. As a result the cells of the bud would have been expected to possess and to retain the tonofilaments and desmosomes that they had developed up to the time of onset of migration. The stability of these structures, especially in the immature form present in these cells is not known but their persistence after injury when most of the other cellular components have disappeared (Figs.15-17) suggests that cellular activity is not necessary for their maintenance. Nevertheless, their presence in the migrating epithelial cells at the leading edge of the bud was the one major landmark that distinguished these cells from other migrating cells in the wound.

The absence of keratoxyaline granules is further evidence that the migrating cells are not derived from the cells of the granular layer. However their failure to appear as stratification is being restored further back along the bud may possibly be explained by the absence of normal cornification at the surface of the bud.

This study of the morphological changes occurring in
epithelial cells during the early phases of wound healing shows that the apparently simple process of epithelial migration is in reality quite complex and that knowledge of the mechanisms behind the majority of the changes that are known to occur is lacking in both detail and clarity.

**SUMMARY**

An injury was inflicted mechanically on the second antemolar palatal ruga either by fine iris scissors or parallel scalpel blades.

The former injury results in necrosis that extends for a variable distance into the marginal epithelium. In this necrotic epithelium the nuclei, protein producing apparatus and other organelles are completely disorganised after 3 hours, but the basement membrane, plasma membranes, desmosomes and tonofilaments can still be recognised. By 6 hours the necrotic epithelium has disintegrated further and has separated from the dermis leaving behind the basement membrane over which the viable epithelial cells have begun to migrate. By 12 hours the leading edge cells have moved into the coagulum in the wound space.

The injury with parallel scalpel blades results in much
less trauma to the marginal epithelium. Migration has begun by 6 hours and by 24 hours has formed a bud of cells which appear to arise from the basal and lower spinous layers of the marginal epithelium and which have moved a variable distance down the inclined connective tissue walls of the wound or directly into the wound coagulum.

Alterations in the fine structure of the migrating cells are evident at all stages. After the first 12 hours the basement membrane is usually absent from beneath the cells forming the lower margin of the tip of the bud. The cells at the leading edge of the bud are separated to varying degrees by distended intercellular spaces that contain various combinations of a fine granular material, migrating white blood cells and debris from the wound. The plasma membranes lining these spaces and those forming the free margins of the cells at the leading edge are heaped up into many irregularly shaped folds. Associated with the folds at the leading edge are vacuoles and vesicles of various sizes. In the cytoplasm, mitochondria are still present but their shape and distribution are more irregular than in normal cells. Rough surfaced endoplasmic reticulum
is also more prevalent. Groups of Golgi membranes are often found in different parts of the same cell. In addition cytoplasmic organelles appear that are not found in the normal cells of the basal and spinous layers. These organelles are described as vesiculated dense bodies and their morphological appearance suggests that they are related to the lysosome group of cytoplasmic organelles. Other inclusions described as uniform dense bodies are also found but their origin and significance were not ascertained.

During the first 24 hours tonofilaments and desmosomes are present but their orientation is different from that in non-migrating cells. The tonofilaments tend to be orientated in the direction of migration. Keratohyaline granules and keratinised cells are not found even though stratification is often partially restored a short distance from the leading edge.

These morphological observations are discussed in the light of current knowledge on epithelial migration during wound healing and the possible significance of the vesiculated dense bodies in the physiology of the migrating cells is analysed.
FIGURE 54. The vesiculated dense bodies stained with the reaction product for acid phosphatase with the Gomori method (modified) in an epithelial cell from a bud 18 hours after injury. No other components of the cytoplasm including the uniform dense body (u) or the nucleus are stained. However some reaction product is evident along the margins of the cytoplasmic processes in the distended intercellular space. Araldite. Lead stain. x 20,000.

FIGURE 55. An epithelial cell from an 18 hour bud showing staining of the vesiculated dense bodies which is more intense than the staining of the inclusion bodies in white blood cells in the nearby distended intercellular spaces. Araldite. Lead stain. x 16,000.
CHAPTER 4

THE NATURE AND FUNCTION OF THE VESICULATED DENSE BODIES IN THE MIGRATING EPITHELIAL CELLS

PART I. THE LOCALISATION OF ACID PHOSPHATASE IN THE MIGRATING EPITHELium.

The purpose of this phase of the investigation was to discover whether the vesiculated dense bodies found in migrating epithelium were related to the lysosomes found in other tissues. The vesiculated dense bodies have already been shown to resemble "derivative" lysosomes in their fine structure. If this similarity in structure could be shown to include similarity in enzyme content then these organelles could be related to functional changes in the migrating epithelium.

RESULTS

The reaction product in the Gomori method for acid phosphatase (lead phosphate) is located in structures with a morphological appearance identical to the vesiculated dense bodies (Figs. 54-56 and 58-61). It is not found in the uniform dense bodies or mitochondria. However, in some sections staining is also present in the distended intercellular spaces and along the plasma membranes lining these
FIGURE 56. The distribution of the reaction product for acid phosphatase is not uniform in the vesiculated dense bodies. Circular areas of various sizes are completely devoid of the stain. Some staining is also evident in the contents of the enlarged intercellular spaces. Araldite. No lead stain. x 40,000.

FIGURE 57. Staining is still present in the intercellular spaces in control tissue incubated with 0.01M sodium fluoride. (This staining is probably due to an "alkaline" phosphatase.) Araldite. Lead stain. x 20,000.
FIGURE 58. Reaction product for acid phosphatase in vesiculated dense bodies in an epithelial cell migrating into a wound 18 hours after injury. The nucleus and other components of the cytoplasm are not stained. Mitochondria (m) can be distinguished from the dense bodies. Araldite. Lead stain. x 20,000.

FIGURE 59. Acid phosphatase staining of the vesiculated dense bodies in a migrating epithelial cell 24 hours after injury. Some diffuse, non-specific background staining has occurred in this preparation but the localisation in the dense bodies is still definite. Araldite. Lead stain. x 20,000.
spaces (Figs. 54 and 56). Nuclear staining in these preparations is infrequent but in some sections a very fine, diffuse lead deposit is evident in the nucleus and in the surrounding cytoplasm. Even in these instances the staining of the vesiculated dense bodies is definitive (Fig. 59).

The distribution of stain in the vesiculated dense bodies is irregular. Some areas in the organelles remain completely unstained. These areas are usually circular in outline and are more electron dense than the surrounding cytoplasm (Fig. 56). Their electron density is very similar to that of the material in the uniform dense bodies nearby. The intensity of staining in the vesiculated dense bodies is sometimes greater than in similar inclusion bodies in macrophages in adjacent, distended intercellular spaces (Fig. 55).

The illustrations demonstrating these staining reactions are taken from tissue blocks. When 50μ frozen sections are incubated, the tissue is more disrupted (presumably by ice crystal formation). When the blocks are left in the incubating medium overnight in the refrigerator it becomes
FIGURES 60 and 61. Long storage of the blocks in the incubating medium (overnight) in the refrigerator has resulted in the staining of other cytoplasmic components (a) in addition to the vesiculated dense bodies (v) in these epithelial cells from a 24 hour bud. However the disruption of the tissue which has concurrently taken place does not allow these components to be accurately recognised. Macrophages (m) in the enlarged intercellular spaces have stained diffusely but the reaction product is concentrated in some areas. Araldite. Lead stain. x 16,000.
apparent that other cytoplasmic components are staining in addition to the vesiculated dense bodies (Figs. 60 and 61). This staining is not diffuse but is deposited in linear arrays especially in areas adjacent to the nucleus (Fig. 60a). However, accurate determination of the components of the cytoplasm that are stained is not possible with this procedure.

CONTROLS. As controls, some of the blocks were incubated in media without substrate to show if spurious lead staining was present. Others were pre-treated with 10% trichloracetic acid to denature the enzyme and in others 0.01 M sodium fluoride was used to inhibit its action (Pearse, 1960).

Acid phosphatase reaction product is completely absent from the cytoplasm and nucleus of all these controls. However, in blocks from the NaF control, staining is evident in the intercellular spaces and on some of the plasma membranes lining distended spaces (Fig. 57).

DISCUSSION

ACID PHOSPHATASE IN NORMAL EPITHELIUM. The distribution of acid phosphatase in various stratified squamous epithelia has been studied with the light microscope. Moretti and
Mescon (1956) showed that in epidermis free phosphate was released after incubation with Gomori's medium and that the released phosphate increased linearly with increase in incubation time. Histochemically, incubation for 1 hour produced staining in the stratum corneum, stratum granulosum and the nuclei of the lower layers. After 12 hours incubation, the cytoplasm of all the layers showed a positive reaction. The authors claimed that control preparations demonstrated that this increased staining was not due to the non-specific "lead effect". Maeir and Angrist (1962) compared the distribution of acid phosphatase and other enzymes in various stratified squamous epithelia, using lead and azo dye techniques. They found that the basal and lower spinous layers were unreactive but that the more superficial layers, especially the stratum granulosum, showed some degree of activity in bladder, vagina, skin and oesophagus. A similar distribution of reaction product was found in the oesophagus by Novikoff (1960). Cabrini and Caranza (1958) demonstrated a similar pattern in human gingiva. All these authors have shown that reaction product is not found in the basal and lower spinous layers of stratified squamous epithelia. In view of these
authors' results with the light microscope the possibility of finding acid phosphatase in the cells of the basal and lower spinous layers of normal epithelium with the electron microscope was considered to be remote. Since the purpose of this phase of the investigation was merely to show whether or not the vesiculated dense bodies (which are not found in these layers in normal epithelium) contained acid phosphatase and since the cells of these lower layers (which apparently provide the cells for the migrating bud) have been shown not to contain acid phosphatase, examination of the distribution of acid phosphatase in normal epithelium with the electron microscope was not carried out.

ACID PHOSPHATASE IN EPITHELIUM DURING WOUND HEALING.

Investigations into the distribution of acid phosphatase in epithelium during wound healing also appear to have been confined to light microscopy and the majority of authors have concentrated on the later phases, i.e., after the first 24 hours. Raekalia (1960) however, noted the paucity of knowledge about the first 1 to 4 days (the supposedly inert "lag" phase) in wound healing in the skin. He found that during this period the uninjured epidermis
adjacent to the wound showed intense activity all through the stratum Malpighii. Carranza and Cabrini (1962, 1963) found that acid phosphatase in wounds of the palate, tongue and skin was markedly decreased in the proliferating epithelium in tissue examined 3 days after injury. The micrographs illustrating their findings were of sections incubated in lead and showed a large amount of nuclear staining and so are not good evidence.

**SIGNIFICANCE OF THE REACTION FOR ACID PHOSPHATASE.** The work of de Duve (1963) and Novikoff (1963) has provided the link between biochemical and histochemical methods that opened the way for many investigations into the distribution, localisation and function of intracellular hydrolases, especially acid phosphatases. However the difficulties inherent in histochemical techniques and especially obvious in the phosphatase techniques utilising lead substrates are increased when applied to interpretations based on localisations at the level of fine structure. Barka and Anderson (1962) considered that demonstration of acid phosphatases with lead techniques was not dependable and became especially suspect when nuclear staining was
present. Holt (1959) on the other hand has shown that if sufficient care was exercised with these techniques the reaction product could be related to the sites of acid phosphatase activity.

It has been conclusively demonstrated that the cytoplasmic structures delineated by these methods correspond with the structures that biochemical analysis has shown to contain acid phosphatase (Essner and Novikoff, 1961, Holt and Hicks, 1961). Barka (1962) in an appraisal of the significance of histochemical localisation of acid phosphatase concluded (in part) that this reaction universally demonstrated structures derived from pinocytotic and reversed pinocytotic processes, and that the acid phosphatase activity of various cells was related to the development and activity of these processes. These conclusions are essentially in agreement with the ideas expressed by Novikoff, 1963.

The presence of reaction product in the vesiculated dense bodies of migrating epithelial cells therefore indicated that these bodies were related to the lysosome group of cytoplasmic organelles as defined by Novikoff
(1961). The uneven staining and the large electron dense areas completely devoid of reaction product suggested that the contents of these bodies included many other components in addition to acid phosphatases. As a result they are not "pure" or "primary" lysosomes but probably fall somewhere in the group of "derivative" lysosomes (Novikoff, 1963).

The absence of reaction product in the uniform dense bodies on the other hand supported the morphological evidence that these structures did not belong to the lysosome group. Nevertheless it is evident that they are related in some way to the functional modification taking place in the migrating epithelial cells but this relationship is at present unknown.

Reactivity in components of the cytoplasm other than the vesiculated dense bodies after incubation of 50u frozen sections stimulated speculations similar to those of Novikoff (1963) when he found some evidence of acid phosphatase activity in elements of the Golgi apparatus. These speculations will be discussed in the final interpretation in Chapter 5.
The reaction product found occasionally along the plasma membranes lining distended intercellular spaces and in the material in these spaces was probably not due to acid phosphatase since it was still present in the NaF control. It is possible that this product is the result of activity similar to that of the so-called "alkaline phosphatase" found on the brush borders of tubule cells in mammalian kidneys. This type of reaction product has been described when the incubation medium is only just on the acid side of neutral pH (Wachstein et al., 1962).

The paucity of nuclear staining in these preparations was a further indication that reasonably accurate structural localisation had been obtained. Investigators are not in agreement about the interpretation of nuclear staining when incubation for acid phosphatase is carried out with substrates based on lead (Deane, 1963). However, if the nucleus does stain it is doubtful whether interpretations applied to the localisation of the reaction product in other sites in the cell has any significance. All the usual controls are inadequate since this staining is apparently the direct result of hydrolysis of the substrate and
diffusion of the resultant reaction product, i.e., the staining is dependent on the presence of both acid phosphatase somewhere in the cell and substrate in the incubating medium (Barka and Anderson, 1962).

In conclusion, therefore, the localisation of the reaction product for acid phosphatase in the vesiculated dense bodies and the morphological appearance of these organelles indicates that they may be classified as lysosomes. The variety in the details of their morphological appearance however, suggests that they are not "pure" lysosomes but probably belong somewhere amongst the "derivative" lysosomes (Novikoff, 1963).

PART II. THE DEMONSTRATION OF THE PHAGOCYTIC ABILITY OF MIGRATING ORAL EPITHELIUM.

The observations and results reported up to this point show that organelles of the lysosome type (the vesiculated dense bodies) appeared in the cytoplasm of the epithelial cells at the leading edge of the migrating bud during the first 24 hours after injury. Since the appearance of these organelles was coincident with the onset of migration I thought that they may be a morphological expression of a
physiological alteration in the epithelial cells at the margin of the wound and that, as such, they could be related in some way to the development of the ability to migrate.

Present knowledge on the role of lysosomes in the physiology of the cell indicates that they are involved in the processes of either cell death (deDuve, 1959) or the intracellular digestion of ingested material (deDuve, 1963). As was stated earlier (see Chapter 3) these organelles were found in cells that both in their position in the bud and their morphological appearance, did not show any indication of actual or imminent death. It became necessary therefore to determine if these organelles were involved in the processes of intracellular digestion.

As a result the possibility that the epithelial cells were phagocytic during migration and that the vesiculated dense bodies were significant in this regard prompted further experimental investigation.

Phagocytosis by mammalian epithelium during wound healing has been suggested by other authors (Forbus, 1952), but demonstration of the phenomenon has relied mainly on
experiments with amphibia. The uptake of injected
carmine granules was shown with the light microscope in
limb bud regeneration in tadpoles by Ide-Rozas (1936).
Singer and Salpeter (1961) have studied limb bud regenerat-
ion with the electron microscope and they concluded from
their morphological studies that the cells of the apical
cap were phagocytic but they did not demonstrate phago-
cytosis experimentally.

Platt (1961, 1963) showed with the light microscope
that guinea pig foot-pad epithelial cells could take up
colloidal material injected directly into blind intra-
epithelial needle tracts. The possibility of confusion
between inter and intra-cellular deposits of colloidal
material is always present when epithelium is examined in
the light microscope because of the closely approximating,
interdigitating, sheet-like nature of this tissue. Platt
was aware of this and his experiments with iron dextran
demonstrated the intracellular position of the injected
material much better than those with Indian ink. However,
with his technique (direct injection of colloidal material
into normal epithelium) the very high concentration of
colloidal particles outside the cells may have resulted in some of these particles being taken up inadvertently during pinocytosis. Since pinocytosis occurs normally in these cells their uptake therefore may not have illustrated any functional alteration in the epithelial cells.

The experiments reported in this part of the chapter were designed to demonstrate the presence or absence of phagocytosis in migrating epithelium while producing the minimum possible deviation from the conditions existing in wounds healing normally.

RESULTS.

DEPOSITION OF THORO TRAST IN THE WOUND. Injected thorotrast becomes deposited in the wound by at least three methods. The most important, from the point of view of these experiments is its intermingling with the wound coagulum as a direct result of the high concentration in the circulating plasma at the time of injury (Figs. 74, 75 and 76). The second method is by passive transport into the wound in macrophages that have ingested thorotrast in the circulation. These cells migrate into the wound during the inflammatory response and carry the ingested thorotrast with
FIGURE 62. Thorotrast in inclusion bodies in a migrating white blood cell in an enlarged space between epithelial cells from a migrating bud 18 hours after injury. Araldite. PTA, uranyl and lead stain. x 20,000.

FIGURE 63. Ingested thorotrast in a macrophage (m) in contact with a migrating epithelial cell at the leading edge of a 24 hour bud. Araldite. PTA, uranyl and lead stain. x 20,000.
them. A few of these migrate into enlarged intercellular spaces in the epithelial bud (Fig. 62) but the majority pass into the cellular exudate on the surface of the wound (Fig. 63m) where many subsequently undergo autolysis and release their thorotrust into the general debris present in this area. The third method is by leakage out of damaged blood vessels in the wound floor. Some of the vessels, especially those that have become occluded by platelet plugs contain high concentrations of thorotrust but only small amounts appear to escape by leakage during the experimental period (Fig. 64). The absolute quantity of thorotrust that becomes exposed to the migrating epithelium as a result of all of these methods is not large.

**UPTAKE OF THE INJECTED THOROTRUST.** The migrating epithelial cells come into contact with the wound coagulum containing the thorotrust as soon as they move into the wound space. The area of contact however is greatly increased when the end of the bud is moving directly into the coagulum, i.e., at the 18 and 24 hour stages.

Eighteen hours after injury the cells at the leading edge of the bud contain small amounts of thorotrust, usually
in membrane bound inclusion bodies (vesiculated dense bodies). The "active" membrane of the leading edge cells occasionally can be seen surrounding small quantities of thorotrast and it is assumed that they are in the process of ingestion (Figs. 65, 66 and 67).

Six hours later, however, there is a definite increase in the quantity of thorotrast in the cells at the leading edge (Fig. 68). Very large masses are found in some of the cells; so large that they indent the nucleus (Fig. 70). These large accumulations are found most often at the pole of the nucleus furthest from the leading edge of the bud (Fig. 68). The thorotrast is densely packed in these sites and resembles closely the appearance of thorotrast in macrophages in the wound (Fig. 63) and in Kupffer cells in the liver. The marker is also found in membrane bound inclusion bodies of different sizes and some of these resemble the vesiculated dense bodies (Figs. 71, 77 and 80). In many instances similarity to the vesiculated dense bodies can only be inferred since the characteristics distinguishing these organelles have become clouded over by granules of thorotrast (Fig. 73). Frequently, small
accumulations of thorotrast lie free in the cytoplasm, apparently without a surrounding membrane (Figs. 70t and 78t).

In the epithelial cells the particle distribution in the inclusions containing thorotrast is much higher than in any equivalent area in the wound coagulum (Figs. 68, 70, 72 and 73). Cells with large quantities of thorotrast often appear to be migrating into areas almost completely devoid of the colloidal marker (Figs. 69 and 80).

The exposed plasma membranes of cells migrating into the wound often appear to be indented by material from the wound coagulum. Deeper in the cytoplasm of these cells, vacuoles that contain material similar in density to the wound coagulum occasionally contain thorotrast particles (Fig. 79c). Large masses of wound coagulum are also sometimes seen indented deeply into the cytoplasm and continuity with coagulum outside the cell can be demonstrated (Figs. 74 and 75). Small vacuoles in the cytoplasm adjacent to these large masses contain thorotrast more closely packed than in the large indentation (Figs. 74v, 75v and 76v). These large masses of wound coagulum indented into the
FIGURE 64. A blood vessel in the floor of a 24 hour wound. It has become occluded by a platelet plug. A high concentration of thorotrast particles is present in the vessel but only a small number (t) appear to have leaked through the vessel wall during the experimental period. The cell at the top left is probably a migrating white cell and contains a few small accumulations of thorotrast particles. Araldite. FTA, uranyl and lead stain. x 8,000.
FIGURE 65. Epithelial cells at the leading edge of an 18 hour migrating bud in contact with the wound coagulum containing small amounts of thorotrast, some of which is in indentations of the plasma membrane of epithelial cells (t). In this micrograph the outlines of the plasma membranes of the epithelial cells (E) are so complex that it is very difficult to determine which plasma membranes are part of the epithelial cells and which are part of the adjacent polymorphs (P). Serial sections showed that the thorotrast particles at t are indented into the plasma membrane of an epithelial cell. Araldite. PTA, uranyl and lead stain. x 25,000.
FIGURES 66 and 67. Thorotrast particles (t) in the intercellular space in Fig. 66 and surrounded by folds of the plasma membrane in Fig. 67 may be in the process of being ingested by these epithelial cells in an 18 hour bud. Araldite. PTA, uranyl and lead stain. x 20,000.
FIGURE 68. Thorotrast particles in a migrating epithelial cell 24 hours after injury. Three large accumulations of densely packed particles (t) are close by each other at the pole of the nucleus distal to the leading edge of the bud. (Direction of migration indicated by arrow). A smaller accumulation is in a membrane bound structure immediately beneath the large uniform dense body (u). At the proximal pole of the nucleus a collection of vacuoles of various shapes and sizes contain material similar to the wound coagulum (c) outside the cell. The largest of these vacuoles also contains a few thorotrast particles (t). Araldite. PTA, uranyl and lead stain. x 18,000.
FIGURE 69. Two small accumulations of thorotrast in the cytoplasm of a migrating epithelial cell 24 hours after injury. The less densely packed group of particles does not appear to be surrounded by a membrane. The wound coagulum through which the cells are migrating is almost devoid of thorotrast particles. (The apparently empty space between the plasma membrane of the epithelial cell and the fibrils of the wound coagulum forming the substratum is well illustrated in this micrograph and the similarity to the relationship of the plasma membrane to the basement membrane in normal epithelium is evident.). Araldite. PTA and uranyl stain. x 20,000.

FIGURE 70. Large accumulations of thorotrast particles at the distal pole of an epithelial cell nucleus. The largest is indented into the nucleus. The thorotrast at t appears to be free in the cytoplasm. Araldite. PTA and uranyl stain. x 20,000.
FIGURE 71. An unusually large collection of uniform and vesiculated dense bodies in an epithelial cell in a migrating bud formed 24 hours after injury. Each one of the vesiculated dense bodies contains a small accumulation of thorotrast particles (t). Some of the uniform dense bodies in this micrograph (especially the one at A) appear to be arising in the vesiculated dense bodies. Collections of granular material (g) interpreted as glycogen and swollen cisternae of rough surfaced ER (e) are also evident. Araldite. Lead stain. x 30,000.
FIGURES 72 and 73. The variety in the distribution of thorotrast in the migrating epithelial cells is illustrated in these micrographs. Large accumulations of densely packed particles are the most prominent. Although a definite membrane cannot be seen around the entire perimeter of these collections the relatively uniform curvature of their outer edges suggests that a membrane is present. Thorotrast particles are in membrane bound bodies associated with less electron dense components (v) and also apparently free in the cytoplasm (t). In the latter instance only a few particles are present and usually one of the other two types of thorotrast accumulations are nearby. Golgi membranes (g) and a large uniform dense body (u) are also present in Fig. 73. Araldite. PTA and uranyl stain. x 40,000.
FIGURE 74. A large mass of wound coagulum containing a relatively high concentration of thorotrast particles is deeply indented into the cytoplasm of a cell on the lower border of a 24 hour bud. Deeper in the cytoplasm are small vacuoles (v) which contain thorotrast particles but little coagulum. A similar mixture of thorotrast and wound coagulum is present in a very large vacuole in an adjacent epithelial cell at the bottom left of the micrograph. The distended intercellular spaces (s) typical of the migrating bud are also evident. Araldite. PTA, uranyl and lead stain. x 18,000.
FIGURE 75. A large vacuole apparently being formed in the cytoplasm of a migrating epithelial cell by the pinching off of wound coagulum indented into the cell. Continuity with the coagulum outside in the wound can be seen. A small vacuole (v) contains thorotrast particles without associated wound coagulum and a few free particles (t) are present in the intercellular space. The direction of migration is indicated by the arrow. Araldite. PTA and uranyl stain. x 20,000.

FIGURE 76. Another area from the same bud as that in Fig. 75. The thorotrast particles outside the cell are intermingled with the wound coagulum whereas those in the vacuole (v) are almost completely free from it. The vacuole at c contains a few thorotrast particles and the granular material associated with them is less dense than the coagulum outside the cell. Araldite. PTA and uranyl stain. x 20,000.
FIGURE 77. Thorotrast particles in membrane-bound bodies in an epithelial cell from a 24 hour migrating bud. Araldite. Lead stain. x 40,000.

FIGURE 78. Thorotrast particles (t) apparently free in the cytoplasm of an epithelial cell. Rough surfaced ER (e) is well developed in this cell. Araldite. Lead stain. x 40,000.
FIGURE 79. Thorotrast (t) associated with material similar to the wound coagulum in a vacuole in an epithelial cell at the leading edge of a 24 hour bud. The external plasma membrane is irregular (p). Araldite. Lead stain. x 40,000.

FIGURE 80. Small quantities of thorotrast intermingled with the wound coagulum (c) into which the epithelial cells are migrating. Thorotrast particles are both in the membrane-bound body (v) and free in the cytoplasm (t). Profiles of rough surfaced ER (e) are scattered nearby. Araldite. Lead stain. x 40,000.
epithelial cells are found most frequently along the deep border of the migrating bud near the leading edge and are extended in the direction of the migration (Fig. 75).

**DISCUSSION**

The first positive evidence in favour of the phagocytosis by the migrating epithelial cells was obtained with colloidal silver. However, its particle size and its distribution in the cytoplasm could lead to confusion with lead stained glycogen. Saccharated iron oxide had the advantage of a small and relatively uniform particle size but the fact that it is slowly (but surely) converted to ferritin in the tissues led to its being abandoned in favour of thorotrast. Colloidal thorium dioxide has the advantages that it is unmistakable in electron micrographs and the accuracy of its localisation is assured by the inability of the tissues to metabolise it. These advantages are partly offset by the toxicity and low level of radioactivity of this compound but the relatively small local dosage and the short experimental period compensate for these disadvantages.

The results of these experiments clearly show that the
migrating epithelial cells are capable of ingesting the
wound coagulum and foreign material that lie close to the
path of their advance. The cells' ability to concentrate
and store non-metabolisable components intermingled with
this coagulum indicates that the processes involved may be
described under the general heading of phagocytosis.

The morphology of phagocytic cells has been studied
with the electron microscope in alveolar macrophages that
had ingested carbon particles (Karrer, 1960), peritoneal
macrophages that had ingested red blood cells (Essner,
1960) and bacteria (North and Mackaness, 1963a, 1963b) and
polymorphs that had ingested bacteria (Goodman and Moore,
1956a, 1956b, Lockwood and Allison, 1963). In all instances
these authors stressed the part played by the surface
membrane. Karrer (1960) interpreted the formation of
"ruffles" and pseudopods as static evidence of the
"activity" of the plasma membrane. The means by which
this membrane "activity" is achieved is unknown but the
frequent association of motility and phagocytic ability
is worthy of note.

The main function of cells that are primarily phago-
cytic appears to be to rid the animal of foreign and
noxious material and their metabolism appears to be organ-
ised towards the most effective means of achieving this
end (Karnovsky, M.L., 1962). The purpose of phagocytosis
in migrating epithelial cells does not appear to be
identical to that in cells that are primarily phagocytic.
In epithelial cells phagocytosis is certainly associated
with an obvious increase in membrane "activity" and the
development of motility, and in these respects the cells
resemble phagocytes in other sites. However, during the
phase of wound healing studied here, the cells do not
appear to seek out debris like normal migrating phagocytes.
The only material ingested appears to be that lying
directly in or adjacent to the path of migration. Of this,
the uniformly electron dense wound coagulum is the material
most frequently observed in the process of ingestion.

The components of this wound coagulum in this site
are not known but the infrequency of electron dense band-
ing suggests that large quantities of other plasma components
are present in addition to fibrin. The morphology of these
components suggests that proteins are a prominent
constituent. The fact that the injected thorotrast becomes intermingled with this wound coagulum also suggests that other plasma components with a molecular size similar to this colloid may be present. It is this complex mixture that has been observed in the process of ingestion (Figs. 74 and 75).

As stated earlier, most of the thorotrast available for ingestion is intermingled with and randomly dispersed in the electron dense components of the wound coagulum. This is in sharp contrast to the compactness of the colloidal particles in the inclusion bodies in the cells and the partial or complete absence of the electron dense material (Figs. 68-70). It is evident that the wound coagulum is being digested and removed so that the non-metabolisable thorotrast ingested along with it becomes more compactly arranged. It is also apparent that the thorotrast ingested in this manner is being pooled in the cytoplasm of the cell and stored (at least for 24 hours) as the large masses distal to the nucleus.

**SUMMARY**

The presence of acid phosphatase in the vesiculated
dense bodies is demonstrated using a modified Gomori method for the histochemical localisation of this enzyme. The combined evidence of the presence of acid phosphatase in these organelles and their morphological appearance allows them to be classified as lysosomes.

Since these organelles are found in actively migrating cells at the tip of the epithelial bud experiments were carried out in an attempt to determine their function in this site.

Electron opaque colloidal markers (of which thorotrast was found to be the most suitable) when injected into the peripheral venous circulation immediately prior to wounding become deposited in the coagulum in the wound. Into this coagulum the epithelial cells subsequently migrate. Twenty-four hours later accumulations of colloidal marker are found in the cells at the leading edge of the bud. Some of these accumulations are intermingled with the components of the vesiculated dense bodies. In large accumulations which are composed almost entirely of thorotrast, the particles are much more densely packed than those in the coagulum into which the cells are migrating.
Occasionally cells are deeply indented by large masses of wound coagulum. Vacuoles around the periphery of these large indentations contain more compactly arranged thorotrast particles.
CHAPTER 5

GENERAL DISCUSSION AND INTERPRETATION OF RESULTS

The study of the relationship between structure and function has received a great impetus since the introduction of the electron microscope as a research tool. The large body of information that is becoming available as a result of the correlation of the findings from various disciplines interested in biologically important macromolecules has stimulated a revival of interest in morphology, especially fine structural morphology. Morphological studies may be used in the light of this correlated information to provide a basis upon which speculations on the functions of various macromolecular systems can be developed and to indicate the direction that future investigation of specific problems may take.

Application of this approach to the problem of epithelial migration during wound healing has led to the formulation of a hypothesis that appears to be consistent with the results reported in this thesis. The hypothesis is one that is capable of further experimental investigation, e.g., with the use of metabolic inhibitors in tissue
Australian Woman Charges Husbands Nailed Her in Closet

ELBOURNE, Australia, April 17 (Reuters)—A 24-year-old housewife told a court here that her husband nailed her into a cupboard for 10 hours to keep her at home while he went to work. Her mother-in-law fed her through a hole in the cupboard with toast cut into strips and sucked through a straw, S. Annabel Mawson said. Her husband, Raymond, 26, pleaded not guilty to charges of assaulting and falsely imprisoning his wife. The hearing was continued.
Immediately after the injury the haemorrhage that occurs from severed blood vessels is promptly controlled by localised vasoconstriction and the formation of a blood clot. This disturbance of the local blood supply produces changes in the availability of metabolites to the viable epithelium at the margin of the wound. It is probable that the local oxygen tension is lowered below normal levels and so the viable epithelial cells would tend to favour the anaerobic pathways to provide for their energy requirements. This could produce a drop in their intracellular pH and result in the stimulation and activation of acid hydrolases. Coincident with this activation of acid hydrolases an increase in membrane activity could occur. If the cells were prevented from coming into contact with ingestible material by adjacent cells that have undergone necrosis then the activation of the acid hydrolases could result in focal cytoplasmic degradation. This could explain the formation of membrane bound structures that contain remnants of other cytoplasmic components, such as mitochondria and RNP particles. When the cell migrates and
comes into contact with the wound coagulum the latter is ingested and becomes the substrate for the acid hydrolases. Hence focal cytoplasmic degradation would not occur. Ingestion would also increase the need for more digestive enzymes and the formation of acid hydrolases would be stimulated. These could be synthesised in the rough surfaced endoplasmic reticulum that develops and be transferred to the Golgi apparatus. Ingestion vacuoles and their contents from the surface of the cell are somehow transported through the cytoplasm to the Golgi zone. The digestive enzymes could become activated and incorporated into the vacuoles containing the ingested material. It appears that the readily digestible components in these vacuoles can be hydrolysed rapidly but other components more refractory to the action of the enzymes and non-metabolisable materials become apparent in the cells in the form of vesiculated dense bodies.

Such a process of ingestion and digestion in the early phases of wound healing could help to explain the rapid onset of epithelial migration. The epithelial cells at the leading edge of the bud were shown to be able to ingest
large quantities of wound coagulum. The disappearance of this coagulum and the resultant condensation of the thorotrast is ample evidence for the digestion of the wound coagulum. This ingestion and digestion could provide the metabolic requirements for the migrating epithelial cells. The means by which these metabolic requirements are met can only be postulated on the evidence available. However, it is possible that the ingested protein is hydrolysed to its amino acid components. These could then be de-aminated and utilised as required in energy production via their respective pathways; the ketogenic amino acids supplying energy through the anaerobic pathway and the glycogenic amino acids through the Kreb's cycle. The availability of oxygen would presumably determine the proportion of each utilised.

Plasma protein catabolism could also help to explain the increase in glycogen observed by other investigators after the first 24 hours. During the early phases when it is likely that the oxygen tension is lowered the cell would be supplying its needs mainly by anaerobic glycolysis. The small proportion of ketogenic amino acids in the plasma
proteins would necessitate the ingestion of large quantities of wound coagulum. Only the cells at the leading edge of the bud in actual contact with the wound coagulum would be able to derive their metabolic requirements directly from phagocytosis. It is possible that the other cells of the bud would need to derive their metabolic requirements, at least in part, from the actively phagocytic cells. Sufficient oxygen would probably be available for a small proportion of the glycogenic amino acids to be utilised for energy production but the rest could be retained and accumulated, probably in the distended intercellular spaces. These excess products from the activity of the phagocytic cells could then become freely available to the other cells of the bud via the distended intercellular spaces. I would expect that an oxygen gradient would exist along the migrating bud, the availability of oxygen increasing as the distance from the leading edge increases. The glycogenic amino acids released from the cells at the leading edge could then be utilised by the cells further back in the bud that have a better oxygen supply. The amino acids in excess of the energy requirements of the
time could then be converted into the glycogen stores that can be seen in the cells.

Without the ability to utilise the ingested plasma proteins the epithelial cells would not be able to move into the environment of the wound, but, like the connective tissue cells, would have to be dependent on the development of an adequate supply of nutrients from ingrowing blood vessels.

Accurate information on the oxygen tension of healing wounds is difficult to obtain but recent developments of polarographic methods may help to fill in this gap. Experiments to determine the oxygen consumption of inflamed gingival tissue (Manhold and Volpe, 1963) have shown that a lowered oxygen utilisation is associated with periods during which exudation and degeneration predominate, i.e., the early phases of wound healing. It has also been known for some time that the pH of healing wounds, especially during the early phases is acid (Tammann, 1931) and that this acidity could be the result of the presence of carbon dioxide and lactic and pyruvic acids due to the disrupted aerobic metabolism (Arey, 1936).
Additional support for the interpretation presented in this thesis may be obtained by comparison with the metabolic patterns found in other phagocytic cells, especially polymorphs and monocytes. At first sight this comparison may not appear to be justified. However, it is evident that the only other active cells present in the environment of the wound into which the epithelial cells are migrating are polymorphs and macrophages. In addition the morphological similarity between the cells of the leading edge of the epithelial bud and the nearby macrophages and the similarity in the function (which has been demonstrated) is considered sufficient for comparisons to be made.

Sbarra and Karnovsky (1959) with guinea pigs and Cohn and Morse (1960) with rabbits, have shown that polymorphs are primarily dependent on glycolysis as a source of energy for phagocytosis. Inhibition of the aerobic pathway had no effect on phagocytosis whereas poisoning of the anaerobic pathway produced a definite reduction in both phagocytosis and motility. Earlier studies had shown that the pH of the cytoplasm of phagocytic cells was below
neutrality (Rous, 1925). Hirsch and Cohn (1960) have related this low pH to the production of lactic acid due to the stimulation of glycolysis during phagocytosis and have shown that it is associated with degranulation of the polymorphs and activation of the hydrolytic enzymes contained in these granules. More recently, Oren et al., (1963) have shown that monocytes from the peritoneal exudate of guinea pigs also rely on glycolysis for phagocytosis.

It therefore appears reasonable to postulate that epithelial cells during migration may derive their energy requirements mainly from anaerobic glycolysis and that this could produce a drop in the intracellular pH sufficient to activate acid hydrolases.

As migration and phagocytosis continue the need for the hydrolytic enzymes would increase. It has recently been demonstrated in the exocrine pancreas, that protein secretion involves assembly of the protein in the rough surfaced endoplasmic reticulum followed by transfer to the Golgi zone where the proteins are apparently concentrated and surrounded by a membrane. The secretion granules
thus formed accumulate at the apex of the cell and eventually discharge their contents into the lumen (Caro and Palade, 1964). I think that the first part of this mechanism is occurring in migrating epithelial cells but that the process is not carried through to the stage of discharge of the protein (hydrolytic enzymes). Instead the enzymes are transferred to ingestion vacuoles that are then gradually transformed into the vesiculated dense bodies. The vesiculated dense bodies therefore are considered to be either digestive vacuoles or residual bodies depending on the stage of their development at the time of examination (deDuve, 1963). The increase in endoplasmic reticulum is interpreted as the morphological expression of the increased production of acid hydrolases for the intracellular digestion of ingested material. It is not considered to be related (at this stage of wound healing) to protein production for excretion (Singer and Salpeter, 1961).

The conclusion that the migrating epithelial cells are utilising ingested wound coagulum to provide metabolic energy is deduced entirely from the morphological
observations. However some support for this interpretation may be obtained from the work of Gilbert (1962). He found that the epidermis of cattle utilised fat and protein as endogenous substrates. The rate of endogenous \( \text{NH}_3 \) formation indicated that protein provided about 25% of the respiratory substrate. It is possible, therefore, that other stratified squamous epithelia potentially have the enzyme systems necessary to use protein for energy production. These enzyme systems may be induced to provide a major proportion of the energy requirements in an environment with a low oxygen tension by an increase in the availability of suitable substrates, i.e., ingested plasma proteins.

The development of the ability to phagocytose material in the path of migration therefore could allow the migrating epithelial cells to "blaze a trail" through the wound coagulum and to "live off the land" as they pass through this otherwise unfavourable environment.

The results reported in this thesis, while throwing some light on epithelial migration in wound healing, do nothing to explain the initiation of migration or the means
by which the actual movement of the cells is achieved.

The "Coaptation" theory of Weiss (1950) is considered by many to explain the means by which the obviously complex processes involved in epithelial migration are set in motion. The basis of this theory is that the cells of a tissue or organ normally maintain a stable and controlled inter-relationship with their surroundings (neighbouring cells, substratum and outer medium) due to "contact inhibition" of the potentially active cells. Removal of this contact inhibition mobilises the inherently active cell membrane which then provides the motive force that propels the cell until contact inhibition is re-established by coaptation with complimentary cells. This theory has gained support from the study of experimental wounds in amphibia (Chiakulas, 1952, Lash, 1956), experimental transplantation in mammals (Herbsman et al., 1957) and from dissociation and re-aggregation techniques (Moscona, 1956). The dependence of contact inhibition on protein synthesis has also been demonstrated (Moscona, 1963).

The frequent relationship between phagocytosis and cell motility indicates that the mechanisms behind these
phenomena may not be basically different. Both appear to be related to and partly dependent on the activity of the plasma membrane. Bellairs and New (1962) showed that phagocytosis of colloidal gold or carmine solution only occurred in chick blastoderm when the edge cells were actively migrating over the vitelline membrane. The evidence at present available also indicates that the metabolic requirements for both types of cellular activity may be similar. Wilde and Crawford (1963) suggested from their observations in amniota that, whereas differentiation and morphogenesis were strictly dependent on aerobic processes, maintenance and movement were probably powered by glycolytic metabolism. Evidence has also been obtained which indicates that ATP derived from glycolysis provides the motive force for protoplasmic streaming in plants (Wohlfarth-Bottermann, 1964). As a result it is tempting to speculate that movement in cells may be dependent on the availability of "free" ATP in the ground substance of the cytoplasm. The presence of the glycolytic enzymes in the ground substance of the cytoplasm and the Krebs cycle and electron transfer enzymes in the
mitochondria (Green and Fleischer, 1963) suggest that the glycolytic pathway, although less efficient in the production of ATP, may be able to provide the ATP in the critical spot at the critical time; i.e., around the contractile protein in the cytoplasm of the cell when the cell is in a temporarily unfavourable environment. In the cells of striated muscle aerobic pathways are the most effective and the preferred method for providing the energy for contraction but in this site a highly developed structural arrangement of mitochondria and sarcoplasmic reticulum has arisen during differentiation (Porter, 1961). When the oxygen supply is not adequate to maintain aerobic metabolism, however, anaerobic processes provide the necessary energy even in this tissue.

In conclusion I consider that, under the conditions present in the material examined in this study, the migration of epithelium into a surgically produced defect is accompanied by alterations in the fine structure of the cells forming the leading edge of the migrating bud. In attempting to interpret these alterations it is postulated that they represent modifications to the functional and
**OBSERVATIONS**

0 hrs.
- Injury
  - Haemorrhage
  - Reduction in blood flow
  - Clot formation
  - Necrosis of marginal epithelium
  - Inflammatory response
  - Appearance of vesiculated dense bodies

3 hrs.
- Epithelial migration into clot
  - Membrane activity
  - Endoplasmic reticulum increase
  - Ingestion of fibrin, 7 other serum proteins and other debris
  - Inclusion bodies containing non-metabolisable components

24 hrs.
- Appearance of intra-cellular glycogen

**INTERPRETATION**

- Localised lowering of oxygen tension
  - Energy requirements partially supplied by anaerobic glycolysis
    - Activation of acid hydrolases
      - Focal cytoplasmic degradation in cells with no ingested material
        - Increased production of acid hydrolases
          - Intracellular digestion of ingested serum proteins
            - Concentration of indigestible debris
              - Release of glycogenic amino acids
metabolic patterns in the cells at the edge of the wound, as a result of, and in response to, alterations in the local environment.

These alterations in fine structure and interpretation of them are summarised in the table opposite.
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