EXPERIMENTAL STUDIES OF WOUND HEALING IN SKIN AND ABDOMINAL HOLLOW VISCERA

by

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"There is no problem of physiological mechanism that does not in the end come down to the dual question of what goes on (chemically) where (anatomically)."

Jonas S. Friedenwald, 1955.

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CHAPTER I

INTRODUCTION

Wound healing is a phenomenon that is appreciated and experienced by both layman and scientist. The surgeon, who deliberately makes wounds under carefully controlled conditions, relies upon the natural processes of repair to produce the results he desires. The tissue of the body that is most liable to injury and most readily available for experimental purposes is the skin, and it is therefore not surprising that most of the available knowledge of the processes involved during wound healing has been derived from this integumentary covering. The reactions to injury of both the epidermis and the dermis have been studied in a number of species under a variety of conditions. Recent commentaries (British Medical Journal, 1956, 1957) indicate that skin is still being chosen for the majority of studies of repair processes. Wound healing in the cornea has also

received a considerable amount of attention. With regard to internal organs, experimental lesions of the stomach and duodenum have been intensively studied, in view of the importance of the problem of peptic ulceration in man. However, comparatively little work has been carried out on repair in the other viscera, and while it has been well established through surgical practice that epithelia and connective tissues normally exhibit sufficient reparative powers, the process of epithelialisation, for example, has not often been the subject of specific study in any particular organ.

The purpose of the work now presented has been to make observations on the behaviour of epithelium and connective tissue during the healing of experimental wounds in abdominal hollow viscera. The organs studied include the oesophagus, stomach, ileum, rectum, urinary bladder and gall-bladder. All experiments have been performed on a single species, the cat - an animal whose organs are of a size suitable for the operative procedures involved. Wounds of the skin, for comparison with those of the viscera, have also been studied

in this animal, and in view of the findings in the cat it was necessary to make skin lesions in rats and guinea-pigs, and rectal wounds in rats, for further comparison. Various histological and histochemical methods have been used, and interest has been centred in the first instance on epithelial behaviour, and secondly on reactions in the connective tissues.

While a precise definition of the term "wound healing" is not important in the present circumstances, it should be noted that confusion has been caused by a number of workers who have considered that when a defect is completely "epithelialised" it is "healed". Obviously this ignores any later changes that occur in subepithelial tissues and which invariably proceed for a much longer time than that required for epithelialisation. All wounds during the process of closure undergo contraction, and it seems that healing can only be considered to be complete when contraction has ceased.

In the report of the Ciba Foundation Clinical Forum on wound healing (British Medical Journal, 1956), it was stated that "To what extent findings based upon the study

of lesions in skin are applicable to other tissues of the body is a question which cannot, as yet, be answered with certainty". It is hoped that the present work makes at least a beginning to an end that is still far distant.

CHAPTER II

HEALING OF SKIN WOUNDS IN THE CAT

The literature on the healing of skin wounds in animals and man is voluminous. The reviews of Arey (1936) and Localio, Casale & Hinton (1943) are sources of abundant information on earlier work on this subject. However, it has not been possible to find reference to the cat as an animal used for such studies. The whole series of experiments now being reported deals mainly with the cat, and it was necessary to confirm that the pattern of healing in skin wounds in this animal is comparable to the known facts in other species, since the findings in experiments on other organs in the cat were to be compared with skin.

Skin consists essentially of an outer epithelial zone overlying a supporting network of connective tissue, the dermis. All the standard textbooks of histology describe the microscopical structure of skin in varying degrees of detail, and two recent monographs are available

(Rothman, 1954; Montagna, 1956) together with review articles (e.g. Carruthers & Suntzeff, 1953; Medawar, 1953). The basic pattern of the epithelium is similar in all mammals but there are, as might be expected, species and regional differences among the various animals and in different sites in the same animal. In view of these differences it is essential to define the normal pattern of the tissue to be used in any series of experiments.

In the present work on the cat, the skin of the lower anterior abdominal wall was used. All layers of the epidermis are here comparatively thin (fig. 1). The stratum lucidum is generally absent but otherwise there is conformity with the usual histological pattern. The basal layer consists of small polyhedral cells with rounded or oval nuclei and mitotic figures are only rarely seen. The stratum spinosum is 2 or 3 cells thick, the cells being mostly flattened with oval nuclei. Surmounting this is a layer of very flat elongated cells with cytoplasmic granules, constituting the stratum granulosum, and above this a layer whose cellularity cannot be distinguished, comprising the

zone of keratinisation.

The dermis consists of interlacing bundles of collagenous fibres in which lie hair follicles, sebaceous glands and sweat glands. The classical division into papillary and reticular layers is poorly marked, but a very narrow region immediately underlying the epidermis where the collagen fibres are less densely arranged may be defined as the papillary layer. In this comparatively thin skin true papillary ridges are not seen. The connective tissue cells that lie scattered in the dermis are chiefly fibrocytes and macrophages. Staining with basic dyes indicates the presence of mast cells, which lie under, and never in, the epidermis, mostly perivascular in position (fig. 2).

Normal skin gives a minimal reaction for alkaline phosphatase, which is present in the stratum granulosum. Glycogen is not usually detectable histochemically but a few granules may be seen in the more superficial cells of the stratum spinosum. Ribonucleic acid (RNA) is present in the basal half of the stratum spinosum. The connective

tissue cells of the dermis do not normally give reactions for glycogen or RNA; blood vessels may give a positive reaction for alkaline phosphatase but the other connective tissue elements are negative. Hair follicles contain glycogen in the external root sheath; alkaline phosphatase is present in active hair papillae, sebaceous glands (fig. 3) and in the myoepithelial cells of sweat glands.

MATERIALS AND METHODS

Wounds were made in 16 adult cats on the anterior abdominal wall about 2 cm. lateral to the midline. Under nembutal anaesthesia the hair was shaved and a shallow oval lesion 0.5 - 1.0 sq. cm. in size was made with a scalpel, involving the removal of the epidermis and a varying amount of dermis and underlying tissue. The precise depth of any particular lesion was not considered to be critical, as it was desirable to study wounds of varying depths.

The animals were killed at varying intervals after operation ranging from 6 hours to 14 days. The site of

the lesion with some normal surrounding tissue was removed, pinned out on cork without tension and fixed in either ice-cold 80% alcohol or ice-cold Carnoy's fluid. Frozen sections were cut from some specimens. After embedding in paraffin wax, serial sections were cut at a thickness of 8μ and every twentieth was mounted and stained with haematoxylin and eosin. Following histological study of these, single serial sections from regions considered to be of particular interest were then mounted on separate slides. The following histological and histochemical techniques were used on these separate sections:

1) Iron haematoxylin and picrofuchsin (van Gieson), for the demonstration of collagenous tissue.

2) Gomori's and Gordon & Sweets' stains for reticulum.

3) The Gomori cobalt sulphide technique for alkaline phosphatase. Sodium-β-glycerophosphate was used as a substrate and Mg ions were added to the incubation medium to activate the enzyme as suggested by Kabat & Furth (1941). The incubation periods varied between 5 minutes and 24 hours. Controls were performed by

omitting the sodium-β-glycerophosphate from the incubation medium.

4) The azo dye method for alkaline phosphatase (Gomori, 1952a) This was performed on frozen sections, and both the Fast blue and Fast red salts were used for varying incubation periods.

5) The periodic acid-Schiff (PAS) method (Hotchkiss, 1948) for the demonstration of polysaccharides. Diastase-labile material, following digestion with saliva for 30 minutes at room temperature, was identified as glycogen. In some instances the acid reducing rinse was not employed; this enhances the demonstration of collagenous tissue, as suggested by Lillie (1953).

6) Toluidine blue (0.5% for 30 minutes) and azure A 0.01% for 5 minutes or 0.1% for 10 minutes) to demonstrate metachromasia.

7) Toluidine blue (0.5% for 30 minutes) and the methyl green pyronin method for RNA. In the latter technique, pyronin Y was used in most instances as advocated by Kurnick (1955). Control sections were stained after treatment overnight with 10% perchloric acid at 4°C (Seigel & Worley, 1951).

8) Feulgen reaction for desoxyribonucleic acid (DNA) (Pearse, 1953).

RESULTS

In the course of this study it became clear that the reactions in the cat's skin were, with one notable exception, closely akin to those that have been described in other species and hence only a brief description of the results is given.

Epithelial Repair

Within 24 hours of creating a breach of continuity, some epithelial cells had begun to migrate from the margin of the wound. The sites of lesions examined after 4 to 5 days (figs. 4 & 5) demonstrated the characteristic features of the regenerating epithelial cells. Those at the margin of the wound and in any hair follicles that may have been immediately adjacent to the wound edge became considerably enlarged. The number of layers of cells was increased and an increased number of mitotic figures was present (figs. 6 & 7).

In some situations the cells of the basal layer became tall columnar in form; intercellular bridges were often very prominent (fig. 8). The cells that were found over the floor of the lesion in continuity with those at the margin showed a similar hypertrophy but no mitoses were found and no keratinisation occurred at this period (fig. 9). As epithelialisation progressed the most superficial cells became flattened. By the tenth day epithelium several layers thick covered the whole wound area, and by the fourteenth day keratinisation was established (fig. 10). By this time all the cells had begun to resume their normal size and shape. In some specimens at the end of the first week, epithelium several layers thick was seen near the wound centre which itself remained without an epithelial covering. Thus it did not appear that all lesions were first covered by a single layer of cells which only became stratified after epithelialisation was complete.

The thickened stratum granulosum gave a reaction for alkaline phosphatase in the granules but the other layers showed no phosphatase activity (fig. 11). With the PAS technique, large quantities of glycogen were found in the

hypertrophic cells, but never in those of the basal layer (figs. 12, 13 & 14). By the fourteenth day when the cells were returning to their normal size and keratinisation had begun again, no glycogen could be demonstrated (fig. 15). The methyl green pyronin technique demonstrated some increase in cytoplasmic RNA in the large type of cell at the ulcer margin (fig. 16), and the nuclei of cells undergoing mitosis showed an increased intensity of staining with the Feulgen reaction.

Connective Tissue Reactions

The reactions that occurred in the floor of the lesion were indicative of the accummulation and maturation of granulation tissue. Mitotic activity was first seen in connective tissue cells 2 days after the infliction of the injury (fig. 17). By the fourth or fifth day many young fibroblasts with slender protoplasmic processes, and sometimes with mitotic nuclei, could be identified together with buds of capillary blood vessels. The amount of infiltration by mononuclear and polymorphonuclear leucocytes was small. Low power views of wounds at this stage demonstrated the

difference between the mature collagen of undisturbed dermis and the fine reticulum that was gathering in the wound area (fig. 5). By the tenth day young collagen fibres could be identified; the cellularity of the area was by now less and a capillary plexus of blood vessels was established (figs.18 & 19).

Staining with toluidine blue showed that mast cells, which were abundant in the normal dermis and subcutaneous tissues (fig. 2), were completely absent in the wound area (fig. 20). They had reappeared in this region after 2 weeks (fig. 21). The ground substance was PAS-positive but metachromasia was minimal. The techniques for alkaline phosphatase revealed no reaction in the young newly forming connective tissue with any of the incubation periods used (fig. 22). This was so at all stages of the healing process examined, and even after the most prolonged incubation (24 hours), young fibroblasts, young blood vessels and extracellular material were still negative.

DISCUSSION

As already noted, the repair processes in skin wounds in the cat closely resemble those found in histological studies in other species (e.g. Dann, Glücksmann & Tansley, 1941; Hunt, 1941; Lindquist, 1946), but the absence of alkaline phosphatase in the newly forming connective tissue is strikingly different from the accummulation of this enzyme that has been found to occur in rodents (Fell & Danielli, 1943). A number of the features of epithelium and connective tissue that are characteristic of the phenomena of repair will now be considered. It is of some importance to note that the skin wounds in the present study are of the "deep excised " variety, as opposed to "incised" wounds and the "shallow excised"type such as are found at Thiersch graft donor sites. The differing reaction patterns in these various forms of wound have been emphasised by Gillman, Penn, Bronks & Roux (1953, 1955a).

The presence of a breach of continuity in the epidermis appears to be the trigger for setting in motion a

series of changes that are characteristically similar in all the mammalian species in which this subject has been studied. But the skin in its normal state is continually undergoing regeneration. Epidermal cells are constantly being shed from the skin surface, to be replaced from below in order that a state of equilibrium can be maintained. Dempster (1954) has pointed out that the active state of regeneration normally displayed by epidermis is probably of evolutionary significance, for skin is continually subject to trauma and without an effective mechanism of quick repair of the outer structure of an individual, survival would be precarious. He suggested that skin might be regarded "as a holocrine gland whose main function is the elaboration of keratin". Some workers have maintained that only the cells of the basal layer exhibit mitotic activity, and that the number seen in division at any one time is not sufficient to replace cells lost on the surface. However, Pinkus (1954) has carefully examined this problem in human skin and concluded that the mechanism of mitotic division is in fact a sufficient explanation for epidermal regeneration. Cells of the

stratum spinosum as well as basal cells are also admitted by Pinkus to be capable of mitosis (fig. 23). A mitotic index as low as 0.17 would not be too low to account for the necessary physiological replacement. On the basis of colchicine studies, Storey & Leblond (1951) have observed the upward migration of the cells in the rat's epidermis. There has been no support for the contention of Andrew & Andrew (1949) that the mitotic figures seen in the epidermis are those of migrating lymphocytes and not of epidermal cells. Andreasen (1952) has stated that the fate of lymphocytes in epidermis is degeneration.

During healing, epithelial cells move out from the wound margin over the denuded surface. The initial movement involves especially the cells of the middle layers of epithelium, and not the basal layer (Forbus, 1952). Arey (1936) maintains that the chief factor responsible for the extension of epithelium over the denuded area is the amoeboid movement of the cells, an explanation first advocated by Klebs (1874). Epithelial cells can produce pseudopodia (Forbus, 1952), and the mere lessening of lateral

pressure that ensues following a solution of continuity no doubt assists in the outward flow of cells (Wright, 1951). The active movement of epidermal cells at a wound edge may be due to two other mechanisms. The first is active migration in response to a chemotactic stimulus emanating from the site of injury (Wigglesworth, 1937); the second is known as thigmotaxis, a phenomenon wherein certain cells, while maintaining contact with one another, apply themselves as closely as possible to adjacent flat surfaces and thus gradually spread over them (Harris, 1954). Forbus (1952) mentions a further possibility, namely phagocytic activity on the part of epithelial cells. Such cells are not normally phagocytic but he states that reproducing cells are, and that when reproducing "certain changes take place in their structure, particularly the surface, which make them sticky. This always accompanies an increase in the fluidity of the cytoplasm. Both these changes, that is, changes in surface tension and fluidity of cytoplasm, surely must favour the process of migration of epithelial cells when tissue equilibrium has become destroyed through destruction of cells".

Cells initially move outwards from the wound margin as a thin single layer (Pinkus, 1954) and stratification is said to be restored only after the mobilised epidermal cells have covered the defect. However, in the present series of wounds, stratification appears to begin before the whole lesion has become covered by a single layer of cells, since the periphery of the wound floor at about the fifth day may be covered with cells several layers thick, while the central region of the wound remained unepithelialised. The migrating cells and those at the wound margin lose the characteristics typical of the normal layer pattern and become considerably hypertrophied, with enlarged vesicular nuclei and enlarged nucleoli. They may even become columnar (fig. 8), and intercellular bridges are often very conspicuous in these hypertrophied cells (Gillman & Penn, 1956). The "tooth root" appearance of epithelium often seen at the wound margin (fig. 5) may be due not to cells growing down into underlying granulation tissue, but to a modification (modulation) of hair follicles adjacent to the wound. The follicles lose their normal structure and their cells contribute to the

epithelialisation process. Sebaceous glands in this region are said to disappear (Argyris, 1956, in the mouse) but Pepper (1954) in a comparable study in the guinea-pig does not mention this.

The absence of increased mitotic activity in the early stages of wound healing is held to be one of the characteristic features of regenerating epithelium. In his extensive review in 1936, Arey states: "Modern investigators agree in failing to find a significant increase in cell division during the earlier stages of epithelial repair when it might theoretically explain the prompt, initial ingrowth over the denuded area". In normal epidermis near the wound margin, mitosis is said to subside or disappear for a period of up to 4 days and then begins again in the cells of the basal layer and of the lower layers of the stratum spinosum. Increased mitotic activity is not seen in regenerating epidermis until several days after the lesion has been made, and even when an increase does occur it is found in the normal epithelium some distance from the margin itself (Loeb, 1920; Hartwell, 1929; Arey, 1936; Bishop, 1945).

In a study of the healing of surgical wounds in man, Hartwell (1929) first noted an increase in the number of mitotic figures on the fifth day. However, recent evidence indicates that increased mitosis in human skin may occur earlier (Gillman et al., 1955a). Cells that have migrated over the wound floor do not show mitosis until several days after they have become firmly fixed to the underlying tissue (Ivy, Grossman & Bachrach, 1952), and this lack of proliferative activity among migrating cells has also been regarded as characteristic of regenerating epithelia.

In the hypertrophic epithelium, intercellular bridges are particularly well marked. A number of authorities, e.g. Maximow & Bloom (1952), and Odland (1954), mention these bridges as being a normal feature of epidermis, and that tonofibrils pass through them from one cell to another, while Albertini(1953) considers that their presence in hyperplastic skin is due to the cells being separated from each other by intercellular fluid. However, Medawar (1953) comments that: ".... it is an orthodox and long-standing belief .... that tonofibrils pass without interruption from cell to cell ....

the intercellular bridges are in fact intracellular structures formed by the withdrawal of cytoplasm from the surface of the cell". He cites the evidence of Pease (1951) who studied skin with the electron microscope.

The time taken for complete epithelialisation clearly depends on the size of the original lesion. In the present series, wounds 10 days old were all covered by epithelium several layers thick.

During the healing of skin wounds in the cat there is some increase in alkaline phosphatase activity in the stratum granulosum. The phosphatase appears to be localised in the keratohyalin granules, an association that has been observed in man (Fisher & Glick, 1947; Pirila & Eranko, 1950), guinea-pig (Bourne, 1944) , rat (Washburn, 1955) and mouse (Hardy, 1952), although Kopf (1957) has claimed that in human skin false positive results are sometimes given by keratohyalin granules. Its presence in this site suggests a relationship between fibrous protein synthesis and alkaline phosphatase activity. This matter is discussed in extenso later (Chapter X). The fact that the stainability of the

granules of the stratum granulosum with basic dyes is abolished following treatment with ribonuclease (Leuchtenberger & Lund, 1951) has led to the belief that the granules contain a high concentration of RNA, which further suggests protein synthesis related to keratinisation. Recent studies by Moberger & De (1955), using x-ray and ultraviolet microspectrophotometry, have shown that the concentration of RNA is much greater in spinous than in granular cells, and they consider that this finding does not support the concept of protein synthesis in the presence of a high RNA content.

The presence of glycogen in skin was noted by Claude Bernard nearly 100 years ago (Bernard, 1859). However, it is not normally detectable histochemically in the skin of mammals, although small amounts may sometimes be revealed in human skin (e.g. Mancini, 1948; Bolliger & McDonald, 1949). During wound healing glycogen has been demonstrated in the hypertrophic epidermal cells in the guinea-pig (Bunting & White, 1950; Bradfield, 1951), rat (Washburn, 1954a) and man (Gillman et al., 1956), and also in the outgrowing cells of human skin autografts and homografts (Scothorne & Scothorne, 1953;

Scothorne & Tough, 1952). The accummulation seen in the regenerating epidermis of the cat is comparable, including the constant and striking absence of glycogen from the basal layer of the epithelium. This matter is discussed further in Chapter X, in the light of the findings in other organs.

The changes in the cytoplasmic RNA are also comparable with those described in other species. The increase in the cells at the ulcer margin is in agreement with the increase found in the growth, as opposed to the migration, phase in healing rat skin (Washburn, 1954b). The work of Caspersson (1950) and Brachet (1950) established the close correlation between the RNA content of a tissue and protein synthesis, and Clement (1944) in the mouse and Firket (1951) in the guinea-pig have demonstrated the expected increase in RNA in proliferating epidermis.

The changes that take place in the subepithelial tissues during the healing of wounds of the type here studied are essentially those involved in the formation and maturation of granulation tissue. Accompanying a variable degree of infiltration by inflammatory cells, there is proliferation of

connective tissue elements resulting in the formation of young fibrous tissue cells, blood vessels, reticulum and collagen fibres, all embedded in intercellular substance that is PAS-positive and metachromatic, indicating the presence of mucopolysaccharides. The origin of new connective tissue cells is still a matter for debate and further investigation (see Chapter X).

A number of workers, e.g. Sylven (1941) have shown that mast cells disappear locally in acute inflammation, and that they reappear with the onset of fibroplasia. Although these cells have been considered to play some part in the formation of new connective tissue (Asboe-Hansen, 1954), their absence from the wound area until after new fibrous tissue is becoming established does not seem to favour this hypothesis. This matter is also discussed later.

In the present experiments on the cat, it has been noted that there was no alkaline phosphatase activity in the young connective tissue. This is in direct contrast to most current views on the relationship between phosphatase and newly forming connective tissue. A number of workers have

provided evidence that phosphatase is concerned with the elaboration of protein, particularly fibrillar protein. For example, Fell et al. (1943) showed that collagen formation in healing wounds of rat skin is associated with an increase in the phosphatase content of the wound. Danielli, Fell & Kodicek (1945) also demonstrated that in vitamin C deficiency there was a close correlation between the degree of vitamin C deficiency, the rate of collagen formation and the level of alkaline phosphatase. On the other hand Robertson, Dunihue & Novikoff (1950) noted the absence of phosphatase in the new connective tissue associated with cellophane perinephritis. These conflicting views on a fundamental metabolic problem will be discussed further in the light of the subsequent findings during healing in the hollow viscera (Chapter X).

CHAPTER III

MUCOSAL REPAIR IN THE OESOPHAGUS

The early experimental studies on the healing potentialities of the oesophagus have been carefully documented by Saint (1929). The increasing interest in the possibilities of oesophageal surgery in man was dependent upon advances in general surgical and anaesthetic techniques. Since surgeons were primarily interested in the longer term results, the very early changes that occurred following interference with oesophageal tissues were not closely studied; it was sufficient to know that after a month or so a wound showed evidence that reparative processes had been taking place. Friedenwald, Feldman & Zinn (1928) studied both microscopically and radiologically the later stages of varying degrees of ulceration in dogs. Among their findings was the significant fact that the repeated introduction of 10% HCl into the oesophagus caused acute lesions to become chronic. The end-to-end anastomosis of cut ends was studied in dogs by Saint &

Mann (1929). Good epithelial repair is illustrated by their figures 8 and 9, taken 79 and 145 days after wounding. Malm (1951) made long term observations on cardioplasty in the surgical treatment of achalasia of the oesophagus, again using dogs, but again no reference was made to the early changes in the epithelium or connective tissue. The most recent experimental work on oesophageal healing appears to be that of Picard, Henry, Cotte & Inglesakis (1956), but their interest lay in the repair of muscular tissue. Schumacher (1927) and Lenkeit (1931) made no reference to regeneration in this organ.

Clinical observations on oesophageal ulceration (e.g. Jackson, 1929; Allison, 1948) while of significance in their own sphere, cannot contribute to histological knowledge, but Lodge's (1955) paper on oesophagitis contained evidence of repair processes, e.g. her figure 9. The recent monographs on oesophageal disease in man by Turner (1946), Franklin (1952), Palmer (1952) and Thorek (1952) make no reference to experimental studies.

From this brief review of the relevant literature,

it is clear that the behaviour of oesophageal epithelium and connective tissue in the first few days after wounding is not known, and the experiments now to be described were designed to make a contribution to this field.

MATERIALS AND METHODS

A total of 31 adult cats was used. Under anaesthesia with intraperitoneal nembutal, the lower end of the oesophagus (i.e. the abdominal part) was approached through an upper abdominal midline incision. A stay suture was inserted proximal to the cardio-oesophageal junction; the oesophagus was then pulled caudally using gentle traction, and opened below the diaphragm by a longitudinal incision in its ventral wall. A piece of mucous membrane about 0.5 sq. cm. in size was then removed from that part of the oesophagus immediately proximal to the incision. The oesophageal wound was closed by a single continuous catgut suture that did not incorporate the mucosa, and the abdomen

closed in layers. After operation the animals were allowed to survive for periods ranging from 6 hours to 1 year. During the immediate post-operative period, the animals were fed with water alone on the first day and milk alone on the second and third days; normal feeding was then resumed.

After sacrificing the animals, the oesophagus was opened and the site of the lesion, together with some undisturbed surrounding tissue, was excised. This tissue was fixed and treated histogically and histochemically in the same manner as the skin lesions (page 9).

RESULTS

The normal oesophageal mucosa

As in other regions of the alimentary tract, the mucous membrane of the oesophagus consists of epithelium resting on a lamina propria, beneath which lies a muscularis mucosae that is thicker in the oesophagus than in any other region of the gut. Submucous connective tissue separates

the muscularis mucosae from the inner layer of muscle of the oesophageal wall (fig. 24).

The epithelium in the lower end of the cat's oesophagus is of the stratified squamous variety in which there is no keratinisation of the surface cells. Sections stained with haematoxylin and eosin (fig. 25) indicate that this epithelium consists of 3 zones of cells, viz. basal, intermediate and superficial. The basal zone consists of 2 to 3 rows of small polygonal cells whose nuclei stain densely. The intermediate zone varies considerably in thickness, consisting of many layers of cells where the epithelium protrudes into the lamina propria, and is formed by large polyhedral cells with faintly staining cytoplasm and nuclei. The superficial zone comprises several layers of flattened cells whose nuclei appear pyknotic. This differentiation of the epithelium into 3 zones can be further demonstrated following histochemical procedures. A reaction for alkaline phosphatase, using the Gomori technique with short incubation periods of 15 to 30 minutes, was confined to the superficial and basal zones, the intermediate zone being invariably negative (fig. 26). This appearance

was similar with the azo dye method (fig. 27), and from this the impression was formed that the reaction was confined to the cell membrane, or intercellular substance, particularly in the basal zone. Staining with toluidine blue again emphasised the division into the 3 zones by the fact that they stained with different intensities (fig. 28). In addition to demonstrating the zoning of the epithelium, toluidine blue (and azure A) revealed the presence of a large number of mast cells. These were found not only in the lamina propria, where they were usually associated with blood vessels, but also in the basal zone of the epithelium (figs. 29 & 30). In no specimen were any mast cells seen in the intermediate or superficial zones. They varied considerably in size and in the extent to which their cytoplasm was filled with metachromatic granules. From the study of the present series it appeared that the number and position of the mast cells in the normal epithelium of the lower part of the oesophagus varied little from one cat to another.

Sections treated by the PAS technique demonstrated that the normal epithelium did not contain any histochemically

detectable glycogen. PAS-positive material other than glycogen was concentrated in the superficial and intermediate zones, and appeared to be intercellular (fig. 31).

When stained by the methyl green pyronin technique for RNA, the normal epithelium gave a strongly positive reaction in the intermediate zone, a faintly positive reaction in the superficial zone and no reaction in the basal zone (fig. 32).

The Healing Wound

Six hours after operation the site of the lesion could be recognised macroscopically by the absence of mucosa and the presence of blood and fibrin clot. During subsequent days reddish granulation tissue was seen, and by the end of 2 or 3 weeks a white, slightly puckered area offered naked-eye evidence of the region of operation. The sites of lesions after 3 and 6 months and 1 year could only be recognised microscopically after sectioning what was considered to be the area concerned.

Histological sections of a 6 hour wound (fig. 33) revealed that the floor of the lesion consisted of the

submucosa and the underlying muscularis externa. The submucous blood vessels were congested and there was some inflammatory exudate on the surface. The margins of the ulcer were formed by the cut edges of the mucosa. The epithelium and lamina propria at the edge tended to fall towards the floor of the wound (fig. 34), thus covering up the cut edge of the muscularis mucosae which may have assisted this process by contracting.

Epithelial Repair

Within 24 hours epithelial cells had begun to spread or migrate from the wound margin over the floor of the lesion. The migrating cells were several layers thick and no longer displayed the normal zoning. By the end of 2 days this migration had increased and there was considerable mitotic activity in the surrounding epithelium. Any high power field of epithelium adjacent to the ulcer margin showed several nuclei in different stages of mitosis (fig. 35), a state in striking contrast to the normal epithelium where very few mitotic figures were seen.

The cells of the spreading epithelium and those at

the margin of the wound were considerably larger than normal, a feature particularly noticeable when normal basal cells were compared with those that now formed the basal layer of the spreading epithelium (fig. 36). Mast cells were never found among the migrating cells.

From the third to the tenth day the process of epithelial migration accompanied by mitotic activity continued. During this period the epithelium became infiltrated by polymorphonuclear leucocytes and lymphocytes. These cells were concentrated chiefly in the upper layers and many were found in spaces that made their appearance during this time in these regions (figs. 36 & 37). The spaces, in which white cells were seen, varied greatly in size and gave a degenerative appearance to the epithelium. They were never seen in the basal layers.

The increased mitotic activity appeared to be confined to the epithelium at the margin of the wound and to the adjacent undisturbed epithelium; only 1 mitotic figure was observed in migrating epithelium (fig. 38).

By the tenth day, the ulcer floor had become completely

epithelialised (fig. 39) and the increased mitotic activity had now declined. At this stage the cells were still considerably larger than normal, and in the more superficial regions some inflammatory cells and spaces were still found (fig. 40).

Subsequent stages in the epithelial repair consisted of a thinning of epithelium due to a decrease in the size of the cells, and a return to the zoning seen in the normal epithelium, with an absence of infiltration. The normal pattern had been re-established within 1 month (fig. 41), and by this time mast cells were again noted in the basal layer of the epithelium now overlying the original wound area.

The normal distribution of alkaline phosphatase was not seen in regenerating epithelium; the enzyme was completely absent from the migrating cells, even up to the time of complete epithelialisation (fig. 42). However, when the stratified pattern of the epithelium was becoming restored (during the second week), a reaction for the enzyme occurred in the superficial zone (fig. 43). From the third week onwards, alkaline phosphatase could be demonstrated in the basal region

also; thus the reaction pattern seen in undisturbed epithelium had now returned (fig. 44).

Alterations in the distribution of PAS-positive material were also noted in the regenerating epithelium. The hypertrophic cells at the wound margins, and those that were migrating, contained abundant quantities of glycogen, with the notable exception of cells that formed the most basal layer, which never contained this substance (figs. 45 & 46). The PAS-positive material (other than glycogen) that was a prominent feature of the superficial zone of undisturbed epithelium, was no longer present in cells at the margin, nor was it found in those that were spreading over the floor of the wound (fig. 47). Following complete epithelialisation and the re-appearance of stratification , glycogen could no longer be detected histochemically but non-specific muco-polysaccharide could be demonstrated again in the superficial zone (fig. 48).

With the methyl green pyronin stain there did not appear to be any significant increase in pyroninophilia in the regenerating cells. Nuclei in mitosis exhibited increased

Feulgen staining. Staining with toluidine blue revealed that the migrating cells reacted less intensely (fig. 37), but the normal pattern was seen again when the zones had become restored (fig. 49).

Connective Tissue Reactions

The changes that occurred in the floor of the lesion indicated the accummulation and maturation of granulation tissue, together with a considerable degree of infiltration with inflammatory cells. Mitotic activity in connective tissue cells was first noted on the second post-operative day, and was most marked by the fourth or fifth day after which it gradually declined (fig. 50). Metachromasia of the ground substance in the wound area became prominent by the end of the first week, was still present at the time of complete epithelialisation, and thereafter disappeared. Mast cells were absent from the wound site until about the end of 1 month, when a few could be detected in the vicinity of blood vessels.

At all stages of the healing process there was a complete absence of alkaline phosphatase in the newly forming connective tissue (fig. 51). This was so even after prolonged

incubation periods of up to 24 hours, when all the tissue elements, including dividing fibroblasts, young blood vessels and fibrous extracellular material, still gave a negative reaction.

After a number of months some wound areas remained relatively flat (fig. 52) while others developed a convoluted pattern more closely resembling the architecture of normal mucosa and submucosa (fig. 53). In all wounds the muscularis mucosae showed no evidence of regeneration even after 1 year (fig. 54) and the cut edges remained to give some indication of the margin of the original lesion (figs. 53 & 54).

DISCUSSION

The results show that, following repair, the site of a small oesophageal wound eventually bears a close resemblance to the normal mucosa, the muscularis mucosae being the only tissue that shows no evidence of restoration.

The repair process in oesophageal epithelium resembles

that in epidermis. In both instances there is a migration of cells from the wound margin, with hypertrophy of the cells concerned, followed by increased mitotic activity in the surrounding epithelium. No convincing evidence of the presence of mitosis in migrating epithelial cells was found in the present study. The presence of only a single mitotic figure in what was presumed to be migrating epithelium, very near a wound margin (fig. 38), can hardly be advanced as evidence of the constant occurrence of such a phenomenon, especially in view of the findings to be described later in other organs. The infiltration of new oesophageal epithelium by inflammatory cells and the presence of spaces which lend to it a degenerative appearance are phenomena not shared with epidermis, but which may be due to the relatively septic environment of the upper part of the alimentary tract compared with the skin. The fact that the inflammatory invasion was less pronounced in the basal layers of cells may indicate that the nutrition and metabolism of these basal cells is less disturbed than that of more superficial cells which are farther removed from the subepithelial tissue fluid, their probable source of nutriment.

The presence and distribution of glycogen in the regenerating epithelium is also comparable with the findings in hypertrophic epidermis, including the absence of glycogen from the basal layers of both types of epithelia. It appears that in the oesophagus there is a reciprocal relationship between the presence of glycogen and of other polysaccharides. When cells are migrating, the PAS-positive material normally present in the superficial zone of the epithelium disappears, and glycogen is then found in most of the enlarged cells; when the glycogen disappears, the PAS-positive material returns to the upper layers of cells. Wislocki, Fawcett & Dempsey (1951) consider that such PAS-positive material (other than glycogen) in squamous epithelia is concentrated in the intercellular spaces and on cell surfaces, and that its presence varies inversely with the amount of keratin in any given epithelium. Since the epithelium of the cat's oesophagus is non-keratinising the present finding of PAS-positive material in the superficial zone is in accord with the observations of these workers.

The finding of alkaline phosphatase in the deep and

superficial layers of the normal epithelium is unexpected. In epithelia, e.g. of the intestine and renal tubules, its presence has been correlated with absorptive processes, but the oesophageal lining is not regarded as a tissue through which the passage of solute normally occurs. No reason can be given for the absence of the enzyme among the migrating cells; a number of as yet unknown physico-chemical changes may be occurring in the cell and in the intercellular substance in order to allow cells the freedom to move, but it is not possible to say whether the absence of phosphatase is correlated with such changes.

The regularity with which large numbers of mast cells occur in the basal layer of normal oesophageal epithelium in the cat is very striking, but Mota, Ferri & Yoneda (1956), who investigated the distribution of mast cells in the digestive tract of laboratory animals (including the cat), do not mention them as being a feature of the epithelium. The presence of mast cells in epithelium is considered to be extremely rare (Asboe-Hansen, 1954), but Carranza & Cabrini (1955) have found them in human gingival epithelium. The function of mast

cells in such sites is unknown.

The reactions in the connective tissue of the floor of the lesion are typical of healing by granulation. Although mast cells have been considered to play some part in the formation of new connective tissue, particularly the metachromatic ground substance (Asboe-Hansen, 1954), their absence from the wound area, as was found in the skin wounds, and the apparent lack of any excessive concentration of them in the undisturbed tissues adjoining the periphery of the granulating area, does not support this hypothesis. In this respect the present work is in accord with the results reported by Taylor & Saunders (1957) who studied the fibrogenesis occurring around implants of gelatine sponge in rats; mast cells were again notably absent from the developing and maturing granulation tissue.

The absence of alkaline phosphatase from the newly forming and newly formed connective tissue is exactly comparable with the findings in the healing skin of the cat. It must be emphasised that this negative reaction was characteristic of all wounds, at whatever time after operation

they were examined, including those studied at daily intervals during the first week, when there was abundant evidence of proliferative activity among the connective tissue cells.

CHAPTER IV

MUCOSAL REPAIR IN THE STOMACH

Ever since the report, by Pavy in 1863, on the healing of experimental mucosal lesions of the stomach, a large number of investigators have devoted attention to this subject (see Ivy et al., 1952, for review of literature). The general picture of healing is one of accumulating granulation tissue in the floor of the defect with contraction, the outgrowth of epithelium from crypts at the wound margin, and the new formation of crypts and glands. According to Williams (1953), the new glands do not show any differentiation of their epithelial lining into chief and parietal cells (his observations being made on rabbits, rats and guinea-pigs), but Myhre (1956) in the fundus of the rat's stomach found chief and parietal cells to be present at wound sites after 21 days.

In the present series of studies, the wounds in the stomach of the cat were the last experiments to be performed,

but they are recorded at this stage in order that the lesions of the alimentary tract might be presented in anatomical sequence. In view of the reports by many investigators on the healing of gastric lesions, attention was focused only on certain histochemical points that were of specific interest in the light of the results obtained in other organs, and only 6 animals were used. Gunter (1950) has already reported on the morphological aspects of healing stomach wounds in the cat.

MATERIALS AND METHODS

Under nembutal anaesthesia, the abdomen was opened through an upper abdominal midline incision. The body of the stomach was identified and an incision made in its ventral wall. An area of mucosa approximately 1.0 sq. cm. in size was removed from the middle of the greater curvature, and the gastric and abdominal wounds closed. The animals were killed on the third, seventh and fifteenth days after operation.

The site of the wound was excised and cut in half through its centre; one piece was fixed in 80% alcohol and the other in Gendre's fluid. Freeze-substitution was used for fixing some specimens - the tissue was dropped into iso-pentane that had been cooled to -180°C. in liquid nitrogen, and after 10 minutes was transferred either to the alcohol or Gendre's fluid that had been cooled to approximately -45°C. by immersion in a mixture of solid carbon dioxide and diethyl oxalate. The tubes of fixative and tissue were left uncovered overnight and by the following morning had attained a temperature of about 0°C. They were then dehydrated and embedded in the usual manner.

After routine staining with haematoxylin and eosin, serial sections from regions of particular interest were stained for alkaline phosphatase and for polysaccharides (page 9).

RESULTS

Specimens of wounds 3 days old showed that at the

wound site the whole thickness of the mucosa, including the muscularis mucosae, had been removed. The defect was filling with granulation tissue that exhibited a considerable degree of leucocytic infiltration. Epithelium in continuity with crypts at the wound margin lay over the periphery of the floor of the lesion (fig. 55), and the cells at this stage were not columnar but cuboidal in shape. By the fifteenth day the wound was completely epithelialised by tall columnar cells, and depressions, that were presumably indicative of new crypt formation, were present over the wound area (fig. 56). No regenerative activity was seen in the muscularis mucosae.

Histochemical Reactions

The epithelium of the crypts and surface of the stomach gave no reaction for alkaline phosphatase either in undisturbed regions or when regenerating. In the normal stomach, subepithelial tissue, including that surrounding the glands, gave a strong reaction (fig. 57). In contrast to this stromal reaction in undisturbed regions, the structures in the floor of the wound were negative (fig. 57), although by the fifteenth day there was a stromal reaction in the immediate

subepithelial area (fig. 58). This, however, served to emphasise the negative reaction at deeper levels, where with haematoxylin and eosin staining maturing connective tissue elements were apparent (fig. 56).

With the PAS technique, the normal surface and crypt epithelium gave a strongly positive supranuclear reaction, demonstrating that a large number of mucin-secreting cells were present. In most specimens, glycogen was found in an infranuclear position (figs. 59 & 60). In the cuboidal cells that were migrating over the floor of a wound on the third day, glycogen and non-specific polysaccharide material were still present but had apparently decreased in quantity (fig. 61). In a few cells glycogen appeared to be absent. In the fully epithelialised wound at the fifteenth day, many of the tall columnar cells contained glycogen in the typical infranuclear position (fig. 62). There was no apparent difference in the amount of glycogen seen after fixation in Gendre's fluid compared with 80% alcohol. Although there may be a variation between one stomach and another, and between different parts of the same stomach, with regard to glycogen content, the impression

was formed that freeze-substituted material showed rather more glycogen than those fixed simply in ice-cold fluids.

DISCUSSION

The small number of lesions examined here affords evidence of the rapid restoration of mucosal continuity that can take place in the stomach. The rapidity of the closure of the defect is emphasised by the degree of repair seen after only 15 days in a lesion that was about 1 sq. cm. in size. The wound must have been considerably reduced in area by the cut edges of the mucosa approaching each other. Not only is epithelialisation complete by the fifteenth day but some new crypt formation is already apparent. Grant (1945) has drawn attention to the rapid migration of which gastric epithelium is capable in the coverage of very superficial erosions, and Gunter's (1950) biopsy wounds, which did not exceed 0.3 cm. in size, were fully epithelialised after 3 days. Such lesions in the experimental animal clearly bear little

relation to chronic peptic ulcer in man, but acute ulcers in the human stomach probably heal in a similar manner.

In their study of the regeneration of the gastric mucosa in the dog and cat, Longmire, Beal, Lipmann & Bishop (1952) found that in the dog the muscularis mucosae is firmly adherent to the underlying submucous layer, so that when attempting to strip off the whole thickness of the mucosa, the muscularis mucosae is invariably left behind. In the stomach of the cat, however, the plane of cleavage lies beneath the muscularis, as it does in the small intestine of both animals. In the dog, epithelialisation of a gastric lesion produced as above is assisted by the remains of the bases of glands lying on the muscularis mucosa, but in the cat epithelialisation can only occur by inward growth from the intact mucosa at the wound margins. The present experiments agree with the findings of Longmire and his associates as far as the cat is concerned. The absence of regeneration of the muscularis mucosae itself has been repeatedly observed both in animals and in man (Ivy et al., 1952).

The migrating epithelial cells show no very striking

histochemical changes. Alkaline phosphatse, as in the normal epithelium, remains absent, while glycogen and other PAS-positive material seem to be diminished in quantity; there is no accumulation of glycogen such as is found in regenerating epidermis or oesophageal epithelium. However, the absence of phosphatase from the maturing granulation tissue in the floor of the wound is comparable with the findings in the other organs so far studied.

CHAPTER V

MUCOSAL REPAIR IN THE SMALL INTESTINE

In their extensive review of wound healing in the intestinal tract, Ivy et al. (1952) state that "....the studies do not approach the accuracy and precision of those on the skin." Of the available papers on this problem, current knowledge of the mechanism of healing of ulcers of the small intestine is presented most concisely by Mann & Bollman (1932) and by Mann (1939). The general picture is one of accummulating granulation tissue in the floor of the defect, with epithelialisation proceeding inwards from the ulcer margin, and with the subsequent development of new crypts and villi. There would appear to be no significant differences between the modes of healing of acute ulcers in man and in those of the cat or dog (Hurst & Stewart, 1929).

In the course of a series of studies on Brunner's glands, Florey & Harding (1935) investigated the healing of

artificial defects of the duodenal mucosa of the cat. In these studies it was found that not only did complete epithelialisation of the lesion occur, but that new villi were formed. In this way an almost normal pattern of mucosal architecture was restored, with the exception of the muscularis mucosae which did not regenerate. These findings were contrary to views then current on the healing of intestinal lesions, which was commonly believed to be confined to a simple epithelial repair overlying scar tissue. Healed lesions of the small intestine in man(excluding the duodenum) are not commonly examined, because their presence is not suspected or their site not known. Brown & Sampson (1930), however, have cited evidence indicating that in certain cases of intestinal tuberulosis, especially where the ulceration has been superficial, a complete epithelial covering has been formed with new, though irregular gland formation. Commenting on the healing of duodenal ulcers in man, Maingot (1955) states that "an inferior mucous membrane, somewhat resembling the original but thinner and less convoluted is reproduced."

The mode of healing of mucosal lesions in the ileum

of the cat has been investigated previously (McMinn & Mitchell, 1954; McMinn, 1955). In those studies, only the morphological aspects were considered and no histochemical tests were performed. A smaller series of similar experiments has since been carried out, in which some histochemical reactions in the regenerating epithelium and connective tissue have been studied. These latter experiments will now be described, reference being made where necessary in the results and discussion to those results that have been published previously, in order that subsequently comparison can be made with the healing of lesions in the large intestine (Chapter VI).

MATERIALS AND METHODS

Twenty-three adult cats were used and were starved for 24 hours before operation. Under nembutal anaesthesia, a mucosal lesion was created in the ileum, approximately 45 cm. proximal to the ileo-colic junction, by removing an area of mucous membrane about 0.5 - 1.0 sq. cm. in size. The whole

thickness of the mucosa, including the muscularis mucosae, was removed. The animals were starved for 24 hours after operation, and thereafter normal feeding was gradually resumed. A continuous Connell suture was used for closing the intestinal wound.

The cats were killed between 1 and 30 days after operation, the majority being sacrificed in the first 14 days; 1 animal was allowed to survive for 3 months. The sites of the lesions were removed and fixed, and the various histological and histochemical techniques that have been described previously (page 9) were carried out, including fixation in alcohol and Gendre's fluid after freeze-substitution (page 47).

RESULTS

Study of the wounds after haematoxylin and eosin staining revealed a pattern of healing similar to that already reported (McMinn et al., 1954; McMinn, 1955), and the results are only very briefly summarised here. By the end

of 14 days, the initial lesion (fig. 63) had become almost completely epithelialised (fig. 64), and granulation tissue was maturing in the floor of the wound (fig. 65). New crypts and villi appeared to be developing near the wound margins (fig. 66). After 3 months, the whole area was covered by crypts and villi, and although the crypts were a little less deep and the villi less closely packed than the normal, the site of the lesion bore a close resemblance to the surrounding undisturbed mucosa (fig. 67). The muscularis mucosae did not regenerate (fig. 68).

Epithelial Reactions

Within 24 hours of operation, epithelial cells had begun to move from the wound margin towards the centre. Such cells were often very much flattened (fig. 69) compared with the normal (figs. 70 & 71). After 2 days this very flat form of the cells was no longer seen, and the cells that from this time onwards lay over the periphery of the wound area were either cubical or columnar (figs. 72 & 73). In all the many sections examined, 2 mitotic figures were observed in spreading epithelium in a wound 4 days old (fig. 74).

The most notable histochemical reactions in the epithelium were found following the tests for alkaline phosphatase and PAS-positive material.

The epithelium of the villi normally gives a very intense reaction for alkaline phosphatase at the free ("brush") border of the cell (fig. 75), a reaction that can be demonstrated with incubation periods as short as 1 minute. In the specimen illustrated (fig. 75) the "bilaminar reaction" described by Johnson & Kugler (1953) is apparent. Some reaction can be found in crypt cells, even at the bases of crypts, but it is much less intense (fig. 76). Rather similar appearances are given after staining by the PAS technique - epithelium covering the villi shows a prominent border (fig. 77), while that lining the crypts gives a weak PAS-positive reaction at the luminal surface of the cell (fig. 78). Glycogen is not present in any epithelial cells.

When the migrating cells were examined for alkaline phosphatase, there was a completely negative reaction after an incubation period of 30 minutes (fig. 79). Even after 3 hours incubation, no typical reaction at the free borders of the cells

was seen; some staining of nucleoli and nuclear membranes that was now present may have been due to diffusion phenomena in view of the very intense reaction given by normal surrounding epithelium after what, for intestinal epithelium, was an excessively long incubation period (fig. 80). By the end of 1 week, columnar cells overlying the wound floor still gave no reaction (fig. 81), but during the second week a typical reaction at the striated border returned (fig. 82), and was seen at all later stages of healing.

The findings with the PAS technique were initially very similar. During the first few days the reaction for PAS-positive material other than glycogen was negative (figs. 83 & 74); but by the seventh day a weakly positive reaction was present again in the position of the striated border. It was also noted that at this time (the seventh day) a few of the migrating cells showed scanty perinuclear granules that were diastase - labile (figs. 84 & 85) and hence identified as glycogen. These granules were most clearly seen in the freeze-substituted material. By the end of 2 weeks, no glycogen could be detected and the PAS-positive

border stained with a normal intensity (fig. 86), remaining so during the rest of the healing process.

The results with the techniques used for the detection of cytoplasmic RNA were inconclusive.

Connective Tissue Reactions

Granulation tissue, with a considerable amount of round cell infiltration, developed in the floor of the lesion. Mitotic activity first became noticeable in connective tissue cells on the second day, and receded after the first week, by which time typical young fibroblasts and extracellular material were present. Metachromasia in this tissue was minimal, and contrasted with the oesophageal wounds where it was pronounced.

The maturing connective tissue again gave a completely negative reaction for alkaline phosphatase at all stages of repair (fig. 82), and at all incubation periods. The intense epithelial reaction that occurred with the longer incubation periods inevitably led to some local diffusion, but even after a 24 hour period, the general connective tissue reaction could still be pronounced completely negative (fig. 87).

Study of undisturbed tissues on the periphery of the wound area revealed that a positive reaction for phosphatase occurred in the blood vessels of the muscularis externa in the region immediately underlying the wound and in the adjoining regions on either side. The reaction faded away some little distance from the wound site (fig. 88). It became present on the third day and remained positive until the second week, when it gradually subsided (fig. 89). It should be pointed out that one specimen (fig. 90) showed a positive reaction in all the blood vessels, whether adjacent to the wound or well removed from it, but this was considered abnormal when compared with all the other specimens examined.

Some metachromasia was present during the first week. Mast cells were not found in the granulation tissue.

DISCUSSION

These results indicate that the histochemical reactions for alkaline phosphatase and PAS-positive material

are no longer present in the epithelial cells that migrate over the floor of a wound during the first week after the infliction of the injury. There is evidence of the accumulation of glycogen in these cells, and hence it would appear that the absence of phosphatase and the presence of glycogen may be regarded as criteria of "new" epithelial cells.

It is of interest now to compare the "new" epithelial cells of a healing wound with normal crypt and villous cells. In the crypts of Lieberkühn, mitotic figures are numerous, and evidence has been presented to indicate that the crypt cells move upwards from the crypts (Friedman, 1945; Leblond, Stevens & Bogoroch, 1948) along the sides of the villi, from the tips of which they are eventually shed (Leblond & Stevens, 1948; McMinn, 1954). The crypt cells may be regarded as a self-replenishing reservoir that is constantly delivering cells to the villous group. Since it has been calculated that, in the cat, the epithelium of the villi is completely renewed in less than 3 days (McMinn, 1954), it follows that such villous cells are as "new" as those that lie over the periphery of a lesion 3 days old or less. However, normal villous cells

always give a positive reaction for phosphatase and polysaccharides at the brush border and never contain glycogen, and it would thus appear that mere youth of cells is not a factor concerned in the accumulation of glycogen or the disappearance of phosphatase. There is probably a considerable metabolic disturbance in the cells that are passing over a floor of developing granulation tissue, since their nutrition in such an abnormal environment can hardly be as adequate as that of the cells passing along the side of a normal villus.

Statements are commonly made to the effect that absorption from the intestine occurs only through the cells of the villi, and not through those of the crypts (e.g. Bockus, 1943). Furthermore, it has been postulated that the presence of phosphatase in the intestine (as in the proximal convoluted tubules of the kidney) is correlated with absorption of sugars and their phosphorylation. These two hypotheses cannot both be entirely correct; either absorption occurs through crypts as well as villi, or else phosphatase at the luminal border of the cell may be concerned with some function other than absorption. As in oesophageal epithelium and others, there

is still much to be learned about the possible significance of the presence of this enzyme.

The findings in the maturing connective tissue with regard to phosphatase are closely akin to the results in the other organs so far studied, and further comment on this matter will be left until later.

The small intestine is the only part of the alimentary tract in the present series of studies that has displayed a positive reaction for phosphatase in the blood vessels of its muscular wall during repair. Attention may be drawn to the distribution of this reaction. It is the small vessels in the vicinity of the wound - in the muscle below the floor and at the margins - that give a reaction, those that are farther removed being negative. While it is well established that many capillaries normally give a positive reaction for phosphatase, it appears that those in the ileum of the cat are normally negative. In seeking a specific reason for the positive reaction in the ulcer region, oedema is at once called to mind as a phenomenon with which it may be associated. If the presence of phosphatase in these

vessels is evidence of the transfer of solute from blood to extravascular tissues, the distribution of the reaction is in keeping with this concept. However, there is no proof for this, and the reason for the state of affairs illustrated in fig. 90, where all vessels are positive, is not known. Fodden (1955) found evidence of increased vascularity in the muscular layers underlying mucosal lesions in the human stomach, and it would have been interesting to know whether these vessels showed an increase of phosphatase. Presumably they did not, for there is no specific reference to phosphatase in those vessels; the Gomori technique was used and it did demonstrate the presence of phosphatase in other parts of the lesions. In the small number of gastric wounds studied here (Chapter IV), the vessels of the muscle coats did not give a positive reaction.

CHAPTER VI

MUCOSAL REPAIR IN THE RECTUM

It has already been noted that investigators of wound healing in the alimentary canal have carried out their experiments mainly in the gastro-duodenal region, in view of the possible relation of such work to the problem of peptic ulceration in man. Very little attention has been paid to repair processes at lower levels of the gastro-intestinal tract; histological investigations on this subject in the large intestine appear to have been carried out only by O'Connor (1954, 1956) and by Lumb & Protheroe (1955). Sircus (1956) studied the ulceration that ensued in portions of colon that had been implanted into the stomach wall in dogs, but his interest lay in the mechanism of ulcer production rather than ulcer healing.

The following experiments were carried out in order to study the repair of mucosal lesions in the rectum. Routine

histological studies were supplemented,as in the other organs, by histochemical observations on both the normal and regenerating tissues.

MATERIALS AND METHODS

A total of 34 animals was used. Under nembutal anaesthesia, a piece of mucous membrane about 0.5 sq. cm. in size was removed per anum from the rectum, at a site on the dorsal wall 2 cm. proximal to the muco-cutaneous junction. The mucosa is very mobile and can be readily picked up with forceps, the required area being cut off with scissors. The animals were allowed to survive after operation for periods ranging from 24 hours to 6 months.

After death by coal-gas poisoning, the site of the lesion was fixed and studied by the methods described previously, including freeze-substitution of some specimens, with fixation in Gendre's fluid.

RESULTS

Histological study of specimens from animals killed 24 hours and later after operation showed that at the wound site the whole thickness of the mucosa, including the muscularis mucosae, had been removed (figs. 91 & 92). The floor of the wound consisted of the inner layer of the muscularis externa, with some overlying submucosa and a variable amount of blood and fibrin clot. The epithelium and lamina propria at the margins had fallen towards the floor of the lesion, thus covering up the cut ends of the muscularis mucosae, which may have assisted the process by contracting. In some specimens a few epithelial cells were found to be overlying the very edge of the wound floor.

By the end of 2 days epithelial cells, in continuity with those of the undisturbed mucosa at the wound margins, were found covering the periphery of the floor (fig. 92). Many of the cells were very much flattened (cf. figs. 93 & 94). During the next few days, the spread or migration of epithelial cells towards the centre of the wound continued, but by the

fourth day the very flat type of cell was no longer seen; all were now cuboidal or low columnar (fig. 95). Among the many serial sections studied from lesions during the first few days after operation, 4 sections were found in which a single mitotic figure was seen among the spreading cells (fig. 96). It was notable that goblet cells were rarely present in the migrating epithelium.

In the floor of the wound there was evidence of proliferative activity in connective tissue cells from the second day onwards (fig. 97). This was not confined to regions near the surface of the wound; mitoses in connective tissue cells were also found in the septa between bundles of muscle fibres in that part of the muscularis externa that lay below the ulcer area (fig. 98). By the fourth day typical young fibroblasts could be recognised (fig. 95).

Wounds examined at the end of 1 week after operation revealed that the epithelium had migrated for about 1,000μ towards the centre of the lesion, and that in some places it dipped down into shallow depressions near the original wound margin (fig. 99). All the epithelial cells were now tall

columnar in form, and goblet cells were still rarely found (fig. 100). Beneath this new epithelium, and in unepithelialised areas in the centre of the wound, typical granulation tissue was apparent, as evidenced by the presence of young fibroblasts with well-marked cytoplasmic processes, and buds of capillary blood vessels. At this time in some specimens at the wound margins, a number of cyst-like spaces were found; they appeared to be the bases of crypts that had become dilated (fig. 101).

Between the second and fourth weeks, some lesions had become completely epithelialised, and showed over the entire wound area numerous shallow depressions (fig. 102), whose epithelium contained occasional mitotic figures (fig. 103) and some goblet cells (fig. 104). These goblets were not as numerous as in normal crypt epithelium. The granulation tissue had become organised and mitotic figures in connective tissue cells were almost absent. On the other hand, other wound sites at similar times after operation showed an exuberance of granulation tissue (figs. 105 & 106); shallow epithelialised depressions were not seen, and deep crypts, with

many goblet cells, abutted against the mound of granulation tissue, the surface of which was not covered by epithelium.

At the end of 3 and 6 months the whole wound area was covered with a mucosa that closely resembled the normal, but the muscularis mucosae was absent from the region of the wound. Some specimens showed strands of fibrous tissue that passed obliquely from the submucosa into the mucosa between groups of crypts (fig. 107). Others exhibited a rather shallow mucosa in which the crypts were a little less deep and less closely packed than normal (fig. 108). The cut edge of the muscularis mucosae remained to give some indication of the original margin of the wound.

Histochemical Reactions

The epithelium of crypts in the undisturbed mucosa gave a strong reaction for alkaline phosphatase at the striated borders of the cells (fig. 109). The reaction could be seen with incubation periods of only a few minutes, and was present even in the deepest parts of the crypts, although it was less intense here than at higher levels. Specimens examined within the first few days after operation showed that

the migrating epithelial cells gave a completely negative reaction for phosphatase (figs. 109 & 110). At the end of the first week the reaction in the new epithelium overlying the floor of the wound was still negative, even in the cells that lined the shallow depressions near the margins (fig. 111). However, during the second week phosphatase began to reappear (fig. 112) and by the end of the third week a reaction of normal intensity was present in all epithelial cells (fig. 113). This degree of phosphatase activity persisted at all subsequent stages of healing.

In the developing and maturing granulation tissue of the wound floor there was a complete absence of phosphatase at all stages of the healing process examined, including those early stages at which proliferative activity of connective tissue cells was observed (figs.111, 112 & 113). Even after prolonged incubation periods of 24 hours this new tissue was still negative. The staining of blood vessels in the muscular wall of the rectum near the wound site was negligible.

With the PAS technique, the epithelium of the crypts

in the normal rectum showed the presence of PAS-positive material in the position of the striated border (fig. 114). As with alkaline phosphatase, the reaction was still present in cells in the deepest parts of the crypts, though of diminished intensity (fig. 115). The material was not diastase-labile following digestion with saliva and hence was not glycogen, nor could the presence of glycogen be demonstrated in any other part of the epithelial cells. The spreading epithelium that was seen in wounds a few days old showed no PAS-positive material at the free borders of most of the cells (fig. 116), though the absence of PAS-positive material was not as complete as the absence of phosphatase. After 7 days a strong reaction had returned to the position of the striated border (fig. 117), and as in the normal rectum the material resisted digestion with saliva. At the same time, a number of small granules, infranuclear in position and diastase-labile and hence identified as glycogen, were revealed in the tall columnar cells of this spreading epithelium (figs. 118 & 119). Some of the cells lining the shallow depressions near the wound margin contained these

glycogen granules (fig. 120). It was not possible to demonstrate glycogen at any later stages but the PAS-positive border was seen in all epithelial cells throughout the remainder of the healing process.

No significant changes were observed in the RNA content of the regenerating epithelial cells. As in the ileum, metachromasia of the ground substance in the floor of the wound was minimal. Mast cells, normally present in small numbers in the submucosa, were absent from the wound area until after the third week.

DISCUSSION

The results indicate that an effective restoration of mucous membrane eventually occurs at the site of a mucosal lesion, but as in other regions of the alimentary tract the muscularis mucosae shows no evidence of restoration. The migration of epithelium from normal mucosa at the wound margins and the accumulation of granulation tissue in the

floor of the wound are phenomena that are typical of wound healing. The main features of interest that arise from the experiments are:

1) the problem of whether the mucosa is made good by the formation of new crypts as in the stomach and small intestine, or by a re-arrangement of "old" mucosa as a result of contraction exerted by the fibrous tissue that develops in the floor of the lesion;

2) the histochemical changes in the new epithelium during the first 2 or 3 weeks;

3) the absence of alkaline phosphatase from developing and maturing granulation tissue.

Concerning the problem of mucosal restoration, it is pertinent to compare the results of the present work with the observations made by O'Connor (1956) on mucosal repair in the large intestine of the mouse. He concluded that the gap in the mucosa was filled by fibrous tissue that contracted and so approximated the cut edges of the mucosa. In the rectum of the mouse, the outgrowth of epithelium from the wound margins never exceeded 600μ, and he found no evidence

of new gland formation.

From the present work on the cat, it could be suggested that the conditions shown in figs. 105, 106 & 107 are comparable with the findings in the mouse. On the other hand, those in figs. 102 & 108 might be cited as evidence of new crypt formation. It could still be argued that the shallow depressions seen for example in fig. 104 are merely old crypts that have been flattened and pulled towards the ulcer centre by the newly-forming fibrous tissue. However, the relative absence of goblet cells, abundant in normal crypts, does not support this contention. Furthermore, the histochemical findings offer evidence that new crypt formation does in fact occur, at least in some specimens, for the following reasons. During the first week "new" epithelium, i.e. epithelium that grows out from the wound margins over the periphery of the floor, gives a negative reaction for alkaline phosphatase, in contrast to the epithelium of undisturbed mucosa that is always strongly positive. By the end of 7 days new epithelium still gives a negative reaction for phosphatase but may now contain glycogen granules that are never present in undisturbed mucosa.

Thus the absence of phosphatase and the presence of glycogen appear to be criteria of "new" epithelial cells in the rectum, as was noted in the ileum (page 62). Such criteria are present in cells lining shallow depressions near the wound margins, and since "old" crypt epithelium always gives a positive reaction for phosphatase and never contains glycogen, the present histochemical findings support the concept of new crypt formation. It is not possible to assess to what extent fibrous tissue contraction may be assisting the closure of the defect. The fact that after the end of 2 to 3 weeks the histochemical reactions of the epithelium had returned to normal renders these tests of no value in determining whether new crypt formation occurred at later stages; deductions can then be made only from morphological appearances.

It should be noted that in those later specimens in which there are no shallow depressions near the ulcer margins (figs. 105 & 106), the granulation tissue is profuse and has reached the level of the tops of the normal crypts, or has even exceeded it. Where such depressions are present and

regarded as the precursors of new crypts, the granulation tissue is less exuberant (fig. 102). It seems possible that an end result, such as is illustrated in fig. 107, where there are fibrous tissue strands between the crypts, may occur when granulation tissue is abundant and subsequently contracting; the shallower type of mucosa illustrated in fig. 108 may be a later development of the pattern of healing seen in fig. 102, which with the histochemical evidence suggests new crypt formation. While either of these two postulated courses of events may occur, the factors that determine which of them takes place in any particular wound are unknown.

At higher levels of the alimentary tract, it would appear that new gland formation is the rule during the repair of mucosal lesions (see Chapters III, IV & V). Since O'Connor (1956) found no evidence of new crypt formation at any stage of healing in the rectum of the mouse, it would be interesting to know what healing process occurs in the stomach and small intestine of this animal. The somewhat variable findings in the cat's rectum, compared with the

constancy of the pattern of repair in other regions of the alimentary tract in this animal, indicate that the regenerative capacity of the rectum is less than at higher levels. It should be noted that Lumb et al. (1955) who studied repair following surgical trauma in the human rectum, considered that outgrowing epithelium was unlikely to form new crypts. They also studied histologically a large number of specimens taken from patients with ulcerative colitis, and considered that small zones of ulceration could be "healed and obliterated completely by fibrous tissue drawing together adjacent areas of mucosa." Elsewhere they state that "repair may be complete and the mucosa reconstituted", but they do not say how this may have occurred. Banks (1943), commenting on ulcers in amoebic dysentery, wrote that the lesions "tend to heal from the edges by ingrowing mucosa restoring the continuity of the normal mucosal surface", but did not venture an opinion on whether or not new crypts were formed. The pattern of mucosal healing in the upper part of the human alimentary tract (e.g. stomach and duodenum) has led to the belief that new gland formation does occur (Ivy et al., 1952),

findings that are similar to those in the experimental animal at comparable sites.

The presence of dilated crypts in a number of specimens at the wound margins is comparable with the findings in the small intestine (McMinn et al., 1954). They appear to arise from a blocking of the necks of the crypts and their presence here, as at the sites of gastro-enterostomy stomata (Rosenow & McDonald, 1944) and in colitis cystica (Goodall & Sinclair, 1957) is compatible with the belief that they are due to obstruction produced by a reparative process (Palmer, 1954).

In view of the fact that the rectum is primarily regarded as a storage organ not concerned with absorption, the presence of a strongly positive reaction for alkaline phosphatase at the striated border of the epithelial cells is surprising. Gomori (1952b),in his brief review of the histochemistry of the gastro-intestinal tract, mentions that phosphatase occurs in the small intestine, but makes no reference to the large bowel. Martin (1951), who studied the distribution of phosphatase in the large intestine of a number of laboratory animals, found that the reaction in the

cat was more pronounced than in the other species that were investigated, and that there was usually a correlation between the presence of phosphatase and the presence of a striated border (though this was not so in the rat where rectal epithelium does not contain phosphatase). No reason can be given for the complete disappearance of the reaction from the migrating cells in the early stages of healing, nor for the concomitant disappearance of PAS-positive material. However, it is noteworthy that the polysaccharide material becomes visible in the new epithelial cells several days before phosphatase can be demonstrated, as in the small intestine. The accumulation of glycogen granules, in epithelium wherein glycogen is not normally detectable histochemically, is comparable with the increase that has been described in some other organs, although the amount seen in the rectal cells is small compared with that in epidermal or oesophageal cells. As in the jejunum and ileum, the epithelium of the crypts of the large intestine normally displays a considerable amount of proliferative activity, but the newly formed cells that result from such mitoses never

contain glycogen, so that here again mere youth of cells would not appear to be a factor concerned in glycogen accumulation. It seems more likely that a nutritional disturbance occurs due to an abnormal environment, although as with the disappearance of phosphatase and PAS-positive material, no information is available concerning the nature of such changes.

No mitotic counts were carried out in the present study, but there did not appear to be any grossly recognisable increase in mitotic activity in epithelium at the wound margins, whereas most epithelia do show such an increase when regenerating. However, on the basis of studies with colchicine, it has been shown that in the ileum of the cat there is no significant increase in mitotic activity at the margin of a healing wound, and that the presence of a gap in continuity does not serve as a stimulus to increased cell division in the epithelium of that organ (McMinn et al., 1954; McMinn, 1954). It may be that the rectum is similar in this respect. The finding during the present investigation of 4 mitoses in a layer of spreading cells indicates that such

cells are capable of dividing.

As in the other organs so far studied, the outstanding feature of the maturing granulation tissue in the floor of the wound has been the complete absence of alkaline phosphatase at all stages of healing.

CHAPTER VII

MUCOSAL REPAIR IN THE URINARY BLADDER

From the point of view of histology and physiology, the bladder has been a neglected organ. Like the rectum, this viscus has been regarded merely as a receptacle for waste products. While a number of workers (e.g. Schiller, 1923; Folsom, O'Brien & Caldwell, 1940; Bohne, Osborn & Hettle, 1955) have demonstrated that the bladder as a whole possesses considerable powers of regeneration, the behaviour of transitional epithelium during repair had not been the subject of a specific study until McMinn & Johnson reported their observations in 1955 (see also McMinn, 1955). The most striking feature of their results was the fact that during regeneration the epithelium displayed a great increase in mitotic activity, not only in the undisturbed epithelium near and at the wound margins, but also in the spreading cells. Among the latter, in wounds 2 days old and throughout the first week or so after the creation

of the lesion, nuclei in all phases of the mitotic cycle were found. In sections from such specimens, there was no difficulty in finding in the migrating epithelium a number of mitotic figures, which were very much more numerous than has been noted in this presentation in the ileum and rectum. Further evidence that bladder epithelial cells were capable of division when migrating was provided by the results of a study of the behaviour of bladder mucosa when implanted into the sheath of the rectus abdominis muscle (Johnson & McMinn, 1955; McMinn, 1955). The implants invariably formed a cyst by the growth of epithelium from the margins of the graft, and in this outgrowing epithelium, numerous mitoses were found. Thus such findings in transitional epithelium in the cat proved to be an exception to the long-standing concept that an absence of mitotic activity was characteristic of epithelia when migrating (McMinn & Johnson, 1956).

In the previous study of the repair of artifical ulcers in the bladder only the morphological aspects were considered. A further series of experiments has since been carried out in order to ascertain some of the histochemical

changes that occur in the epithelium and connective during the repair process.

MATERIALS AND METHODS

The operation, which was carried out in 14 adult cats under nembutal anaesthesia, involved the removal of an area of bladder mucosa, approximately 0.5 sq. cm. in size, from the dorsal wall of the organ. The mucosa is very mobile, and the required area can be readily cut off with scissors after elevating it with forceps from the rest of the bladder wall. A single Connell suture sufficed to close the bladder wound. The animals were killed at intervals between the first and thirtieth days after operation.

The histological and histochemical techniques used were the same as those employed for the other organs, and included freeze-substitution of some specimens, with fixation in Gendre's fluid.

RESULTS

The normal epithelial cells of the urinary bladder gave a reaction for alkaline phosphatase that was rather patchy in distribution. When present, it was seen in all layers of cells with the exception of the most superficial layer which was invariably negative. The positive reaction appeared to be most prominent at the cell surfaces or in the intercellular substance. The longer incubation periods (3 hours or more) emphasised the positivity of the reaction and the negativity of the superficial layer, but a band of stromal tissue immediately underlying the epithelium was now strongly positive (fig. 121).

In the hypertrophic cells that were migrating over the floor of the wound, the phosphatase reaction was almost completely negative (fig. 122), but after complete epithelialisation when the cells had returned to their normal size, the positive reaction was again apparent (fig. 123).

Normal epithelial cells stained by the PAS technique revealed the presence of glycogen in all layers of cells except

the basal, in which glycogen was never found (figs. 124 & 125). Most specimens also showed a variable amount of PAS-positive material that resisted diastase digestion and that was concentrated in the upper and middle layers of cells; in some cases this material was abundant with very little accompanying glycogen (fig. 126).

In migrating cells, the distribution of glycogen was confined to a few scanty granules in some cells, including on occasions those cells that formed the basal layer (figs. 127 & 128). A great increase in glycogen accumulation, such as has been noted in the skin and oesophagus, was never found in the urinary bladder (cf. fig. 127 with figs. 12 & 45). PAS-positive material other than glycogen was still found in hypertrophic cells, but stained less intensely than in the normal cells (fig. 128) and was often present in the form of rounded granules that varied greatly in size. At completely epithelialised wound sites PAS-positive material and glycogen were noted in their normal distribution but only after the cells had returned to their normal size (fig. 129).

In the granulation tissue in the floor of the wound,

the most striking histochemical finding, as in the wounds of other organs, was the complete absence of alkaline phosphatase at all stages of the healing process examined. In normal subepithelial tissues, mast cells were scanty, and were absent from the wound area, where there was some metachromatic ground substance during the first week of repair.

DISCUSSION

A number of investigators, e.g. Mende & Chambers (1957) working with species other than the cat, have noted the presence of phosphatase, PAS-positive material and glycogen in transitional epithelium, and no doubt many other constituents of these epithelial cells will be revealed as further histochemical tests are performed. The reasons for the presence of such substances are as yet unknown, but it may well be that the bladder is far from being a passive receptacle. Nemoto (1954) claimed that Indian ink and fat could be absorbed by transitional epithelium, and further studies of

absorption by the bladder may prove of great interest. This would seem to be a suitable field for exploration by a technique such as Wiseman (1953) devised for investigating absorption through intestinal epithelium.

The changes that occur in the regenerating cells consist of the disappearance of phosphatase, and what may amount only to a re-distribution of PAS-positive material. The less intense staining of the material other than glycogen may be a dilution effect due to the large size of the cells. It would appear that there has been some decrease in the total amount of glycogen present. It is clear that there has been nothing like the dramatic accumulation of glycogen such as has been found to occur in regenerating epidermis or oesophageal epithelium.

If the phosphatase of transitional epithelium, like that of the oesophagus, is in fact primarily a constituent of the intercellular substance, its absence from the regenerating epithelium may be concerned with the mobility of the cells. Its absence from the maturing granulation tissue in the floor of the wound is exactly comparable with the findings in the other organs that have been studied.

CHAPTER VIII

MUCOSAL REPAIR IN THE GALL - BLADDER

Experimental investigations on the gall-bladder have dealt mainly with physiological and pathological problems. The physiologist and radiologist have combined to study the role of the gall-bladder in digestion, while the pathologist is well versed in the manifestations of disease processes. Although a certain amount of work has been carried out on the cytology of the epithelium, this tissue has not often been the subject of experimental study. There is general agreement that it secretes various substances, but controversy has been centred on the problem of whether it secretes or absorbs cholesterol. As a result of investigations on cholesterosis, Elman & Graham (1932) championed the cause of secretion, while Illingworth (1929) and Mackey (1937, 1941) have presented evidence that cholesterol is absorbed from material in the lumen of the organ. Experiments on

epithelial permeability have been carried out by Winkenwerder (1929) and Cooke (1932), while the mucous cells of the epithelium have been the subject of study by Ito & Nagahiro (1942) and Gallinaro (1947).

Wound healing in the gall-bladder does not appear to have been investigated previously, and it therefore seemed opportune to complete the present series of studies on hollow viscera in the cat by creating mucosal lesions in this viscus.

MATERIALS AND METHODS

A total of 25 cats was used. The animals were anaesthetised with intraperitoneal nembutal, and the abdomen opened through an upper midline incision. The gall-bladder, with 2 adjacent lobes of the liver, was delivered through the wound (fig. 130) and gauze packs were inserted. The wall of the gall-bladder was incised longitudinally and the fundus was invaginated by the finger-tip of the operator. Although the wall of this viscus in the cat is extremely thin, it was

found possible with the aid of a sharp scalpel to scrape away a rectanular area of mucosa approximately 0.5 sq. cm. in size. The visibility of the papillary ridges of the invaginating finger through what remained of the gall-bladder wall proved to be a useful indication of the completeness of the removal of the mucous membrane (fig. 131). Following this the viscus was closed using a single, continuous, catgut suture and the abdominal wall closed. The animals were allowed to survive for the following periods of time: 18 hours and 1, 2, 3, 4, 5, 6, 7, 14 and 30 days. They were then killed by coal gas poisoning and the gall-bladders removed for histological and histochemical study, using the techniques already described (page 9).

RESULTS

The earliest period studied was 18 hours after operation. When the gall-bladder was opened, the site of the lesion was identified by overlying blood clot.

Microscopically, the floor of the lesion was seen to consist of the muscularis and the underlying serosa, the epithelium and subepithelial connective tissue having been removed (fig. 132). The margins of the wound were formed by the undisturbed mucous membrane. There was no evidence of mitosis in the connective tissue or in the surrounding epithelium, but some oedema was present. The normal epithelium did not appear to possess a striated border (fig. 133). The appearances after 1 day were similar to those seen at 18 hours but there was now a striking increase in mitotic activity. This was apparent both in the epithelium near the wound margins (fig. 134) and in fibrous tissue cells in the floor (fig. 135). The activity in these two sites was especially noteworthy since in these tissues mitosis is seldom seen in the normal gall-bladder. It now became evident that epithelial cells were beginning to migrate towards the centre of the floor of the wound.

By the second and third days the spread of epithelial cells over the wound floor had become more apparent (fig. 136), and while in general the cells were of normal size and shape,

they were occasionally much flattened (cf. figs. 137 & 138). This period was further characterised by the fact that the cells taking part in the migration showed numerous mitotic figures (figs. 139, 140 & 141). All phases of the mitotic cycle were seen, and proliferative activity continued in the epithelium surrounding the ulcer. In the floor of the lesion the appearance of new blood vessels and active connective tissue indicated the formation of granulation tissue.

The processes that have been described continued until the sixth or seventh day, by which time the epithelium had grown and spread over the entire wound floor. Mitotic activity had now reverted to the normal low level. Due to the presence of large amounts of granulation tissue the floor was elevated above the level of the surrounding epithelium; its contour was flat, being devoid of the fingerlike processes seen in normal gall-bladder mucosa (fig. 142). No longer were flattened epithelial cells seen; all cells were now of the tall columnar variety (fig. 143).

In the later periods studied (2 and 4 weeks after

operation) the epithelial cells had reverted to the normal size. Owing to organisation of the granulation tissue, the elevation of the ulcer region had been lowered to that of the surrounding mucosa. Although there were slight projections of the mucous membrane, there was no reconstitution of the typical convoluted pattern of the mucosa by the end of 1 month (fig.144).

Histochemical Reactions

The epithelium, both in the undisturbed regions of the gall-bladder and at the wound sites, always gave a negative reaction for alkaline phosphatase even after prolonged incubation. This also applied to the loose subepithelial tissue that is found in the fingerlike processes of the normal gall-bladder mucosa. The deeper, more condensed connective tissue, however, showed a marked reaction for this enzyme (fig. 145), as did the blood vessels therein. In contrast to this strong reaction,which persisted in the marginal zone bordering the wound, the connective tissue in the floor of the wound itself was almost completely negative (fig. 145) apart from some positive blood vessels. This latter region

was that in which mitosis in fibroblasts was observed with haematoxylin and eosin staining. Some metachromatic ground substance was present.

When the epithelium in undisturbed regions was studied by the PAS technique, it was found that the cells contained scanty glycogen granules that were infranuclear in position (fig. 146). In addition the supranuclear cytoplasm usually gave a strong reaction that did not disappear following incubation with saliva (fig. 147). In contrast to this, the migrating epithelium and the large columnar cells that covered what had been the floor of the wound contained a relatively large amount of glycogen which was perinuclear and infranuclear in position (figs. 148 & 149). PAS-positive material other than glycogen was not so abundant in these large cells as in the normal.

In the study of nucleic acids with toluidine blue and methyl green pyronin there did not appear to be any significant change in the amounts of these substances in the regenerating epithelium or active connective tissue as compared with the normal surrounding tissues.

DISCUSSION

The results show that wound healing in the gall-bladder of the cat occurs in a rapid and effective manner. It follows the generally accepted pattern of ulcer healing, in that there is migration of epithelial cells from the wound margins, and a filling of the wound floor with granulation tissue, the latter to be followed by organisation. There are, however, a number of differences when compared with other tissues.

The incidence of mitosis in the epithelial cells is an interesting finding in view of its very early onset and its occurence in migrating cells as well as in the surrounding mucosa. In the gall-bladder of the guinea-pig, Jacoby (1953) noted a similar outbreak of mitotic activity that was well established within 24 hours of producing distension in the viscus as a result of ligating the common bile duct. He attributed this response to a functional hyperplasia. His findings, together with those of the present work on the cat, would seem to indicate that the early onset of increased

proliferative activity is an inherent characteristic of gall-bladder epithelium. None of the other epithelia studied in this presentation have displayed such an increase earlier than 2 days after operation.

The presence of dividing cells in spreading epithelium has already been noted in the urinary bladder (page 84), and a similar occurance in the viscus now under discussion supports the need for some revision of existing concepts with regard to mitosis in migrating epithelium.

The absence of a positive reaction for phosphatase in the epithelium of the gall-bladder of the cat emphasises the species differences that have been noted by others who have investigated the distribution of the enzyme in this site. Jacoby & Martin (1951) found a patchy distribution in the epithelium of the dog's gall-bladder, while that of the rabbit and guinea-pig was negative. Bourne (1944) had previously reported that the guinea-pig's gall-bladder did contain phosphatase in the free borders of the epithelial cells, and he postulated that this was the source of the biliary alkaline phosphatase of this species. Clearly the work of Jacoby et al.(1951)

cannot be made to subscribe to Bourne's theory. In view of the possible absorptive function of the epithelium, it is interesting to observe whether there is any correlation between the presence of phosphatase and the presence of a striated border. A well defined striated border can be found in the gall-bladder epithelium of man (Ferner, 1949; Ralph, 1950) and in the monkey (Ralph, 1950), mouse (Dalton, Kahler, Striebich & Lloyd, 1950) and dog (Jacoby et al., 1951), while Togari & Okada (1954) could not detect it in the newt Triturus. In the cat, neither phosphatase nor a striated border was present. Only in the dog can phosphatase be found associated with a striated border, and this suggests a functional significance which is further substantiated by the accumulation of fat droplets in the epithelium after fatty meals (Pfuhl, 1932). Thus the presence of a striated border, phosphatase and fat may all be related, a state of affairs that may have been foreseen unwittingly a hundred years ago by Virchow (1857) who noted, particularly in the dog, a structural resemblance between intestinal and gall-bladder epithelium. It must be borne in mind that the epithelium of the cat, rabbit, guinea-pig and newt has been

studied only with the bright field microscope, and that a definitive statement concerning the presence or absence of a striated border can only be made after further work with phase contrast and with the electron microscope.

The increased accumulation of glycogen in the regenerating epithelium follows the now familiar pattern seen in the skin and oesophagus. Concomitant with the increase of glycogen, there is a decrease in polysaccharide material other than glycogen. Thus this pattern of metabolic activity in the simple epithelium of the gall-bladder parallels that found in the stratified types in the skin and oesophagus.

Mitotic activity in the connective tissue cells of the wound floor is well established at a relatively early stage (within 24 hours). Jacoby (1955) found mitotic figures in the connective tissue cells of the gall-bladder wall 2 or 3 days after ligating the common bile duct in guinea-pigs. No reason can be given for the very early onset of the fibrous tissue activity recorded here, although the manner of production of the ulcer, involving a scraping

of the wall of the viscus, may have been more stimulating than that employed in creating excision ulcers in other organs. However, in this connection it is interesting to note that in their study of healing in the common bile duct of the dog, Douglass, Lounsbury, Cutter & Wetzel (1950) concluded that the amount of fibrosis that ensued during repair was excessive when compared with the amount occurring in intestinal wounds. They showed further that the amount could be diminished by diverting bile from contact with the wound area. In view of this latter finding, it would not appear that the early mitotic activity per se has a bearing on the subsequent excessive fibrosis. It is not known whether bile can stimulate mitosis in a wound. Despite the rapid epithelialisation of the denuded area, the site can still be easily recognised 1 month after operation, due to the lack of re-formation of the normal, convoluted mucosal pattern.

The absence of phosphatase from the newly forming connective tissue in the floor of the wound is once again apparent at all stages of healing.

CHAPTER IX

FURTHER EXPERIMENTS ON WOUND HEALING IN THE CAT, RAT AND GUINEA - PIG

It has already been mentioned (page 25) that the negative finding for alkaline phosphatase in the new connective tissue of skin wounds in the cat is at variance with the histochemical results of Fell et al. (1943), who showed that there was a considerable increase in the amount of alkaline phosphatase associated with maturing granulation tissue during the healing of skin wounds in the rat. They confirmed the increase biochemically. It seemed important, therefore, to compare the reactions of wounds in the rat and guinea-pig with those of wounds in the cat and the following experiments were carried out with this in view.

METHODS AND RESULTS

The methods and results are recorded here under a

single heading, since some of the experiments to be described are a logical follow-up of previous results.

Skin lesions in the rat and guinea-pig were produced as in the cat, a total of 15 being available for study. The animals were killed between the fifth and fifteenth days after operation, and the wound sites were dealt with in the manner already described (page 9).

The general appearance of sections of wounds from both the rat and guinea-pig closely resembled those of the cat. The lesions were fully epithelialised by the tenth day, and the underlying young connective tissue (fig. 150) could not be distinguished from that of the cat, when haematoxylin and eosin, reticulum or PAS staining was used. Examination of these wounds for phosphatase (Gomori technique) revealed that the new connective tissue gave an intense reaction for this enzyme (figs. 151 & 152). The positive reactions could be demonstrated with an incubation period as short as 7 minutes. The reaction was strictly localised to the regenerating area and on close examination was seen to be present in the fibroblasts, young collagenous tissue, blood

vessels and infiltrating cells. The reaction in the fibroblasts was mainly cytoplasmic. In the normal skin surrounding the lesion, all structures (apart from sebaceous glands, hair follicles and the stratum granulosum) were negative with periods of incubation up to 24 hours.

The positive findings obtained with the Gomori technique were confirmed in both species with the azo dye method.

Wounds representative of visceral lesions were made in 4 rats by removing small areas of mucosa from the rectum. The technique was similar to that used in the cat, though the wounds were only a few square millimetres in area. The animals were killed on the tenth or fifteenth day after operation, and the wound sites examined by the usual methods.

Histological examination of these rectal wounds revealed that a breach of mucosal continuity was present, filled with developing granulation tissue that gave a strongly positive reaction for phosphatase (fig. 153). The rectal epithelium in this species gives no phosphatase reaction (Martin, 1951).

One final experiment on the cat, involving only a single animal, is included in this Chapter in view of the known relationship between vitamin C and wound healing. It is well established that in healing wounds of scorbutic guinea-pigs, where collagen formation is retarded, little or no phosphatase is found (Bourne, 1944; Danielli et al., 1945; Bunting et al.,1950; Robertson & Schwartz, 1953). The cat is most unlikely to be deficient in vitamin C since, unlike man, other primates and the guinea-pig, it can synthesise its own requirements (Linton, 1950). It has recently been shown that primates and the guinea-pig are unable to convert L-gulonolactone to L-ascorbic acid, the last step in the biosynthesis of ascorbic acid from D-glucose (Burns, 1957). Despite the unlikelihood of C-deficiency, 4 skin wounds were made in a cat that had received 100 mgm. ascorbic acid by mouth daily for 2 days before operation and until it was killed on the tenth day. There appeared to be no significant difference in the histological (fig. 154) or histochemical picture of the wounds in this cat compared with those in all the other cats that had received no dietary supplement of the

vitamin. Phosphatase was still absent from the connective tissue of the wound site (fig. 155), and such an absence is therefore not due to avitaminosis C.

Thus, while in wounds of the cat the developing connective tissue has been consistently negative for alkaline phosphatase, the enzyme was always present in the rodent wounds examined.

In view of this striking difference between the cat and the rodents in the phosphatase reaction of connective tissue during repair, some additional experiments were carried out in order to compare cat and rat tissues with regard to their potential affinity for substances used in or formed during histochemical procedures.

Experiment I was designed to determine the affinity of cells in the rat and cat skin wounds for phosphatase itself. Sections of rat skin ulcers, in which the phosphatase had been inactivated either by immersion in water at 90°C. for 10 minutes or in Lugol's solution for 10 minutes, were placed in a buffered solution that contained either an homogenate of the mucosa of rat's duodenum (a rich source of the enzyme) or

commercial alkaline phosphatase, for 90 minutes. It was considered that any cellular element that had an affinity for phosphatase would take up the phosphatase and hence, during the subsequent performance of the histochemical test, would then give a positive reaction. After removal from the buffered solution, the sections were thoroughly washed in running water, and transferred to normal substrate for incubation for 3 hours. It was found that not only did the fibroblasts show an affinity for the enzyme, but so also did the epithelial cells (fig. 156). In both types of cell it was the nucleoli and nuclear membranes that gave the greatest intensity of reaction, rather than the cytoplasm.

When experiments of the same nature were carried out on sections of skin ulcers from the cat, the results were similar (fig. 157). Both fibroblasts and epithelial cells gave a positive reaction, chiefly nuclear. Thus the cat and rat tissues showed a comparable affinity for phosphatase.

A further series of experiments (II) also determined the affinity of both rat and cat tissue cells for phosphatase, the enzyme in this instance being made available through the

phenomenon of diffusion from tissue sites of high activity. Sections of rat skin wounds, or of rat kidney cortex (both of which contain abundant phosphatase), were superimposed on sections from rat skin wounds in which the "native" phosphatase had been inactivated (by immersion in hot water or Lugol's solution, as above). These combined sections were then incubated for periods up to 48 hours and taken through the rest of the histochemical technique in the usual manner. On examination it was noted that after 24 and 48 hours incubation, fibroblasts and epithelial cells, in that part of the inactivated section that was adjacent to the margin of the overlying section, gave a positive reaction (fig. 158). Thus there had been a diffusion of some of the products of reaction during the histochemical procedure, although with incubation periods of less than 24 hours diffusion was negligible.

Similar experiments were performed with sections of skin wounds of the cat, i.e. rat skin wound or rat kidney cortex sections were superimposed upon sections of cat skin wounds (inactivated as above in order to remove phosphatase

from sebaceous glands, hair follicles and the stratum granulosum). The results (fig. 159) appeared to be identical with those described above in the rat, showing again that the phosphatase affinity of both rat and cat tissues is similar.

A third series of experiments (III) determined the affinity of rat and cat tissues for calcium phosphate. Sections of rat and cat skin wounds, all of whose phosphatase had been inactivated as above, were incubated in normal substrate to which either hydrogen peroxide or commercial alkaline phosphatase had been added. Following incubation, the sections were thoroughly washed and the remainder of the histochemical technique performed. The hydrogen peroxide or commercial phosphatase that had been added to the incubation medium caused the formation of a precipitate of calcium phosphate in the solution. It was expected that any element having an affinity for calcium phosphate would adsorb it, and then when the sections were taken into cobalt nitrate and ammonium sulphide, a positive reaction would occur in those elements. Examination of the sections did reveal that in both species all cells, both of the connective tissue and of the epithelium,

had a considerable affinity for phosphate, and that the reaction, as in the experiments showing an affinity for phosphatase itself, was localised to nuclear membranes and nucleoli (fig. 160). Thus the calcium phosphate affinity of rat and cat tissues is similar.

DISCUSSION

During wound healing in the cat, no evidence of phosphatase activity, as demonstrated by histochemical tests, has been found in the maturing connective tissue in any of the organs studied. In contrast, fibroplasia in the skin of the rat and guinea-pig and in the rectum of the rat is accompanied by considerable enzymatic activity, which in the present experiments was very well visualised after 30 minutes incubation. Longer periods merely emphasised the positive result without increasing the area in which activity could be found.

Gold & Gould (1951) questioned the significance of the apparent relationship between high phosphatase activity

and fibrogenesis. They suggested that the positive reaction for phosphatase was due to the fibrous proteins at some stage in their formation adsorbing the enzyme from surrounding tissue fluids and concentrating it so that histochemical methods visualise it. Biochemical tests, such as were carried out by Fell et al. (1943) to confirm the increase that had been demonstrated histochemically, cannot of course determine whether the phosphatase present has been adsorbed, for example from tissue fluid (a possibility of which those authors were aware), or whether it is "native" to cells or fibres. It could be contended that if adsorption of the enzyme does occur, structures that are normally positive for phosphatase, such as sebaceous glands, would provide a readily available source of the enzyme. However, experiments I and II have demonstrated that both the fibroblasts and epithelial cells of the cat have an affinity for phosphatase equal to that of the fibroblasts and epithelial cells of the rat, so that these cells in both species would appear to be capable of adsorbing the enzyme. In the rat, a structure such as epidermis that is normally negative (apart from the

stratum granulosum) is still negative in the healing wound, despite the fact that the new connective tissue immediately underlying it is strongly positive. If in the rat wounds the phosphatase was present in maturing fibrous tissue as a result of adsorption, it would have been expected that the epithelial cells would also have shown a positive reaction. Furthermore, in the experiments demonstrating adsorption, the reaction was mainly nuclear and nucleolar; in the rat wounds it was essentially cytoplasmic. It would appear, therefore, that adsorption of phosphatase itself is not the reason for the positive reaction in rat wounds. Similarly, the staining in experiment III, demonstrating the adsorption of calcium phosphate, is also nuclear and rules out artefact due to calcium adsorption. These findings are confirmed by the results with the azo dye method, where calcium phosphate is not involved.

From the above observations, it may be concluded that the presence of phosphatase in the rat wounds is "true" and not due to adsorption in vivo or to artefact arising from the histochemical techinque. The difference between the

phosphatase reactions in the normal wounds of the cat and rat, when compared with the similarity between the cellular reactions of the tissues of the two species under the experimental conditions noted above, indicates that there is a true difference between the cat and the rat with regard to alkaline phosphatase activity in new connective tissue.

CHAPTER X

CONCLUSIONS

In commenting upon the results of the repair processes in the various organs that have been studied, the epithelial and connective tissue reactions will be considered separately. While this is convenient in the present circumstances, it does not deny the influence that epithelium may have on connective tissue or vice versa, influences that have been stressed by Gillman et al. (1953) and which in the case of skin may be of importance in the pathogenesis of cancer (Gillman et al., 1955 b).

EPITHELIAL REACTIONS

In all organs, epithelialisation has occurred by the outgrowth of cells in continuity with the undisturbed

epithelium at the wound margins. From time to time reports have appeared suggesting that metaplasia of stromal cells into the appropriate epithelial type can occur (Papanicolaou (1933), in the uterus; Levander (1950, 1953) in the skin and stomach). There has been no evidence of such metaplasia to assist surface coverage in any of the organs studied here, but Brunschwig & Robbins (1954) considered that the mesoblastic tissues underlying the peritoneum have the potentialities for re-forming peritoneum.

The changes that occur in the regenerating epidermis of the cat call for little to be added to what has already been said. In general they confirm the similarity with other species. Pinkus (1954) has stated that: "Usually a thin single layer of flat cells is formed ..... once the mobilised epidermal cells have covered the defect, the epidermis again becomes stratified." However, this did not always appear to be the case in the epidermis, and certainly in the oesophagus and urinary bladder it is not true to say that the whole lesion is first epithelialised by a single layer, with later stratification of this covering. The advancing edge

of the epithelium in these organs usually consisted of hypertrophic cells several layers thick, and no specimens, either of the oesophagus or bladder, could be said to conform to Pinkus' description, which appears to be the common one when reference is made to repair in the epidermis.

During the first or second day, the cells that advance over the floor of a lesion are flattened in form compared with the normal, and this feature of regenerating epithelia is very well exemplified by all the simple epithelia of this study (figs. 69, 94 & 137). Such an attempt to cover as much of a lesion as quickly as possible in the early stages of repair was regarded by earlier workers, e.g. Loeb (1920), as a minor factor contributing to wound closure.

The recent observations of Weiss & Matoltsy (1957) on skin healing in chick embryos are of great interest in view of the important role that is usually assigned to cell migration in the process of epithelial repair. They found that in wounds made before the twelfth day there was abundant mitotic activity that resulted in the heaping up of epithelial cells at the margin, but there was no migration over the wound

surface. Only in wounds made after the twelfth day did migration occur; thus the cells of the earlier embryos appeared to be incapable of migrating. The experiments of Hess (1954) dealt with the skin of foetal guinea-pigs, all wounds healing as in the adult, but there was little age variation in his series, and it remains to be seen whether the foetal cells of embryos of any other species apart from the chick exhibit a behaviour pattern that displays such a clear time relationship between mitotic activity and the onset of migration.

All specimens that were examined within 24 hours of the creation of a breach of continuity showed that a few epithelial cells had begun to migrate over the floor of the lesion, and such findings therefore differ from those of Howes (1943) who noted in regenerating epidermis a quiescent period of several days before epithelial migration occurred. Williams (1953), in gastric wounds of small laboratory animals, also found evidence of migration within 24 hours. In the light of these observations, the term "lag phase", that is often applied to the first few days of a healing wound, seems inappropriate.

The occurrence of mitosis in migrating epithelial cells in the urinary bladder and gall-bladder is at variance with the concept that has been perpetuated by Arey's (1936) review of wound healing (see page 20). The finding of occasional dividing cells in migrating epithelial sheets in the small and large intestines shows that the lining cells of these organs are also capable of proliferating at an early stage in the coverage of a lesion. Wilhelm (1953) found 2 mitotic figures in migrating sheets of regenerating tracheal epithelium in the rat (in animals that had received colchicine), though in a synopsis of Wilhelm's article Cameron (1955) leads one to believe that mitotic division in spreading cells was more frequent. In the light of these recent observations, it seems necessary to emphasise that the remarks in Arey's (1936) review, and those for example made more recently by Hadfield (1955), should refer to skin only, and should not be taken as generalisations that are applicable to epithelia in general. It is perhaps unfortunate that one of Arey's own articles (1932) was entitled "Certain basic principles of wound healing", since the experiments outlined

therein were performed on the skin of marine animals, whose tissues may show environmental adaptations (cf. Montagna & Harrison, 1957, on seal skin), and the references in Arey's article to other work deal only with skin.

In connection with the proliferative activity noted in the migrating epithelial cells of the urinary bladder and gall-bladder, it may be mentioned that these epithelia appear to be the only epithelia that are capable of inducing the formation of heterotopic bone. Huggins (1931) was the first to demonstrate the association of such bone formation with bladder mucosa, using dogs. Subsequent studies confirmed his observations in dogs (Regen & Wilkins, 1934; Huggins, McCarroll & Blocksom, 1936; Gomori, 1943; Abbott & Stephenson, 1945; Marshall & Spellman, 1954; Boyarsky & Duque, 1955), and the phenomenon has also been found to occur in rats (Huggins et al., 1936), guinea-pigs (Huggins et al., 1936; Gomori, 1943; Loewi, 1954; Kurokawa, 1955), rabbits (Huggins, 1931; Gomori, 1943) and cats (Johnson & McMinn, 1956). Gall-bladder mucosa has induced bone formation in the dog and guinea-pig (Huggins & Sammett, 1933). It remains to be seen

whether there is any correlation between the high degree of proliferative activity of transitional and gall-bladder epithelia and the induction of bone formation. There is no logical reason for supposing that there may be, but all attempts to explain the phenomenon have so far failed. Indeed, the "trigger" for osteogenesis at the sites of development of embryonic bones is also unknown. It should be noted that there are no reports confirming the work of Huggins et al., (1933) with gall-bladder mucosa. Work by the writer at present in progress on gall-bladder implants in cats has not yet resulted in bone formation (8 animals used to date).

The regenerating oesophageal epithelium presents features similar to those of skin, e.g. the changes in cell size and the accumulation of glycogen, though that of the urinary bladder, while showing cellular hypertrophy and a disappearance of phosphatase comparable with the findings in the oesophagus, does not display an increased accumulation of glycogen.

The oesophageal and transitional epithelia of the cat both possess non-specific polysaccharide material in the upper layers of cells. The distribution of this PAS-positive

material is distinctly different in the two types.In the oesophagus it appears to be intercellular, whereas in the bladder it is intracellular. In both types it disappears during regeneration. Moog & Wenger (1952) examined a number of tissues and claimed that "a mucopolysaccharide is generally concomitant with sites that are rich in alkaline phosphatase". It so happens that they studied neither oesophagus nor bladder. In the oesophagus, while there is phosphatase both in the superficial and deep layers, it is most pronounced in the deep, where there is no PAS-positive material. In the bladder, where PAS-positive material is prominent in the superficial layers, phosphatase is always absent from the topmost layer.

While there are reasons for correlating the presence of the alkaline phosphatase in the columnar epithelium of the intestine with the transport of organic molecules (Bradfield, 1950) the occurrence of this enzyme in the stratified epithelia of the oesophagus and urinary bladder has yet to be explained. It has been speculated here that its absence from the regenerating cells may be correlated with their mobility, but it must be

admitted that very little is known about the precise function of this ubiquitous enzyme in many parts of the body. In some sites, such as ossification centres, phosphatase is present in situations where transport is not apparently involved; its possible correlation with the manufacture of fibrillar protein is discussed below.

The disappearance of phosphatase from regenerating intestinal cells, with the concomitant disappearance of PAS-positive material, and then its re-appearance, is of interest when compared with developing embryonic tissue. In the gut of the chick embryo, polysaccharide material appears slightly before phosphatase (Moog et al., 1952), and in the regenerating cells of the small and large intestines observed here, the polysaccharide and the enzyme return in a similar sequence.

The absence of phosphatase and PAS-positive material from regenerating intestinal cells is coincident with the apparent absence of the striated border in sections examined with the light microscope after routine staining with haematoxylin and eosin. Granger & Baker (1950) studied

the striated border of normal intestinal cells with the electron microscope and found that it consisted of large numbers of narrow, finger-like (filiform) processes, possibly 3,000 to each cell. Such findings have been confirmed by Dalton et al. (1950) and others, and are comparable with the "microvilli" described by Boyd & Hughes (1954) in chorionic villi. The precise location of the histochemical reactions in the region of the striated border has not yet been correlated with the picture seen with the electron microscope. It would be of great interest to know what happens to the filiform processes in migrating epithelium, and an investigation into this problem using the electron microscope is planned as a future research project.

For the preservation of glycogen for histochemical studies, a number of workers, e.g. Culling (1957) and Hale (1957), recommend fixation in Gendre's fluid. In the work reported here, 80% alcohol appears to be as efficacious as Gendre's fluid for glycogen demonstration, but freeze-substitution is indicated when small amounts are to be detected.

The phenomenon of glycogen accumulation in the

regenerating epidermis of animals apart from the cat has already been noted (page 23). In the cat's oesophageal epithelium a similar increase occurs, and in both tissues the glycogen is always absent from the basal layer of cells. It has only been possible to find 2 reports of the presence of glycogen in adult basal epidermal cells. In the first Lobitz & Holyoke (1954) induced minor trauma in human skin by the "strip method" of Pinkus (1951), which involved the repeated application and stripping off of Scotch tape. This caused hypertrophy and hyperplasia of all layers of epidermal cells without producing a breach of continuity. Within 4 hours of carrying out this procedure, glycogen was found in the basal cells only, and had disappeared within 24 hours. The characteristic accumulation in the stratum spinosum began after 2 days. From these findings it is clear that the glycogen demonstrated in the stratum spinosum is not found there simply because basal cells containing glycogen had migrated into that stratum, since there was a period between 24 and 48 hours in which increased glycogen could not be demonstrated in any of the cells of either layer. Attempts

by the writer to reproduce these results in the cat, using the "strip method" and by scraping the epidermis in various ways, were not successful. Similar attempts in the oesophagus - by vigorous scraping of the mucosal surface - also revealed no glycogen accumulation after 6 hours. When Bourne (1956) stated that "many cells accumulate glycogen before entering into a phase of rapid multiplication and/or synthesis or differentiation (the cells of the basal layers of the skin are one example)", he was presumably referring to the paper of Lobitz et al. (1954) that has just been mentioned, although he does not say so.

Secondly, Washburn (1954a) was able to demonstrate glycogen in all the cells, including those that formed the basal layer, that were outgrowing from a culture explant of skin, but there was none in the explant itself. The glycogen that is normally present in transitional epithelium in the cat is found in the middle layers of cells, but in other animals, e.g. the rhesus monkey and goat, glycogen is abundant in all layers except the most superficial (recent personal observations).

There is no consistency in the pattern of distribution of glycogen in the normal epithelia, or in the pattern of glycogen accumulation in the regenerating epithelia, that have been studied here. Those of skin and the oesophagus, which rarely show any in histochemically detectable quantities under normal conditions, accumulate large amounts; those of the bladder and stomach, which normally contain glycogen, seem to lose most of it and certainly never accumulate quantities comparable with skin and oesophagus; that of the gall-bladder, which normally contains it, shows a considerable increase; and small amounts appear in regenerating ileal and rectal cells, wherein it is normally absent. Warbrick (1955) found none in the regenerating endometrium of the post-partum rat. It is curious to note that while developing osteoblasts in growing bone contain glycogen, the young osteoblasts that are found at the site of repair of a fracture contain none (Pritchard, 1956).

A number of theories have been advanced in an endeavour to explain the reason for glycogen accumulation in regenerating epidermis. Reduced carbohydrate utilisation with ensuing storage has been considered an unlikely cause by Scothorne et

al. (1953) in view of the "histological signs of increased cellular activity". Presumably "increased cellular activity" refers to the increase in size of the marginal and migrating cells of wounds and grafts; these cells (in skin) do not show increased mitotic activity. Against the theory of storage is the fact that there is little variation of epidermal glycogen with variations in blood glucose (Cornbleet, 1940). It has been shown by Montagna, Chase & Hamilton (1951) that glycogen accumulates where keratinisation is slowed, and in healing skin wounds glycogen disappears when keratinisation begins (cf. figs. 14 & 15). These facts suggest that glycogen may provide the energy for the synthesis of keratin from the RNA that is present in the stratum granulosum (Bradfield, 1951; Firket, 1951; Scothorne et al., 1953). It might have been expected that the maximum concentration of RNA would be found in this stratum but, as already mentioned (page 23), Moberger et al. (1955) have found that the concentration of RNA is in fact less in this layer than in the stratum spinosum. However, this does not alter the fact that glycogen may be providing energy for

metabolic processes, though it does imply that metabolism is altered and hence the glycogen is visualised histochemically, whereas under normal conditions the glycogen is either used up or manufactured at a rate that does not allow sufficient accumulation for histochemical detection. In support of this concept, Washburn (1954a) states that "the soft palate epithelium which does not keratinize contains glycogen while the keratinizing hard palate contains none". Studies by the writer that are at present in progress do not confirm the presence of glycogen in the soft palate (in the cat and rhesus monkey); it has already been shown that the cat's oesophageal epithelium, which closely resembles that of the soft palate, does not contain glycogen. The statement of Montagna, Chase & Lobitz (1952) that the stratified squamous epithelium of mucous membranes is "rich in glycogen" (and a similar statement by Montagna alone in 1952), is a misinterpretation of the article by Wislocki et al. (1951) to which the former authors refer. Wislocki and his colleagues studied vagina, gingiva, oesophagus, tongue, lip and skin from 2 rhesus monkeys, and vagina and skin from human subjects. The epithelium of

the vagina was indeed rich in glycogen, but the oesophagus contained "mere traces" and in the other epithelia "some glycogen" was present. Montagna et al. (1952) discovered that glycogen was present in hair follicle cells just after a phase of proliferation and mitotis, whereas the accumulation noted by Lobitz et al. (1954) suggested to them that the glycogen might be there to supply energy for the subsequent mitotic activity. Montagna et al. (1952) concluded that mitotically active cells use glycogen rapidly and do not accumulate it. The studies reported here on transitional epithelium were at first thought to support this latter concept; the normal, non-keratinising, mitotically inactive cells contain glycogen, whereas the regenerating and migrating cells that display considerable mitotic activity show little or no glycogen. However, regenerating gall-bladder epithelium, which is also capable of mitotic activity during migration, contains glycogen apparently in excess of its normal content.

The possibility that the presence of glycogen in skin is a degenerative phenomenon has been deduced by comparison with the accumulation that occurs in tumours (Ewing, 1942) and

in the pro-oestrus rodent vagina (Tribby, 1943), both of which phenomena may be due to reduced blood supply. In skin grafts, however, glycogen accumulation outlives the period of poor vascularity (Scothorne et al., 1953), and in culture only the proliferating and migrating cells contain glycogen (Washburn, 1954a). These facts indicate that environmental changes are not a likely explanation; rather there is synthetic activity by the cells themselves. The basal cells that Lobitz et al. (1954) found to contain glycogen after epidermal strippings were about to mitose, so that degeneration can hardly be advanced as a suitable explanation in this instance. In view of the normal, relatively short life of epithelial cells in the small intestine (McMinn, 1954), it might be argued that cells which were seen over a wound site after 1 week had outlived their normal life span of less than 3 days and might therefore be degenerating. However, there is no histological evidence for this.

Dempsey & Wislocki (1944) considered whether reduced oxygen tension might account for glycogen accumulation. They speculated that in the relative absence of oxygen or with

inadequate aerobic oxidative mechanisms, cells adopted anaerobic fermentative mechanisms. Previous workers had shown that epithelial cells normally require glycogen for both aerobic and anaerobic glycolysis (Dickens & Weil-Malherbe, 1943; Berenblum, Chain & Heatley, 1940). The anaerobic mechanisms are less efficient and require larger quantities of substrate. Bradfield (1951), who studied regenerating epidermis in the guinea-pig, thought that the new, thick epidermis was avascular and that its cells, being poorly supplied with glucose and oxygen, were required to adopt anaerobic mechanisms for the breakdown of sugar. It is well known that foetal epidermis, together with many other embryonic organs, contains histochemically detectable glycogen (Borghese, 1957), possibly due to relative intra-uterine anoxia. However, Scothorne et al. (1953) have pointed out that the source of the glycogen is unknown; presumably it is derived from blood glucose, and if tissues are relatively avascular it is difficult to see how they can accumulate large quantities of glycogen or why they do not utilise glucose directly. The basal cells of Lobitz et al. (1954)

are nearer their blood supply than those at more superficial levels, but yet they accumulate it.

It is clear that there is so far no satisfactory explanation for glycogen accumulation in epithelial cells. The present histochemical studies have demonstrated that there is a lack of uniformity in the behaviour of different regenerating epithelia with regard to the presence of glycogen, and the elucidation of the reasons for the differences probably requires further studies of a biochemical rather than a histochemical nature.

While the results of the Feulgen reaction for DNA in regenerating epithelia have usually shown the expected increased intensity of nuclear staining in mitosis, the results for RNA have been disappointing and inconclusive. The delicate staining with the methyl green pyronin technique rarely gives satisfactory photographic differentiation in monochrome, and it cannot be claimed that the present work has made any contribution to the study of the behaviour of nucleic acids during wound healing. This is unfortunate in view of the fundamental importance of these substances in cell metabolism.

CONNECTIVE TISSUE REACTIONS

All the wounds studied here have been examples of healing by granulation, with the subsequent development of mature connective tissue. Apart from the conditions seen in some specimens of the healing rectum, there has been a notable absence of excessive fibrosis in the bases of the lesions. This is at first sight surprising in view of the fibrosis that is seen in the various forms of gastro-intestinal ulceration in man, but these are accompanied by varying degrees of endarteritis and other complicating factors of disease. It may be that the experimental wound, uncomplicated by such factors, is able to elicit an optimum response from the regenerating tissues, with an end result as near to the normal as is possible.

Although abundant evidence of mitosis can be found in the early stages of healing in all the organs studied, the question as to what precise type of cell is undergoing mitosis remains unanswered. The formation of new connective tissue cells from pre-existing connective tissue cells is not universally acknowledged. A mitotic figure seen in a cell in the floor of a granulating wound may be that of an activated fibrocyte,

or of a migrating mononuclear cell from the blood. It would appear that most pathologists incline to the view that fibroblasts arise from pre-existing fibrous tissue cells, while admitting that there is some evidence of metaplasia from mononuclear reticulo-endothelial cells (Cameron, 1952). However, Wright (1951) considers that the fibroblast is a fully differentiated cell, with distinctive properties, that never arises by transformation from other types. The generally accepted situation has been summarised by Florey (1954) as follows: "...it would be a very convenient and relatively satisfactory explanation of the presence of lymphocytes in healing wounds and chronic inflammatory lesions, if it could be clearly and unequivocally shown that a lymphocyte could develop into a monocyte or a macrophage; that it could, perhaps by a transformation to a macrophage, become a fibroblast... unfortunately none of these changes has been unequivocally shown to occur, and until such a transformation can actually be seen in vivo (as opposed to being deduced from a series of fixed specimens) it is difficult to think of a way in which the question can be finally settled." However, from their

examination of Thiersch graft (split skin) donor sites, Gillman et al. (1955a) concluded that: "Connective-tissue regeneration apparently takes place from the blood cells which immigrate into the subepithelial exudate, from the blood-stream itself, and from the previously occurring perivascular cuffing of round cells, and not by the activation of previously existing fibrocytes." Their evidence was derived from fixed specimens, and thus does not achieve the criterion laid down by Florey, as quoted above, for accepting the transformation of a mononuclear cell of the blood into a fibroblast. However, Maximow et al. (1952) believed that small lymphocytes and monocytes could indirectly develop into macrophages and fibroblasts, a theory re-echoed on the basis of colchicine and radiation studies by Allgower (1956), while Hartwell (1955) thought that lymphocytes developed into macrophages which then disintegrated, the resulting protoplasmic mass containing the chemical substances from which pre-collagen was made and subsequently converted by unknown means into collagen. He quotes Harper (in a personal communication) as having studied new tissue formation in transparent chambers on

rabbit ears and "watched the lymphocytes go through their entire life history, confirming the sudden disintegration of their macrophagic forms with the appearance of pre-collagen fibrils in their place which in turn rapidly change to larger, more stress-resistant, collagen fibers." If this could be confirmed, a significant step forward would have occurred not only in knowledge of wound healing but in elucidating the function of lymphocytes, a rational account of whose behaviour in pathological lesions is impossible in the present state of knowledge (Yoffey & Courtice, 1956). It seems likely that the use of radio-active tracers may help to solve this problem in the future.

A number of workers have drawn attention to the apparent correlation between alkaline phosphatase activity and the synthesis of fibrous protein, (see the review of Bradfield, 1950). Others, however, e.g. Bern & Levy (1952), have had reason to doubt such an association. In order to classify the opinions of some of the investigators who have studied the possible correlation during wound repair, Table I has been compiled.* In ascertaining the relevant literature for this

* page 139.

Table, a number of misquotations or misinterpretations have been noted. Rouiller (1956) and Buck (1953) have included the work of Gould & Gold (1951) in lists of references supporting the part played by phosphatase in collagen synthesis. However, these latter workers, who applied phosphatase to wounds of scorbutic animals, and who injected phosphatase inhibitors into wounds where there was rapid fibre formation, considered that phosphatase plays a doubtful role in fibrogenesis. Rouiller also quotes Frieden & Hisaw (1951) in a similar context, but they found little change in phosphatase in their experiments (which were not concerned with wounds) on relaxation of the pubic symphysis, although in their review article in 1953 they do mention an increase of phosphatase. Also, Buck mentions Hollinger & Rossiter (1952) as having found no phosphatase during collagen formation in regenerating nerve; although this was so in the early stages, they detected biochemically an increase after 96 days of regeneration, by which time fibroblastic activity was probably still evident. In view of these conflicting results, their paper is not included in the Table.

Perusal of Table I makes it clear that there is by no means universal agreement on the possible role of phosphatase in the fibrogenesis occurring during wound repair. The

Table I

WOUND HEALING AND ALKALINE PHOSPHATASE

Animal	Tissue	Author
(a) Phosphatase correlated with protein synthesis.		
Rat	Skin	Fell & Danielli (1943).
	Tendon	Buck (1953).
	Liver	Sulkin & Gardner (1948).
Guinea-pig	Skin	Danielli, Fell & Kodicek (1945).
	Bone	Bourne (1944, 1948).
Man	Skin	Fischer & Glick (1947).
	Stomach	Fodden (1953).
(b) Doubtful correlation of phosphatase with protein synthesis.		
Rat	Skin	Gold & Gould (1951).
		Gould & Gold (1951).
Guinea-pig	Skin	Bunting & White (1950).
(c) Phosphatase absent.		
Guinea-pig	Perinephric	Robertson, Dunihue & Novikoff (1950)
Rabbit	Nerve	Marchant (1949).
Cat	Various organs	The present studies.

findings in the cat have been consistent, and it seems that in this animal phosphatase is unlikely to play a significant part in the development of either the cells or fibres of healing wounds in the organs studied under the conditions of the present experiments. A statement such as that made by Fawcett (1954) in one of the newer textbooks, to the effect that in areas of connective tissue proliferation fibroblasts give a strong reaction for alkaline phosphatase, is now misleading in the light of the work reported here. Danielli et al. (1945) were careful to point out that it was not known whether the association between phosphatase activity and the regeneration of fibrous tissue existed in species other than rodents. The present findings in the cat suggest that species differences do exist with regard to the fibrogenesis in healing wounds, although the presence in cat skin of phosphatase in hair follicles, for example, does not question the validity of the association of phosphatase with some other forms of fibrillar protein. It may be noted that Henrichsen (1956) found no phosphatase in chicken heart fibroblasts in culture; the subsequent appearance of phosphatase was coincident with

degenerative changes in the cytoplasm and nuclei.

The metachromasia of the ground substance seen in the healing wounds of the oesophagus was more pronounced than in wounds of other organs in this study. This may be thought significant in view of the large number of mast cells that are found in this viscus, and in view of the possible contribution of such cells towards the formation of ground substance (Asboe-Hansen, 1954). On the other hand, there is no general agreement concerning the origin of ground substance constituents from mast cells; some workers, e.g. Meyer (1947) and Curran & Kennedy (1955) support the theory of their secretion by fibroblasts, while others, e.g. Perez-Tamayo & Ihnen (1953) discount a cellular origin from either mast cells or fibroblasts. Although it must be borne in mind that mast cells act by the formation and release of humoral agents (Fulton, Maynard, Riley & West, 1957), it might be expected that if they were intimately concerned with repair processes in a localised area, they would gather in that vicinity in greater concentration. If this premise is correct, the absence of mast cells from the wound areas in the tissues of

the present study in which they are normally most prolific - skin and oesophagus - speaks against their participation on any significant scale in the formation of new tissue constituents. In contrast, Asboe-Hansen (1957) has found in skin wounds in rabbits, whose tissues normally contain very few mast cells (Riley, 1955), an increase of such cells in metachromatic areas. But Wichmann (1955), who counted mast cells in the vicinity of incised wounds in rats, came to no conclusions about their presence in young connective tissue, and stated that they did not always appear in such tissue. Holczinger & Devenyi (1955) found no increase in granulation tissue. However, Wichmann (1955) did find an increase in the undisturbed tissue adjoining the incised wound, and suggested that their function in such a site was to liberate heparin for the purpose of preventing the clotting of blood in newly formed capillaries. Fulton et al. (1957) have drawn attention to the need for exercising caution where the counting of mast cells is concerned owing to the great variations in normal material. Riley's (1954) "riddle of the mast cells" remains unsolved.

While all healing wounds undergo contraction to a greater or lesser extent, the present studies have been mainly concerned with certain histochemical changes in both epithelium and connective tissue, and no attempt has been made to assess the amount of contraction occurring in this series. Skin wounds are pre-eminently suitable for such studies, since the same wound can be subjected to visual measurement at chosen time intervals. There is no doubt that there is great variation in the rate of contraction that occurs in different organs. A gastric mucosal wound that is 1 cm. square at the time of removal of the mucosa is barely recognisable with the naked eye 2 weeks later, whereas a similar lesion in the rectum would be very obvious, with a considerable granulating area plainly visible at its centre. There must of course be a distinction between the sliding of mobile mucosa towards a wound centre in the early stages of repair and the contraction that is due to the activity of maturing fibrous tissue. There is also an essential difference between contraction and later cicatrisation (Brenk, 1956). Some recent studies on the phenomena of

contraction in skin wounds have been made by Abercrombie and his associates (Abercrombie, Flint & James, 1954, 1956; Abercrombie & James, 1957), who suggested that it is not the fibres of fibrous tissue that cause contraction but an active contractility on the part of the cells of the tissue. In scorbutic animals where fibres are deficient, they found just as much contraction as in normal animals. Billingham & Russell (1956) considered that collagen fibres might serve "to temporarily anchor the margins of the wound" as they are drawn inwards by forces originating principally within the cells, though this is not to deny the part played by collagen fibres in increasing the tensile strength of a healing wound, a role in which they are well established (Dunphy & Udupa, 1955). The problem of contracture in visceral wounds needs investigation.

The main features of interest that have accrued from this series of experiments are:

1) the occurrence of mitosis in migrating epithelial cells,
2) the variations in the glycogen content of the different types of epithelial cells, and
3) the species differences that appear to exist with regard

to the association of alkaline phosphatase with fibrogenesis.

It is emphasised that generalisations derived from skin may be misleading when applied to other tissues that have been less intensively studied. The reports of Gillman et al. (1953, 1955a, 1956) have even cast doubt on some of the hitherto accepted reactions of skin following injury. The relatively new methods of histochemistry are opening up a wide field for further exploration in cytology, and the combined attack on anatomy at the cellular level that is being made by biochemistry and by research on ultrastructure must inevitably result in a better understanding of cell behaviour under a variety of conditions. Despite the continued progress that has been made in the understanding of cellular activity during the past 100 years since Virchow (1858) published "Die Cellularpathologie", much remains to be discovered concerning the metabolism of normal cells, quite apart from the modifications that ensue in the region of a wound. The more cell behaviour is understood, the more logical will be the attempts of the physician and surgeon to modify disease processes.

In view of what remains to be accomplished, it may be pertinent to close this study with the words of Evarts Graham (1955):

> "Some day we shall know more about the fundamental chemical factors involved in the healing of a wound - about the enzymes that construct fibrous tissue from the fibroblasts and that stimulate the epithelium of the skin and mucous membranes to cover the fibrous tissue and then stop growing."

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EXPLANATION OF FIGURES

All photomicrographs are of cat tissues unless otherwise stated. (F) indicates fixation in Gendre's fluid after freeze-substitution.

Fig. 1. Skin of the anterior abdominal wall, showing the thin epidermis. H. & E. x 750.

Fig. 2. Skin of the abdominal wall, showing mast cells in the subepithelial tissues; note that they are not present among the epithelial cells. Toluidine blue. x 300.

Fig. 3. Sebaceous glands showing a strongly positive reaction for alkaline phosphatase. Gomori technique, incubation time 3 hours. x 500.

Fig. 4. A skin wound 4 days after operation. This small lesion is completely epithelialised. H. & E. x 35.

Fig. 5. A skin wound after 5 days. A scab overlies the wound area. The epithelium at the wound margin is hypertrophic, including that of hair follicles immediately adjacent to the margin. Fine reticulum is developing in the floor of the lesion. Gomori's reticulum stain. x 25.

Fig. 6. Epithelium from the margin of a skin wound after 5 days. Compare with fig. 1, and note the increase in size of the cells and in the number of layers of cells. Two nuclei in the metaphase stage of mitosis are seen in the basal layer. H. & E. x 750.

Fig. 7. Hypertrophic epithelium at a wound margin after 4 days. Note the nucleus in prophase in the basal layer. Feulgen. x 750.

Fig. 8. Epithelium from the margin of a skin wound after 5 days. Here the basal cells have become tall columnar in form. H. & E. x 750.

Fig. 9. Non-keratinising epithelium overlying part of a wound site after 4 days. Compare with figs. 1 & 6. H. & E. x 750.

Fig. 10. Epithelium from the centre of a fully epithelialised wound after 14 days. Compare with fig. 9 and note that a stratum granulosum is now apparent, with overlying keratinisation. Compare also with figs. 1 & 6 and note that the cells are beginning to revert to their normal size. H. & E. x 750.

Fig. 11. Hypertrophic epithelium at a wound margin after 5 days, showing a reaction for alkaline phosphatase in the granules of the stratum granulosum. Gomori technique, incubation time 3 hours. x 750.

Fig. 12. Hypertrophic epithelium at a wound margin after 5 days showing abundant quantities of glycogen, but note its absence from the basal layer. PAS. x 750.

Fig. 13. An adjacent section to that illustrated in fig. 12 showing that the material indentified as glycogen in fig. 12 has now been removed. PAS after saliva digestion. x 750.

Fig. 14. Migrating epithelium over a wound after 5 days, showing glycogen in the upper layers of cells. There is no keratinisation in this epithelium. PAS. x 750.

Fig. 15. Epithelium from a fully epithelialised wound after 14 days. A stratum granulosum and keratinisation are present but glycogen can no longer be demonstrated. Compare with fig. 14. PAS. x 750.

Fig. 16. Hypertrophic epithelium at a wound margin after 5 days. The slightly darkened areas of cytoplasm indicate some increase in cytoplasmic RNA. Methyl green pyronin. x 750.

Fig. 17. Connective tissue near the surface of the floor of a skin wound after 2 days. There is infiltration with polymorphonuclear leucocytes and lymphocytes, and the nuclei of 3 connective tissue cells are undergoing mitosis. H. & E. x 750.

Fig. 18. Young connective tissue underlying new epithelium in the centre of a skin wound 10 days after operation. The new fibroblasts have a well defined cytoplasm with fine, elongated processes. H. & E. x 640.

Fig. 19. Similar tissue to that illustrated in fig. 18, showing subepithelial connective tissue cells and extracellular material. PAS without acid reducing rinse. x 425.

Fig. 20. Centre of a newly epithelialsed skin wound at 10 days. Although some dark-staining round cells are found in the subepithelial tissues, no mast cells are present. Compare with fig. 2. Toluidine blue. x 300.

Fig. 21. Centre of a skin wound 14 days after operation, showing the re-appearance of mast cells. Compare with figs. 2 & 20. Toluidine blue. x 300.

Fig. 22. Region of a wound margin after 10 days. On the left is the undisturbed skin with sebaceous glands and hair follicles; on the right is the young connective tissue of the wound such as is illustrated in figs. 18 & 19. Note that while the sebaceous glands and some large blood vessels give a strong reaction for alkaline phosphatase, the new connective tissue gives a completely negative reaction. Gomori technique, incubation time 3 hours. x 66.

Fig. 23. Mitosis in an epidermal cell of the stratum spinosum. H. & E. x 640.

Fig. 24. Section through the wall of the lower end of the oesophagus at low magnification. Note the thickness of the muscularis mucosae. H. & E. x 25.

Fig. 25. Normal oesophageal epithelium. Note the small basal cells, the larger cells of the intermediate zone, and the layers of flattened cells that form the superficial zone. There is no keratinisation. H. & E. x 170.

Fig. 26. Normal oesophageal epithelium, showing the presence of alkaline phosphatase in the basal zone, and to a lesser extent in the superficial layers. Gomori technique, incubation time 30 minutes. x 170.

Fig. 27. Normal epithelium, showing a similar distribution of alkaline phosphatase to that seen in fig. 26. Azo dye method. x 170.

Fig. 28. Normal epithelium, showing the 3 zones of cells. Toluidine blue. x 170.

Fig. 29. Mast cells lying in the basal layer of oesophageal epithelium. Note that they are absent in more superficial regions. Azure A. x 540.

Fig. 30. Mast cells in the basal layer. The granules are strongly metachromatic. Azure A. x 1400.

Fig. 31. Normal epithelium, showing PAS-positive material in the superficial and intermediate zones. PAS after saliva digestion. x 170.

Fig. 32. Normal epithelium, showing that cytoplasmic pyroninophilia is most marked in the intermediate zone and absent from the basal layer. Methyl green pyronin. x 500.

Fig. 33. Oesophageal wound 6 hours after operation. The floor of the lesion consists of the submucosa, the whole thickness of the mucosa, including the muscularis mucosae, having been removed. H. & E. x 25.

Fig. 34. The right margin of the wound illustrated in fig. 33, showing the cut edge of the muscularis mucosae. H. & E. x 100.

Fig. 35. Epithelium near a wound margin after 2 days, showing a number of mitotic figures. H. & E. x 500.

Fig. 36. Epithelial cells migrating towards the right over the floor of a wound after 3 days. Note the size of the basal cells compared with those in fig. 25, the cellular infiltration and the spaces in the upper layers. H. & E. x 170.

Fig. 37. Epithelium migrating towards the left over a wound floor after 3 days. Note that the cellular infiltration of the epithelium is most marked in the upper layers. Compared with fig. 28 the cells of the epithelium stain less intensely. Toluidine blue. x 170.

Fig. 38. Epithelium spreading towards the left from the wound margin on the right, after 3 days. One mitotic figure (indicated by the arrow) is seen in the basal layer of spreading cells. H. & E. x 170.

Fig. 39. Completely epithelialised wound site after 10 days, showing the thickened epithelium overlying maturing granulation tissue. H. & E. x 30.

Fig. 40. Hypertrophic epithelium overlying a wound area after 10 days. Compare with figs. 25 & 36, at the same magnification. Some inflammatory cells and spaces are still present. H. & E. x 170.

Fig. 41. Epithelium from the centre of a wound area 1 month after operation, showing the return of the normal zoning. Compare with figs. 25 & 40. H. & E. x 170.

Fig. 42. Epithelium from an epithelialised wound after 10 days. Compare with fig. 26 and note the absence of alkaline phosphatase. Gomori technique, incubation time 30 minutes, lightly counterstained with haematoxylin. x 120.

Fig. 43. Epithelium from a wound site after 15 days. Compare with fig. 42 and note that the superficial zone now gives a reaction for alkaline phosphatase, but the basal region is still negative. Gomori technique, incubation time 30 minutes, lightly counterstained with haematoxylin. x 120.

Fig. 44. Epithelium from a healed site after 6 months, showing a normal distribution of alkaline phosphatase. The reaction in the superficial zone is not usually as strong as in this specimen. Compare with fig. 26. Gomori technique, incubation time 30 minutes. x 120.

Fig. 45. Epithelium from a wound margin after 3 days, showing abundant quantities of glycogen, but note its absence from the basal layer. PAS. x 750.

Fig. 46. An adjacent section to that illustrated in fig. 45, showing that the material identified as glycogen in fig. 45 has now been removed. PAS after saliva digestion. x 750.

Fig. 47. Section of a wound margin after 10 days. Normal epithelium, above, gives a PAS-positive reaction in the superficial zone. The large, migrating cells, below, contain glycogen. PAS. x 120.

Fig. 48. Epithelium from a wound site after 6 months, showing a normal distribution of PAS-positive material other than glycogen. Compare with fig. 31. PAS after saliva digestion. x 170.

Fig. 49. Epithelium from a wound site after 6 months, showing the restoration of the 3 zones. Compare with figs. 28 & 37. Toluidine blue. x 170.

Fig. 50. Section from the centre of a wound area after 10 days, showing typical young connective tissue underlying the new epithelium. One connective tissue cell is in the metaphase stage of mitosis. H. & E. x 640.

Fig. 51. Part of a wound area after 15 days, merging with undisturbed tissue (on the left). While the epithelium and some blood vessels (on the left) show a positive reaction for alkaline phosphatase, the young connective is completely negative. Gomori technique, incubation time 3 hours. x 66.

Fig. 52. Wound area 6 months after operation. The site has remained relatively flat. Note the absence of the muscularis mucosae in the wound area. H. & E. x 25.

Fig. 53. Wound area 6 months after operation. The mucosa here has developed a convoluted pattern. Note the cut edges of the muscularis mucosae. H. & E. x 45.

Fig. 54. Wound area 1 year after operation. A space due to artefact is seen where the muscularis mucosae is absent, indicating the region of the original lesion. The cut edges of the muscularis mucosae are indicated. H. & E. x 35.

Fig. 55. Margin of a wound of the stomach after 3 days, showing epithelium migrating over the wound floor. H. & E. x 35.

Fig. 56. Site of a wound of gastric mucosa after 15 days. Note the cut edges of the muscularis mucosae, and the new crypts in the centre, dipping down into maturing granulation tissue. H. & E. x 35.

Fig. 57. Margin of a wound of gastric mucosa after 3 days. Above, normal mucosa, showing a positive reaction for alkaline phosphatase in the stroma but no reaction in the epithelium. Below, the migrating epithelium and subepithelial tissues are all negative. Gomori technique, incubation time 3 hours. x 100.

Fig. 58. Epithelium and underlying tissue from the centre of a gastric lesion after 15 days. Note that the positive reaction for phosphatase is confined to stromal tissue immediately under the epithelium, and that the deeper tissue is negative. Gomori technique, incubation time 3 hours. x 100.

Fig. 59. Normal surface epithelium of the stomach. PAS.(F). x 640.

Fig. 60. An adjacent section to that illustrated in fig. 59, showing that the infranuclear granules seen in fig. 59 are diastase-labile and hence identified as glycogen. PAS after saliva digestion. (F). x 640.

Fig. 61. Migrating epithelial cells after 3 days. The perinuclear granules are of glycogen. PAS.(F). x 640.

Fig. 62. Epithelium from the centre of a wound site after 15 days. Compare with fig. 59. PAS.(F). x 640.

Fig. 63. Section through the margin of a mucosal lesion of the ileum 24 hours after operation, showing that the mucosa, and in this case the submucosa also, has been removed. H. & E. x 17.

Fig. 64. Epithelium overlying maturing granulation tissue near the centre of an ileal wound after 14 days. H.& E. x 95.

Fig. 65. Wound of ileum 14 days after operation. Note the degree of epithelialisation, the granulation tissue and the suggestion of new crypt formation. H. & E. x 17.

Fig. 66. Wound of ileum after 7 days. The centre is beyond the left border of the illustration. Note that as the margin is approached (towards the right) there is a pattern of increasingly deepening depressions. H. & E. x 70.

Fig. 67. Site of ileal wound 3 months after operation. Note the degree of reconstitution of the mucosa; the whole area is covered by crypts and villi which closely resemble the normal. H. & E. x 17.

Fig. 68. Margin of a 3 month old ileal wound, showing the cut edge of the muscularis mucosae that has not regenerated. H. & E. x 40.

Fig. 69. Flattened epithelial cells overlying the periphery of a wound after 1 day. Compare with figs. 70 &71. H. & E. x 760.

Fig. 70. Normal epithelium of villi in the ileum. H. & E. x 760.

Fig. 71. Normal epithelium of crypts in the ileum. H. & E. x 760.

Fig. 72. Migrating epithelial cells near the margin of a wound after 2 days. H. & E. x 760.

Fig. 73. Epithelial cells overlying granulation tissue in a wound. 10 days old. Compare with figs. 69, 70 & 72.

Fig. 74. Spreading epithelium from a wound after 4 days, showing an epithelial cell in the metaphase stage of mitosis. Note that the cells do not display a PAS-positive border. PAS. x 640.

Fig. 75. Normal epithelium from villi in the ileum showing a strong reaction for alkaline phosphatase in the brush border. Gomori technique, incubation time 30 minutes. x 640.

Fig. 76. Normal epithelium from crypts in the ileum, showing a weak reaction for alkaline phosphatase. Compare with fig. 75. Gomori technique, incubation time 30 minutes. x 640.

Fig. 77. Normal epithelium from villi in the ileum, showing a PAS-positive border. PAS after saliva digestion. x 640.

Fig. 78. Normal epithelium from crypts in the ileum, showing a rather poorly defined PAS-positive border. PAS after saliva digestion. x 640.

Fig. 79. Spreading epithelium from a 3 day wound, showing a completely negative reaction for alkaline phosphatase. Gomori technique, incubation time 30 minutes. x 640.

Fig. 80. An adjacent section to that illustrated in fig. 79, after longer incubation. The nuclear staining may be due to diffusion, in view of the very intense reaction in normal epithelium, a fragment of which is seen in the top left corner. Gomori technique, incubation time 3 hours. x 640.

Fig. 81. Spreading epithelium after 7 days. The reaction for alkaline phosphatase is still negative. Gomori technique, incubation time 30 minutes. x 640.

Fig. 82. Below, epithelium overlying granulation tissue after 10 days. Above, normal villi. Note that the striated border in the new epithelial cells is now positive. The maturing granulation tissue remains negative. Gomori technique, incubation time 30 minutes. x 250.

Fig. 83. Spreading epithelial cells from a wound after 3 days. The PAS-positive border is absent. PAS.(F). x 640.

Fig. 84. Spreading epithelium after 7 days. Note that a PAS-positive border is now present and that some granules are seen in the perinuclear region of some cells. PAS. (F).. x 640.

Fig. 85. An adjacent section to that illustrated in fig. 84. showing that the PAS-positive border remains but the granules have disappeared. PAS after saliva digestion. (F). x 640.

Fig. 86. Epithelium from a wound 15 days old, showing a normal PAS reaction at the cell borders. There are no glycogen granules. PAS.(F). x 640.

Fig. 87. Healing wound after 21 days. Note that all the epithelium gives an intense reaction for alkaline phosphatase, while the underlying tissue (apart from some local diffusion) is negative. Gomori technique, incubation time 24 hours. x 50.

Fig. 88. On the left and in the centre is seen the undisturbed intestinal wall; on the right is seen the wound margin, and granulation tissue that is negative for alkaline phosphatase, after 3 days. Note the distribution of the positive reaction in the blood vessels of the muscular wall of the intestine. Gomori technique, incubation time 3 hours. x 15.

Fig. 89. Healing wound after 15 days. The lesion is completely epithelialised, and there is virtually no phosphatase reaction in the blood vessels. Compare with fig. 88. Gomori technique, incubation time 3 hours. x 15.

Fig. 90. A specimen that is considered abnormal, showing a positive reaction for phosphatase in most of the blood vessels, whether they are near or far removed from the wound area. Gomori technique, incubation time 3 hours. x 15.

Fig. 91. Normal rectal mucosa. H. & E. x 35.

Fig. 92. Margin of a wound after 2 days. Epithelial cells are growing from the margin towards the left. Note the cut edge of the muscularis mucosae. H. & E. x 65.

Fig. 93. Typical epithelial cells of the normal rectal mucosa. H. & E. x 640

Fig. 94. Flattened epithelial cells near the margin of a wound after 2 days, at the same magnification as fig. 93. H. & E. x 640.

Fig. 95. The epithelial cells overlying typical young fibroblasts after 4 days are becoming cuboidal or low columnar. H. & E. x 640.

Fig. 96. Migrating epithelium after 3 days showing a nucleus in prophase. PAS. x 1340.

Fig. 97. A connective tissue cell in the metaphase atage of mitosis near a wound margin after 2 days. H. & E. x 640.

Fig. 98. Two connective tissue cells in metaphase, lying deep to a bundle of muscle fibres of the muscularis externa below the floor of the wound after 2 days. H. & E. x 640.

Fig. 99. Margin of a wound after 7 days, showing epithelial cells that have grown from the margin (on the left) dipping down into granulation tissue. Note that goblet cells are scanty in the new epithelium. H. & E. x 70.

Fig. 100. Typical spreading epithelium after 7 days. Compare with figs. 93 & 94, and note the columnar shape of the cells and the absence of goblets. H. & E. x 640.

Fig. 101. Dilated crypts at a wound margin after 7 days. H. & E. x 70.

Fig. 102. Wound site after 4 weeks, showing that the whole area is covered by shallow epithelialised depressions. H. & E. x 35.

Fig. 103. A mitotic figure in the epithelium lining a shallow depression in a wound site after 2 weeks. PAS. x 640.

Fig. 104. An adjacent section to that illustrated in fig. 102, at higher magnification. On the right is the mucosa of the original wound margin; on the left is the spreading epithelium lining shallow depressions and showing some goblet cells. H. & E. x 100.

Fig. 105. A wound site after 4 weeks showing exuberant granulation tissue that is not covered by epithelium. Compare with fig. 102. Van Gieson. x 35.

Fig. 106. A further wound site after 4 weeks, illustrating the lack of shallow depressions and the absence of complete epithelialisation, with exuberant granulation tissue. H. & E. x 50.

Fig. 107. Wound site after 3 months. A strand or septum of fibrous tissue is passing from the submucosa between groups of crypts towards the surface. H. & E. x 35.

Fig. 108. Site of a lesion after 6 months. The position of the cut edges of the muscularis mucosae is indicated. The area of the original wound is occupied by crypts that are less deep and less closely packed than normal. H. & E. x 15.

Fig. 109. Above, normal rectal epithelium, showing a strong reaction for alkaline phosphatase at the brush border. Below, migrating epithelium after 3 days, giving no phosphatase reaction. Gomori technique, incubation time 30 minutes. x 640.

Fig. 110. Margin of a rectal wound, after 4 days, illustrating the continuity between normal epithelium, above, that gives a strong phosphatase reaction, and migrating epithelium, below, that is negative. Gomori technique, incubation time 30 minutes. x 255.

Fig. 111. Epithelialised depressions from a wound after 7 days, giving no reaction for phosphatase. Note that the underlying granulation tissue is also negative. Gomori technique, incubation time 30 minutes. x 255.

Fig. 112. Epithelium from the centre of a wound after 15 days, showing that a phosphatase reaction is returning to the brush border. The subepithelial tissues are negative. Gomori technique, incubation time 30 minutes. x 640.

Fig. 113. Epithelium from the centre of a wound after 3 weeks, giving a phosphatase reaction of normal intensity. The subepithelial tissues are again negative. Gomori technique, incubation time 30 minutes. x 640.

Fig. 114. Epithelium near the surface of a crypt, showing the PAS-positive border. PAS. x 640.

Fig. 115. Epithelium near the base of a crypt, with a PAS reaction less intense than that in fig. 114. PAS. x 640.

Fig. 116. Migrating epithelium after 3 days, showing that a PAS-positive border is almost completely absent. PAS (F). x 640.

Fig. 117. Migrating epithelium overlying granulation tissue in a wound after 7 days. A PAS reaction is present at the free border of the cells. PAS after saliva digestion. x 255.

Fig. 118. Migrating epithelium from a 7 day wound, showing infranuclear PAS-positive granules. PAS.(F). x 640.

Fig. 119. An adjacent section to that illustrated in fig. 118. The infranuclear granules are no longer present. PAS after saliva digestion. (F). x 640.

Fig. 120. Epithelial cells with some glycogen granules lining a depression in a wound site after 7 days. PAS. x 640.

Fig. 121. Normal bladder epithelium. Note the absence of a phosphatase reaction in the superficial layer of epithelium, and the positive stroma. Gomori technique, incubation time 3 hours. x 640.

Fig. 122. Migrating epithelium after 5 days, showing an almost complete absence of phosphatase. The subepithelial tissues are negative. Gomori technique, incubation time 3 hours. x 640.

Fig. 123. Epithelium from the centre of an epithelialised wound after 3 weeks, showing a return of the normal phosphatase pattern. Gomori technique, incubation time 3 hours. x 640.

Fig. 124. Normal bladder epithelium. PAS. x 640.

Fig. 125. An adjacent section to that illustrated in fig. 124, showing that the dark-staining material in that figure is diastase-labile and hence identified as glycogen. PAS after saliva digestion. x 640.

Fig. 126. Normal bladder epithelium in which almost all the PAS-positive material, including the large, dark granules, is resistant to diastase. PAS after saliva digestion (F). x 640.

Fig. 127. Margin of a wound of bladder mucosa after 3 days. On the left is the undisturbed normal epithelium, that contains PAS-positive material and glycogen granules. In the centre are hypertrophic epithelial cells that are pale staining, with negligible glycogen. Compare with figs. 12 & 45. PAS.(F). x 270.

Fig. 128. Hypertrophic migrating cells after 5 days, showing scanty glycogen granules in some cells, including those at the basal layer. Relatively pale staining PAS-positive material other than glycogen is also present. PAS.(F). x 640.

Fig. 129. Epithelium from the centre of a wound site after 3 weeks, showing some return of glycogen to its normal distribution. Compare with fig. 124. PAS. x 640.

Fig. 130. Gall-bladder and 2 adjacent lobes of liver. x 7/8.

Fig. 131. The gall-bladder has been incised and an area of its mucosa is being scraped away. x 1.6.

Fig. 132. Section through the middle of a wound 18 hours after operation. H. & E. x 18.

Fig. 133. Normal gall-bladder epithelium. H. & E. x 600.

Fig. 134. Normal mucosa near a wound margin after 24 hours, showing a number of mitotic figures in the epithelium. H. & E. x 450.

Fig. 135. A connective tissue cell in the anaphase stage of mitosis in the floor of a wound after 24 hours. H. & E. x 600.

Fig. 136. Epithelium spreading from a wound margin (on the left) over the floor of the lesion after 2 days. H. & E. x 75.

Fig. 137. Migrating epithelial cells after 2 days. H. & E. x 600.

Fig. 138. Migrating epithelial cells after 3 days, with normal overlying epithelium. H. & E. x 600.

Fig. 139. Migrating epithelium after 2 days, showing 3 mitotic figures. That on the left is not in focus in the photomicrograph, but under the microscope it is clearly mitotic. H. & E. x 500.

Fig. 140. Epithelial cell in anaphase at the advancing edge of an epithelial sheet after 3 days. H. & E. x 600.

Fig. 141. Migrating epithelial cell in anaphase. H. & E. x 600.

Fig. 142. Wound site after 6 days, showing epithelium overlying granulation tissue. H. & E. x 18.

Fig. 143. Large columnar epithelial cells from the centre of a wound site after 7 days. Compare with fig. 133 at the same magnification. H. & E. x 600.

Fig. 144. Wound site after 1 month. Apart from some minor elevations, there is no evidence of a return to the normal convoluted pattern. H. & E. x 18.

Fig. 145. Region of a wound margin after 3 days. On the left is the undisturbed gall-bladder wall, showing a negative reaction for phosphatase in the epithelium and loose connective tissue, but a positive reaction in the deeper stroma. On the right is the wound area, whose tissues are negative for phosphatase, apart from some blood vessels. Gomori technique, incubation time 24 hours. x 43.

Fig. 146. Normal gall-bladder epithelium, showing infranuclear glycogen granules. This specimen shows very little PAS-positive material other than glycogen. PAS. x 600.

Fig. 147. Normal epithelium showing PAS-positive material other than glycogen in the supra-nuclear region of the cells. PAS after saliva digestion. x 450.

Fig. 148. Large columnar cells from a wound site after 6 days, demonstrating large quantities of glycogen in the peri-nuclear and infra-nuclear regions. PAS. x 600.

Fig. 149. An adjacent section to that illustrated in fig. 148, showing that the material identified as glycogen is now absent. PAS after saliva digestion. x 600.

Fig. 150. Young connective tissue and overlying epithelium from the centre of a wound site in rat skin, after 10 days. Compare with fig. 18 and note the similarity between these 2 figures. H. & E. x 640.

Fig. 151. Site of a healing skin wound in the rat after 10 days. Note the strongly positive reaction for alkaline phosphatase in the granulation tissue. The epithelium and the surrounding subepithelial tissues are negative, apart from the stratum spinosum and hair follicles. Compare with fig. 22. Gomori technique, incubation time 30 minutes. x 25.

Fig. 152. Site of a healing skin wound in the guinea-pig after 10 days, showing a similar phosphatase reaction to that seen in the rat. Gomori technique, incubation time 30 minutes. x 25.

Fig. 153. Margin of a rectal wound in the rat after 10 days. On the right is the undisturbed mucosa, showing some stromal reaction for phosphatase. On the left is a mass of unepithelialised granulation tissue, giving a strongly positive reaction. Gomori technique, incubation time 30 minutes. x 35.

Fig. 154. Connective tissue from an epithelialised skin wound after 10 days, in a cat that had received a dietary supplement of vitamin C. Compare with figs. 18 & 150. H. & E. x 640.

Fig. 155. Margin of a skin wound after 10 days in a cat that had received a dietary supplement of vitamin C. In the undisturbed tissue on the left, hair follicles and sebaceous glands are strongly positive for phosphatase. The maturing granulation tissue on the right is negative. Compare with fig. 22. Gomori technique, incubation time 24 hours. x 66.

Fig. 156. Section of a wound of rat skin, after 10 days, showing that both epithelial and connective tissue cells show an affinity for phosphatase. For technique, see text. x 345.

Fig. 157. Section of a wound of cat skin, after 10 days, showing that epithelial and connective tissue cells have an equal affinity for phosphatase. Compare with fig. 156. For technique see text. x 345.

Fig. 158. Section demonstrating diffusion of products of reaction from an overlying section of kidney into inactivated tissues of a rat skin wound. For technique see text. x 260.

Fig. 159. Similar experiment to that illustrated in fig. 158, showing diffusion into tissues of a cat skin wound. For technique see text. x 260.

Fig. 160. Section illustrating the affinity of epithelial and connective tissue cells of the cat for calcium phosphate. For technique see text. x 345.

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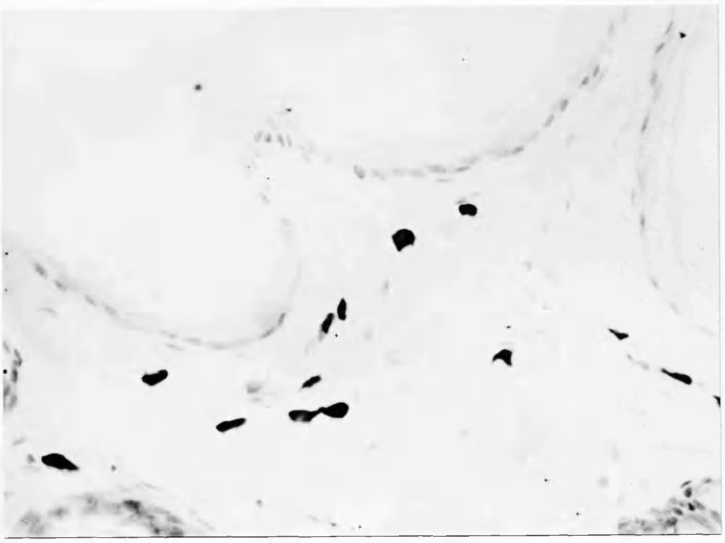

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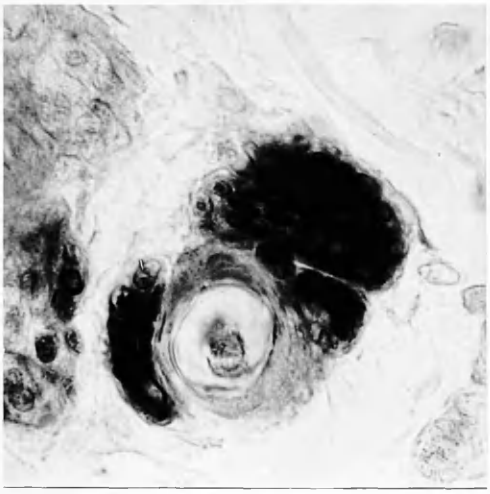

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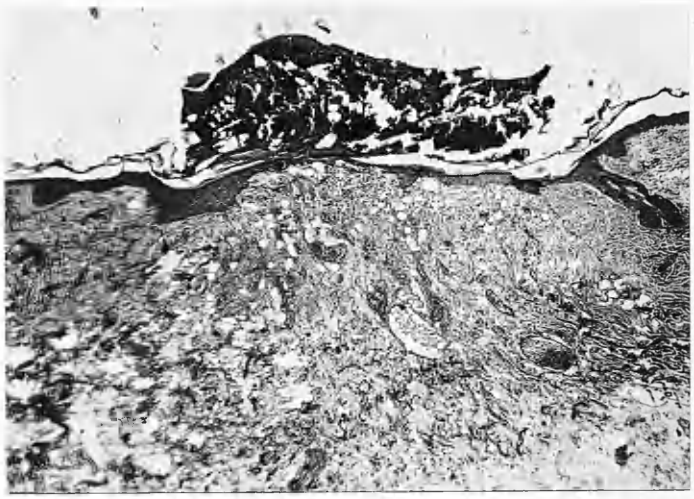

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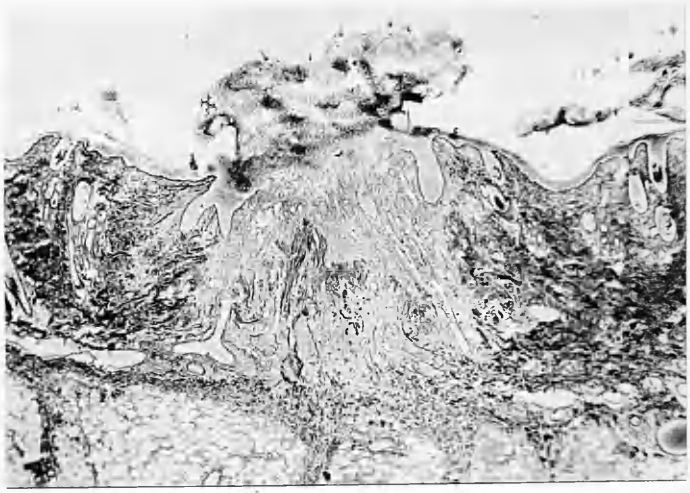

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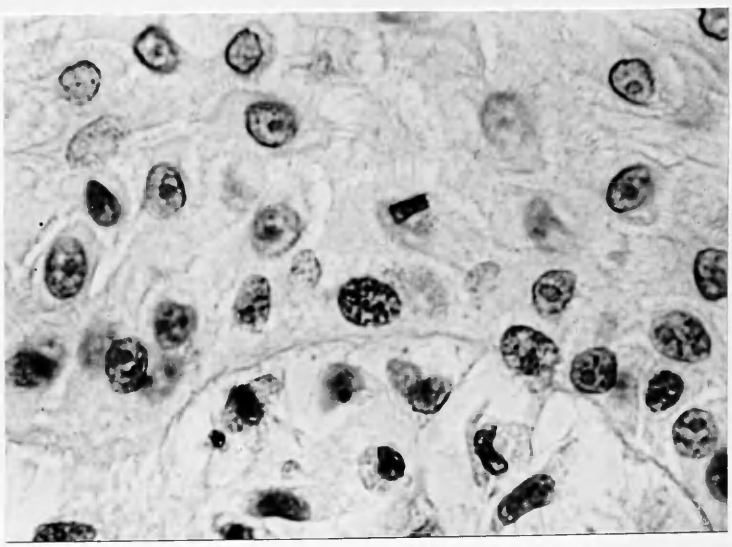

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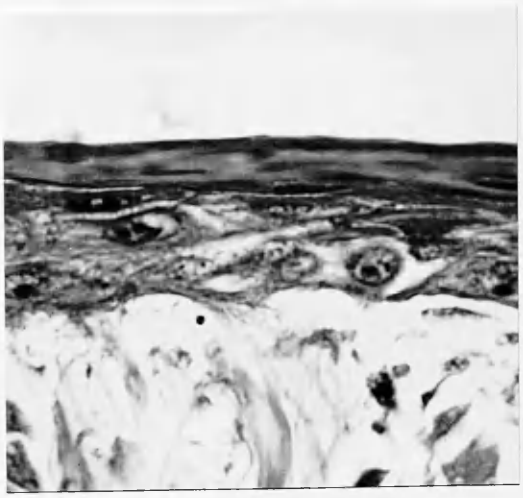

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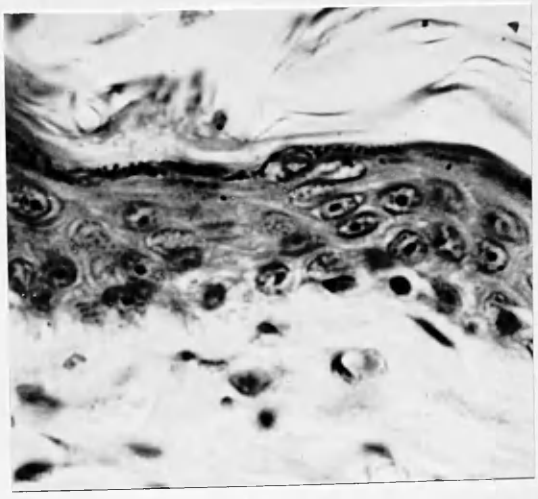

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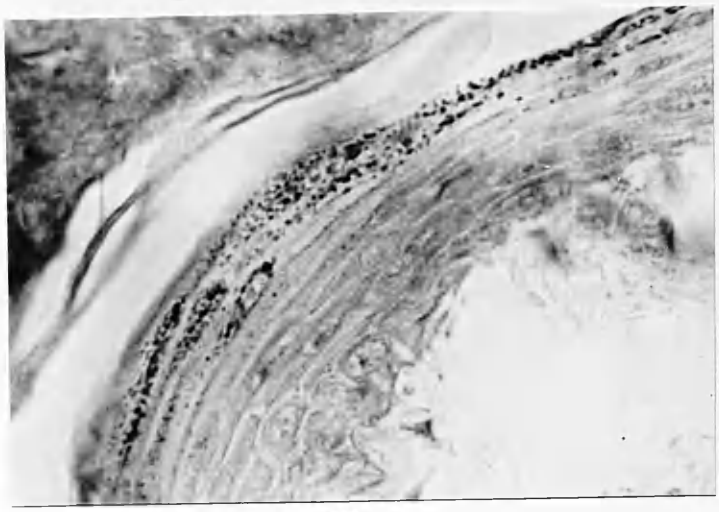

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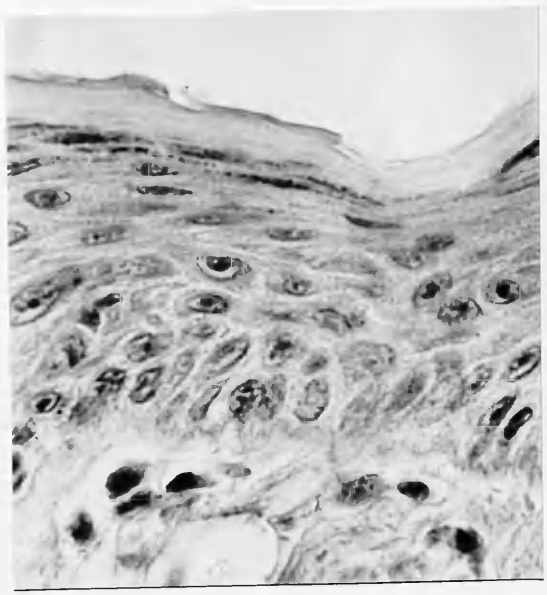

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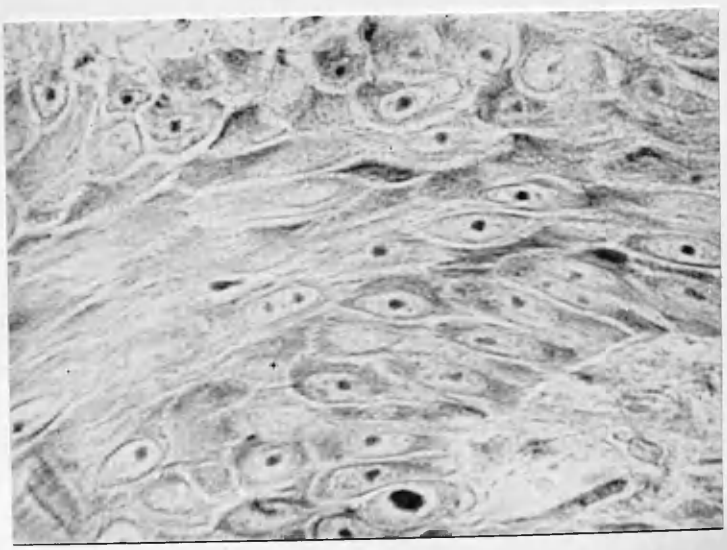

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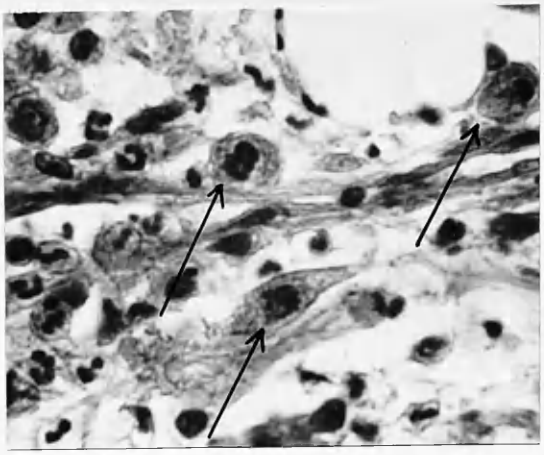

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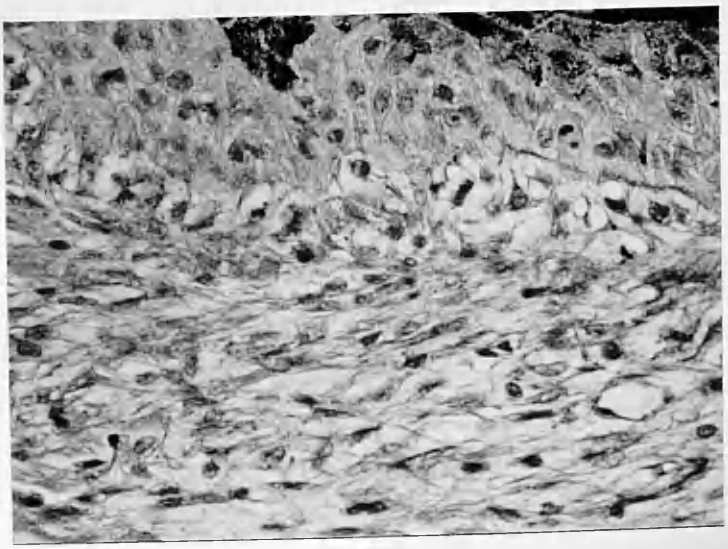

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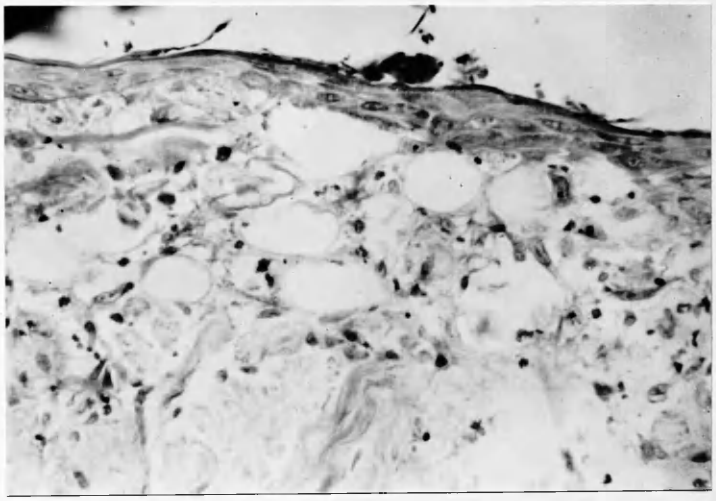

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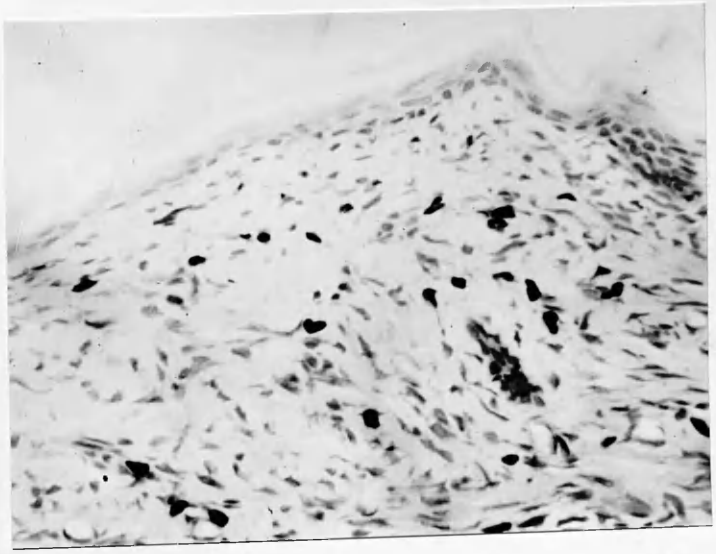

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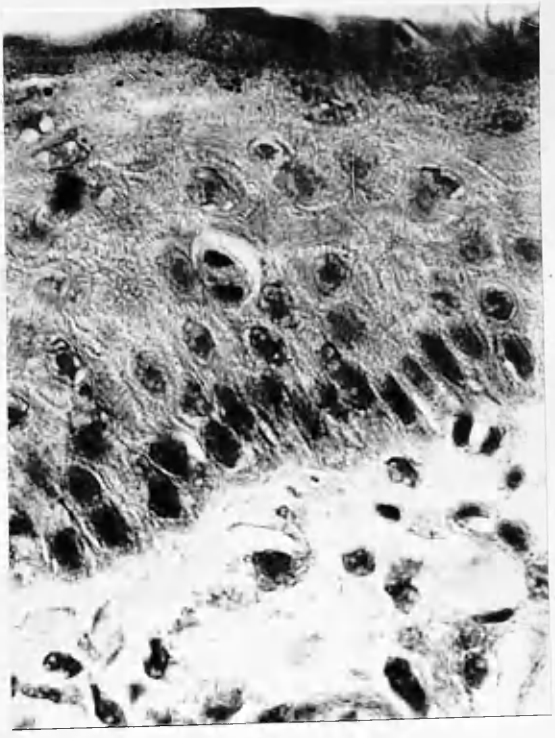

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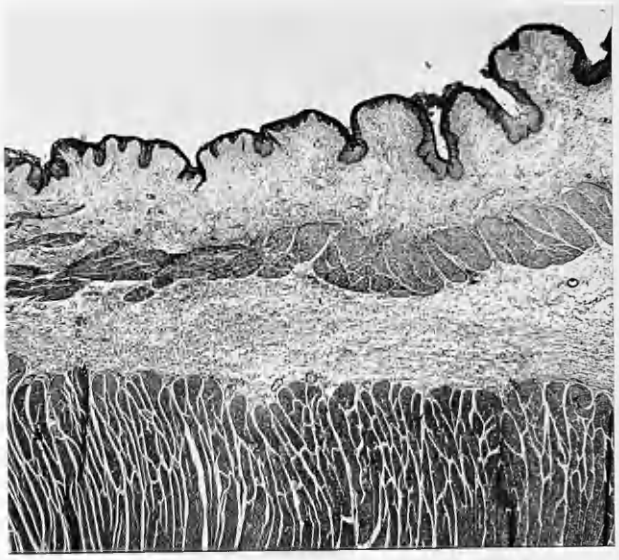

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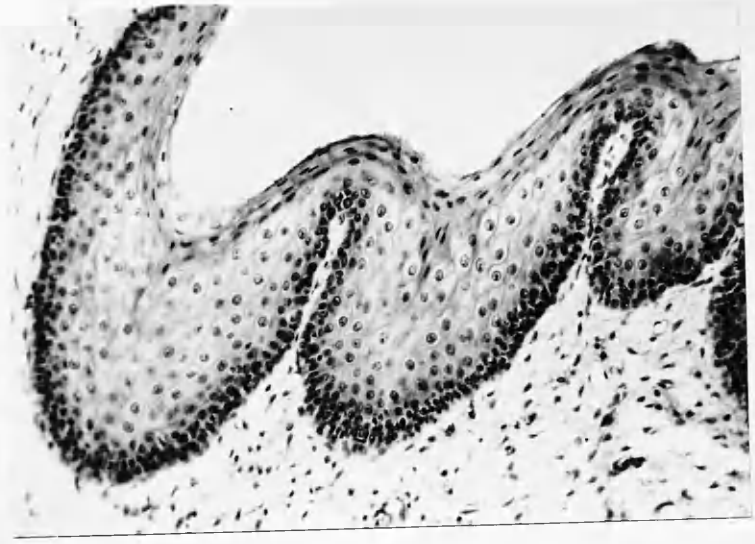

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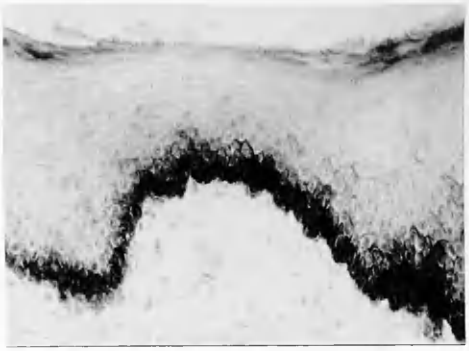

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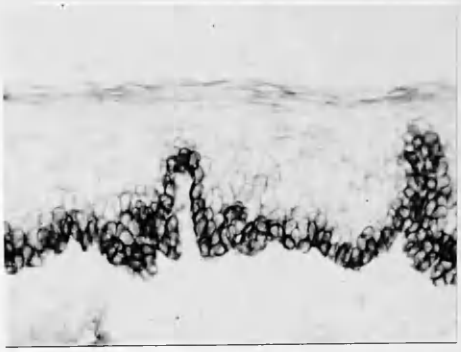

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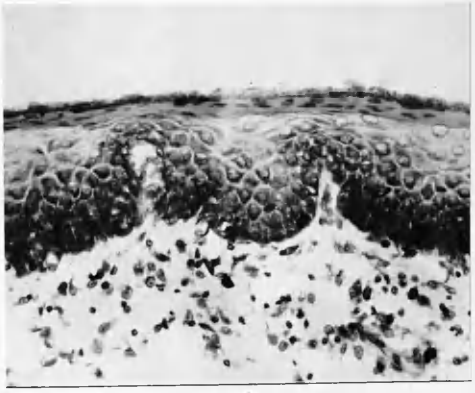

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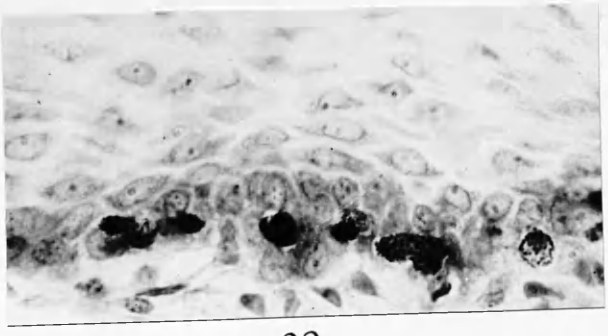

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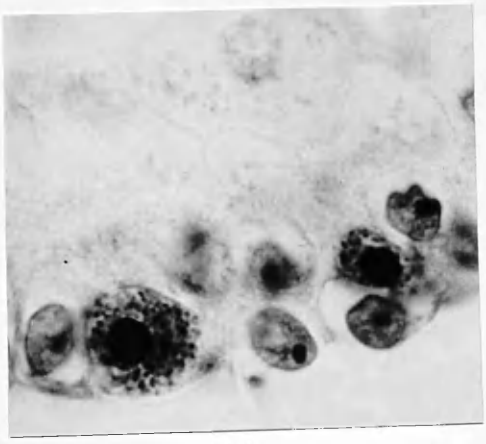

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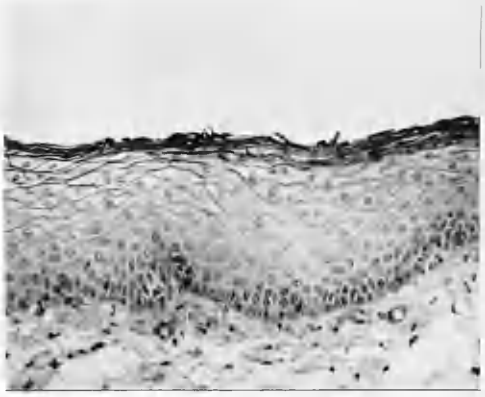

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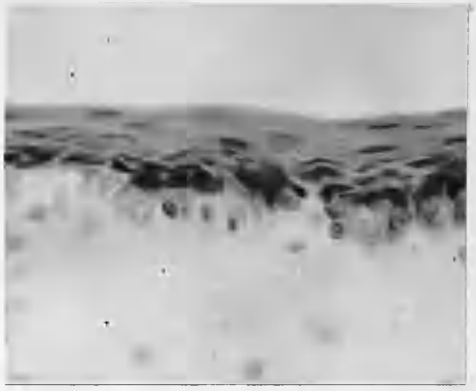

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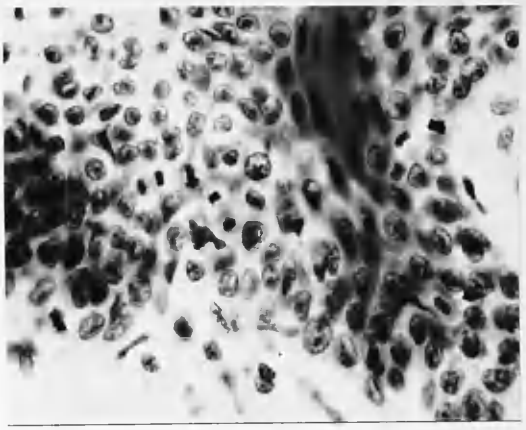

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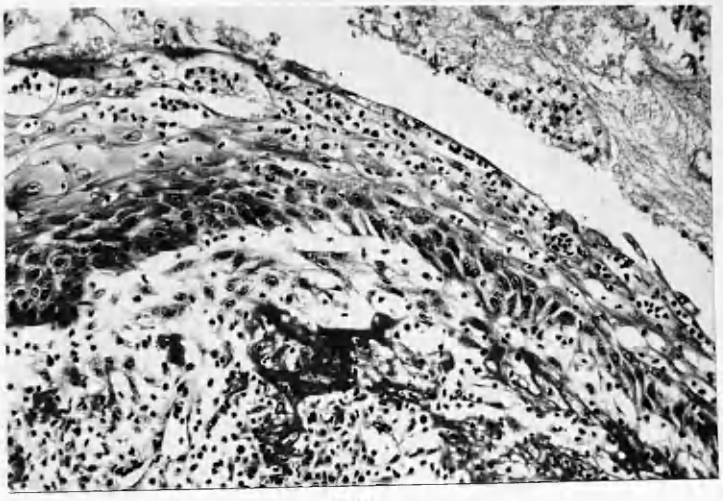

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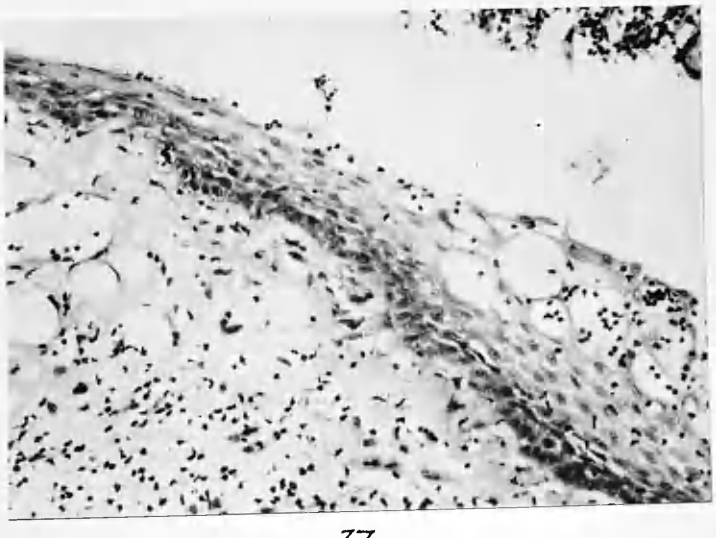

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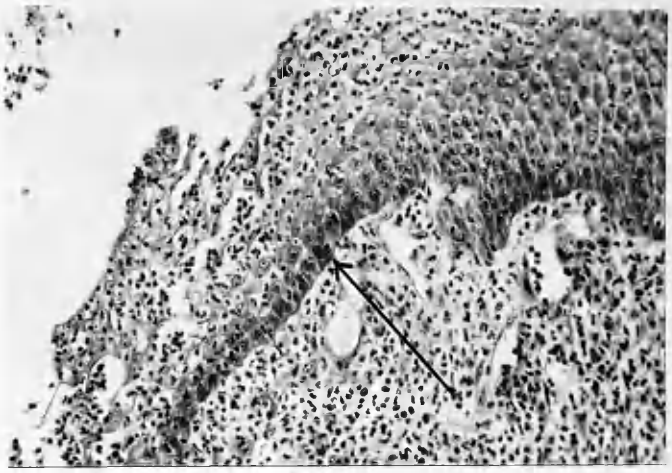

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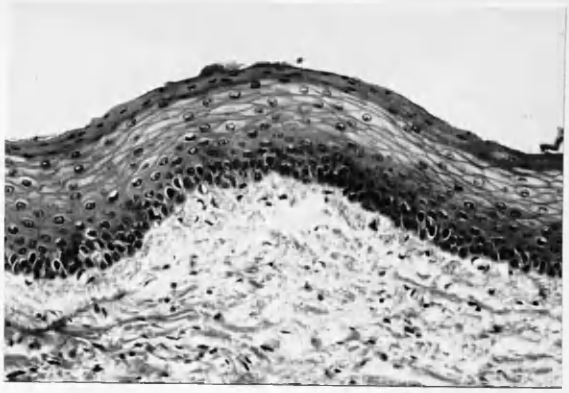

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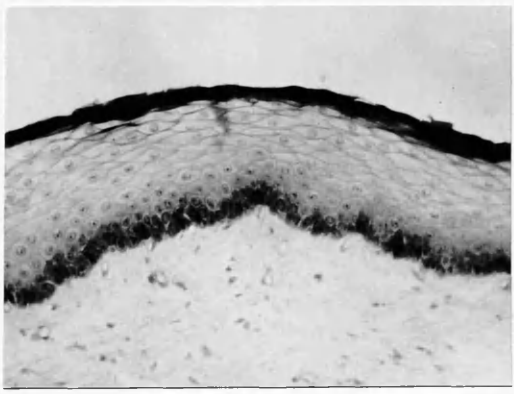

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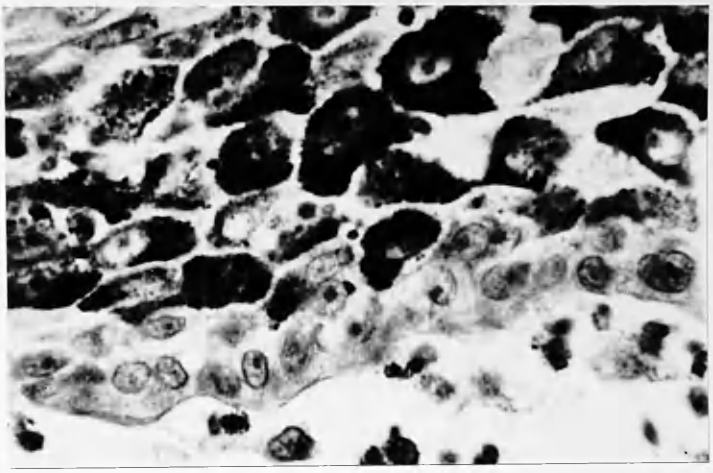

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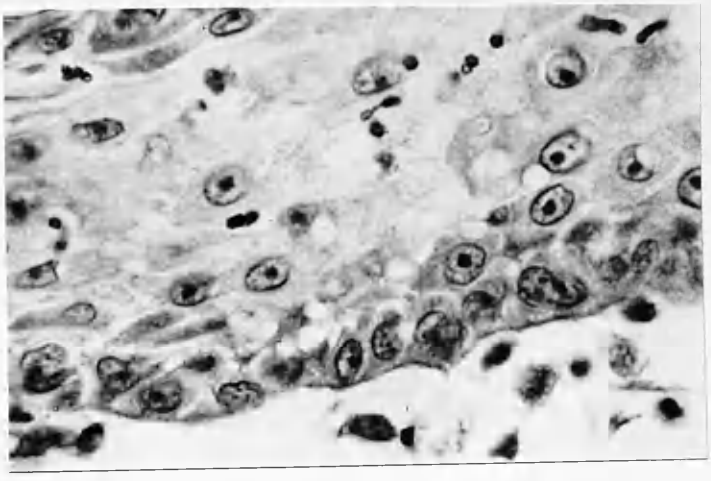

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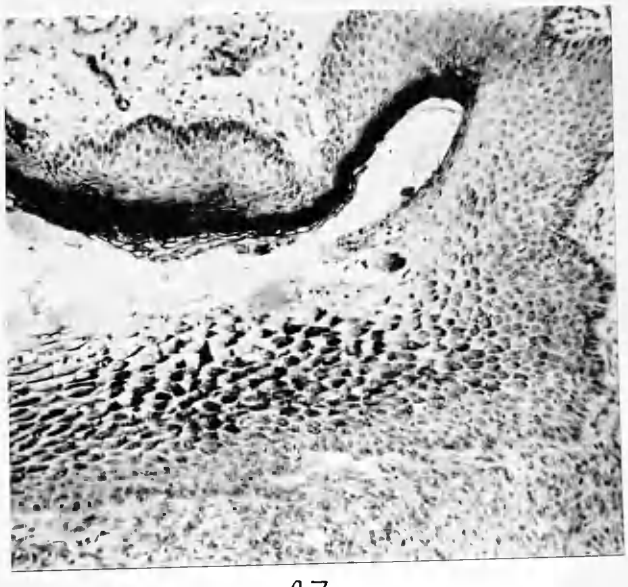

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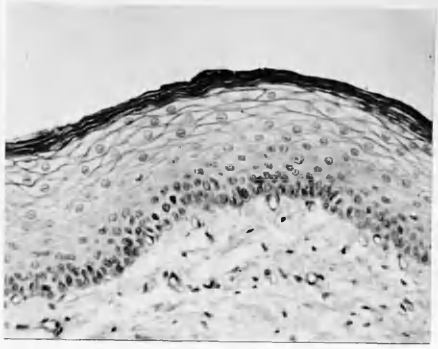

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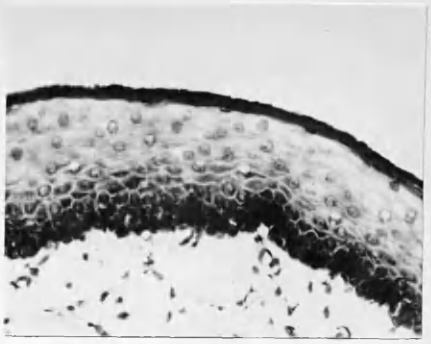

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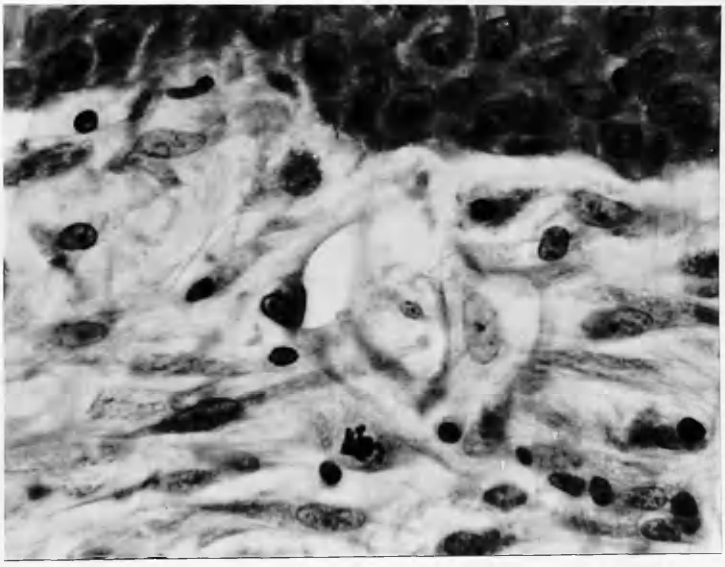

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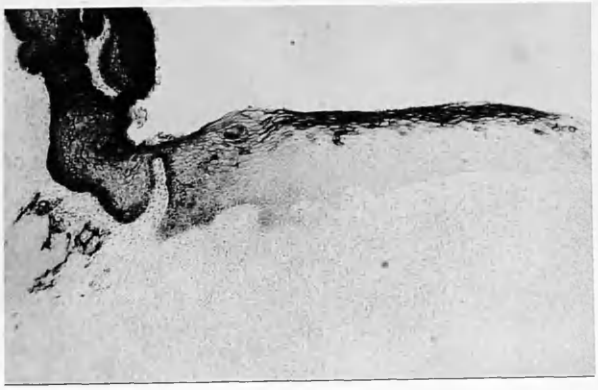

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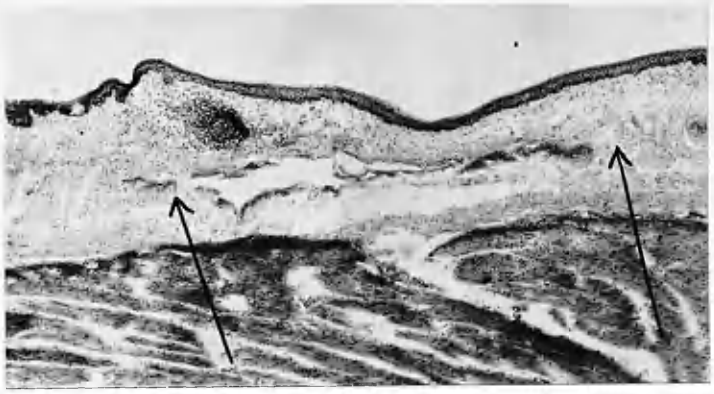

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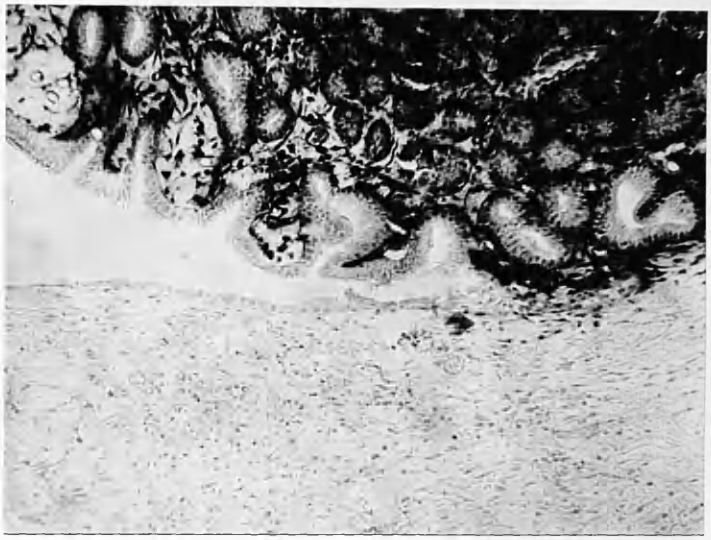

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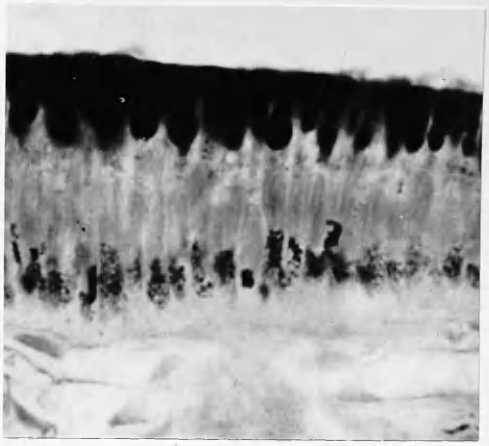

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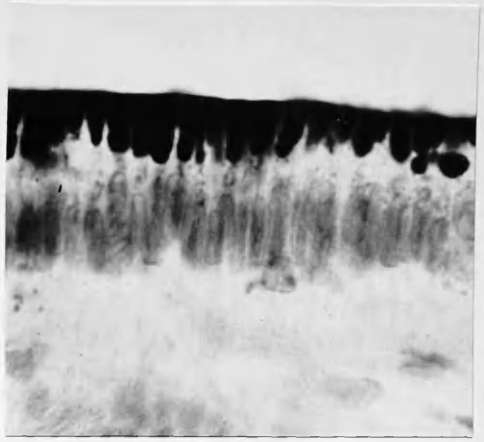

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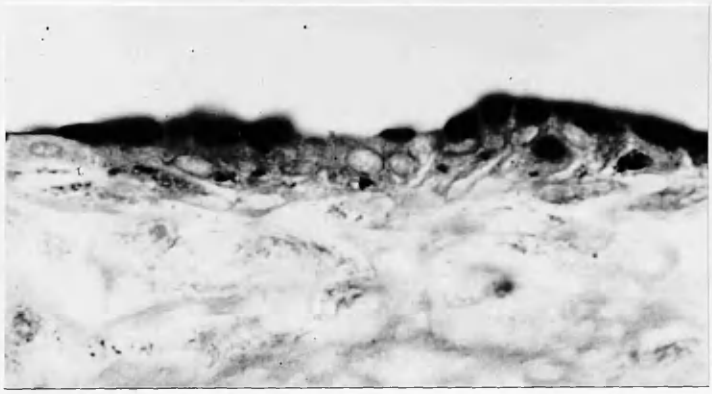

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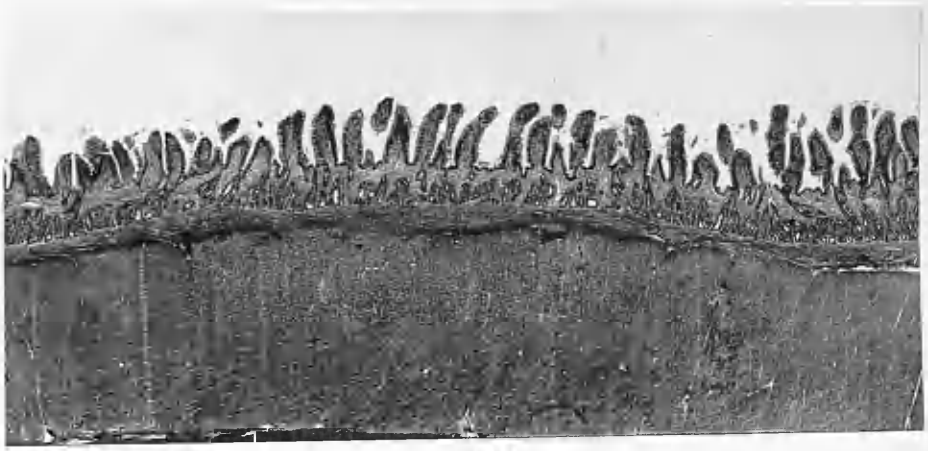

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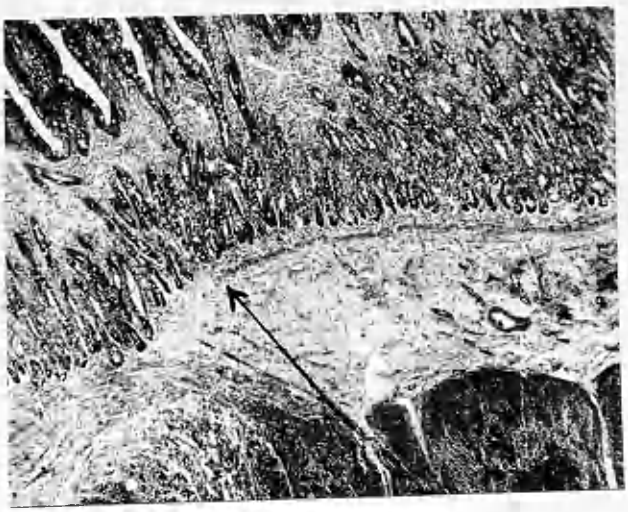

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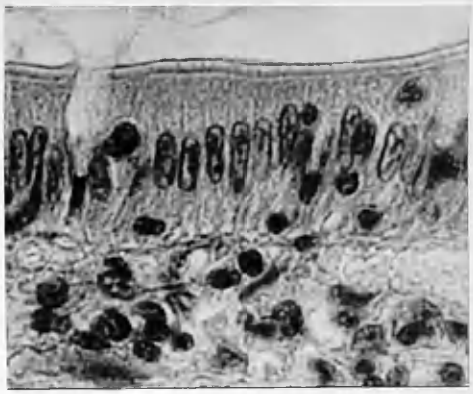

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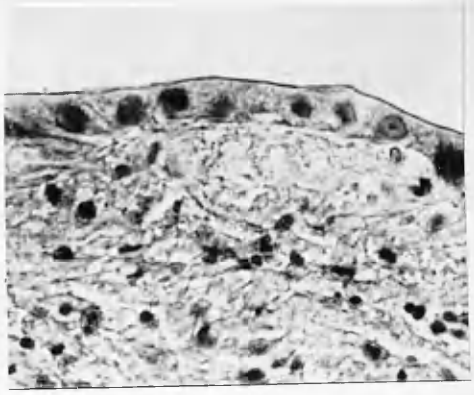

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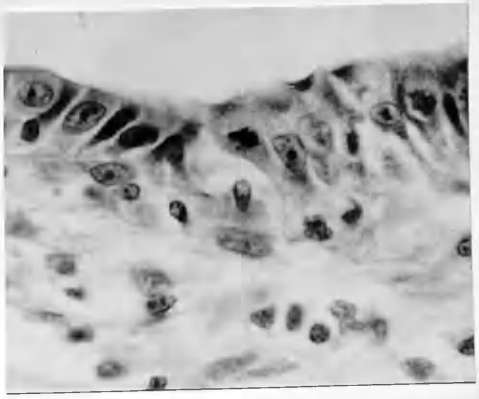

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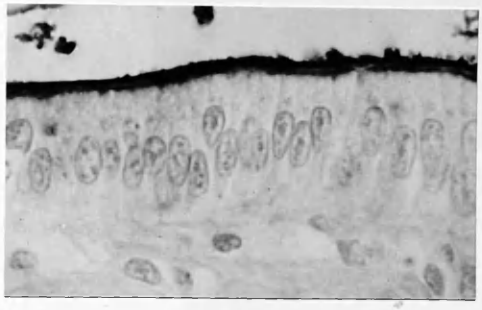

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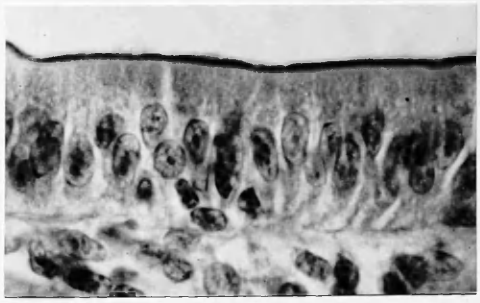

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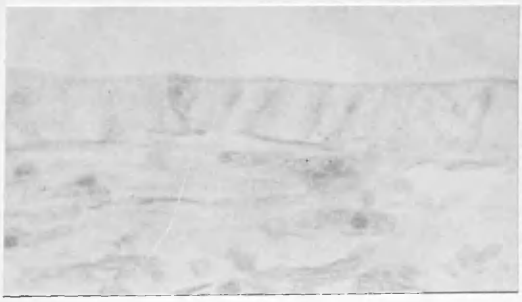

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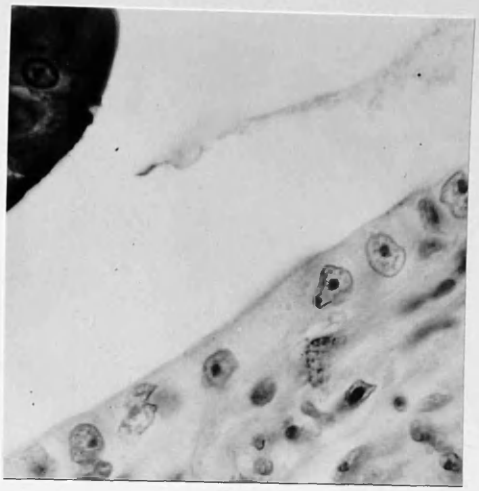

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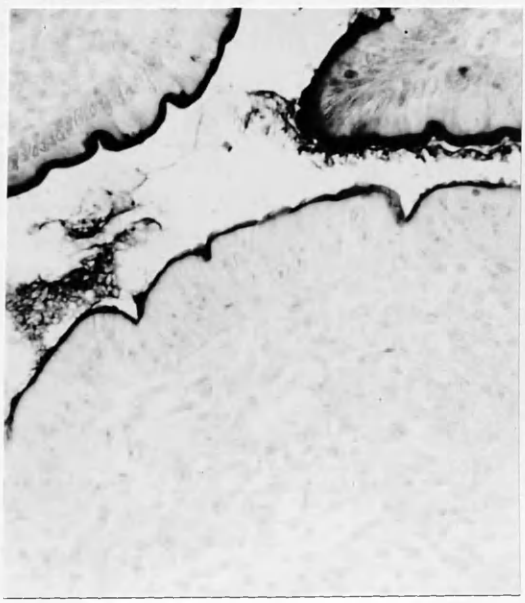

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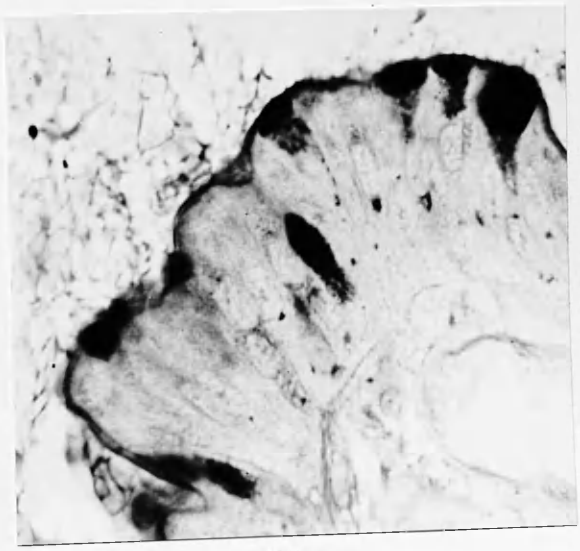

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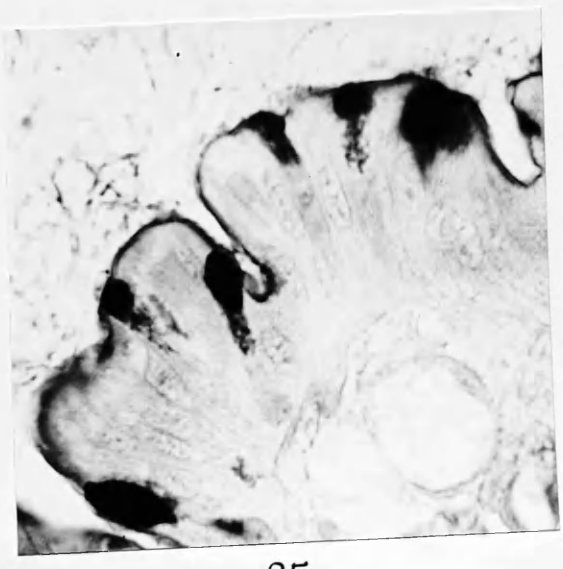

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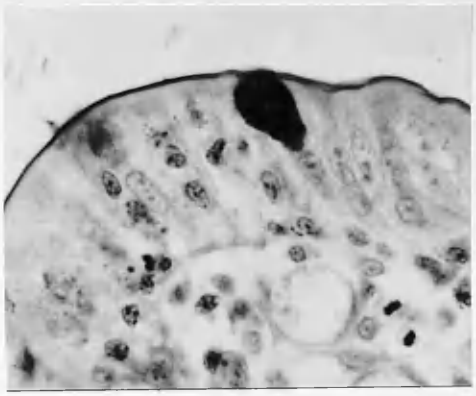

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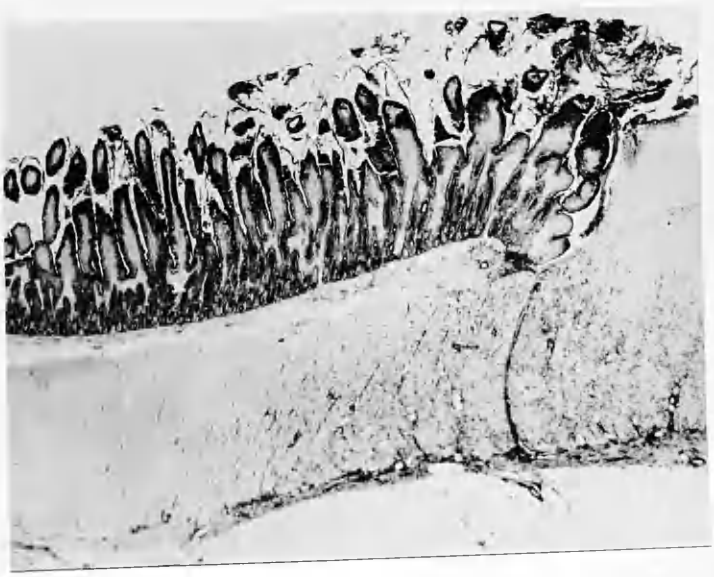

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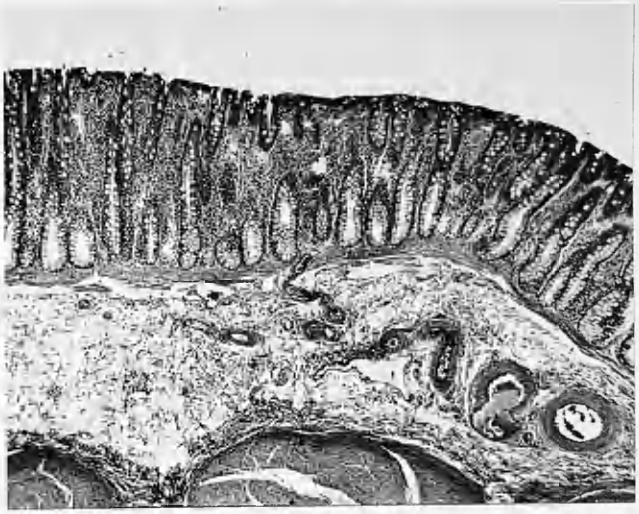

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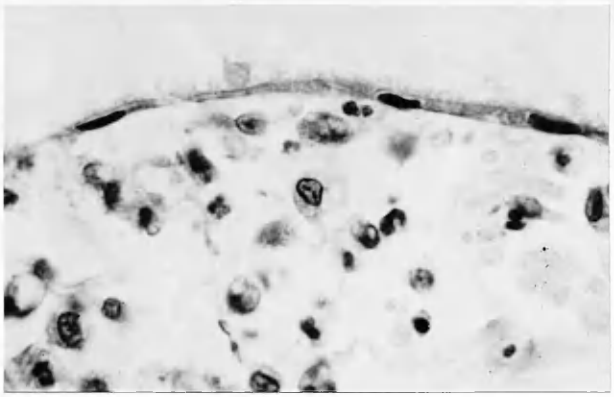

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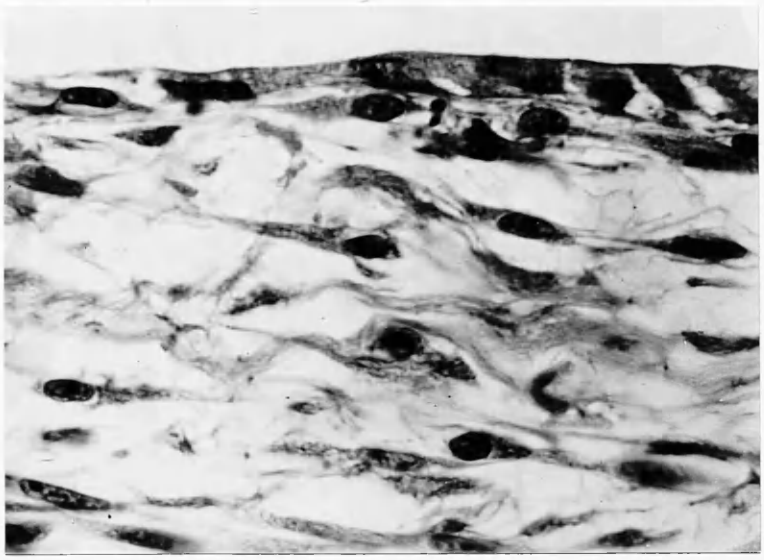

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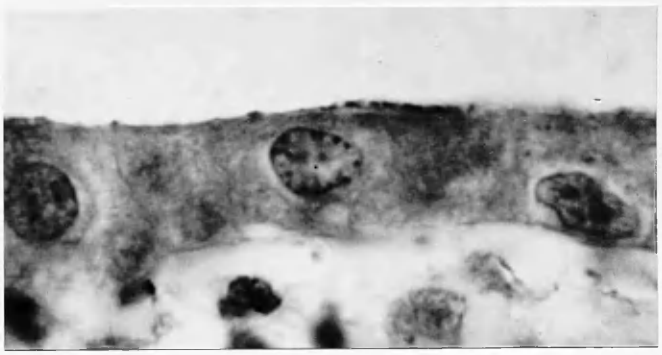

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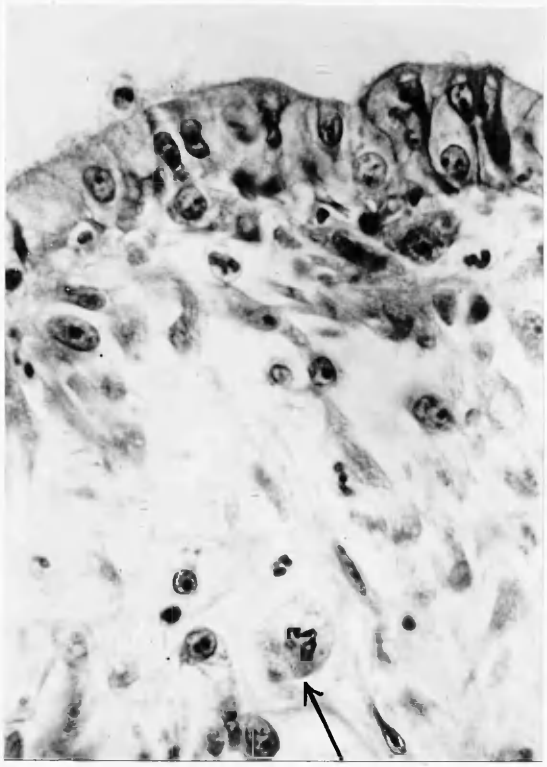

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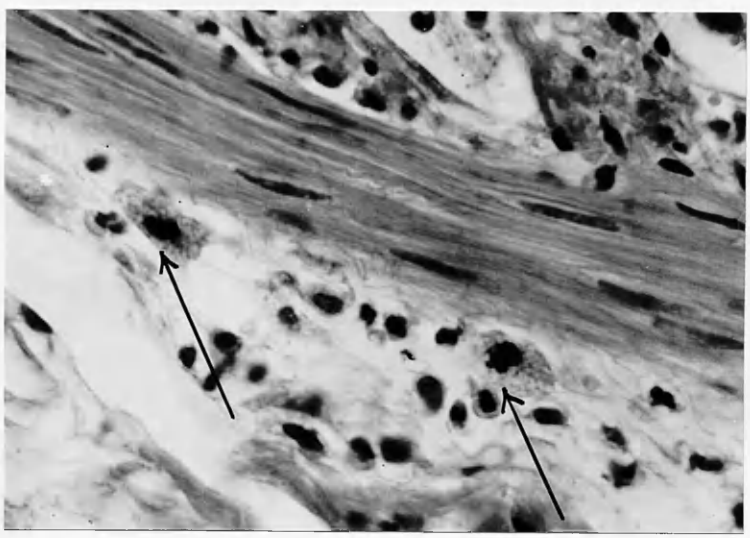

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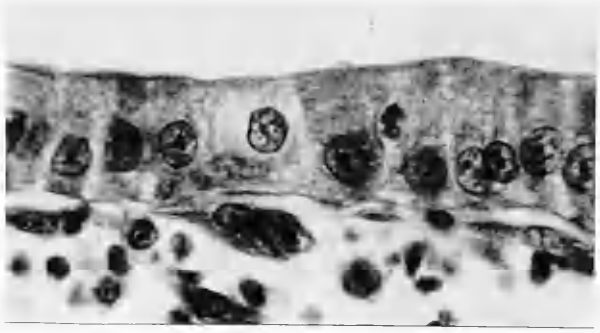

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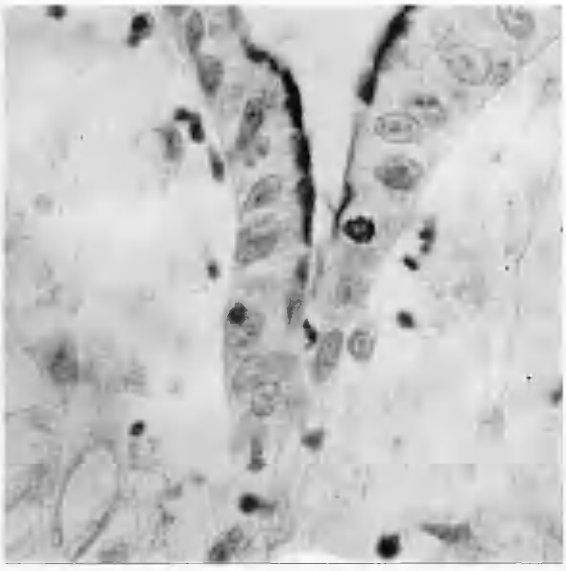

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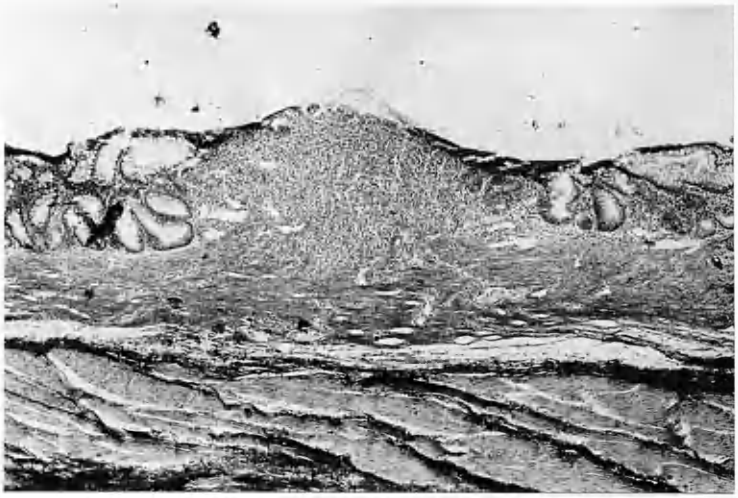

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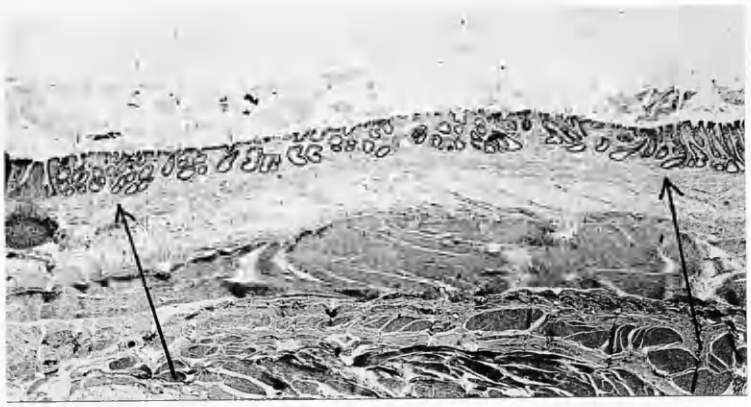

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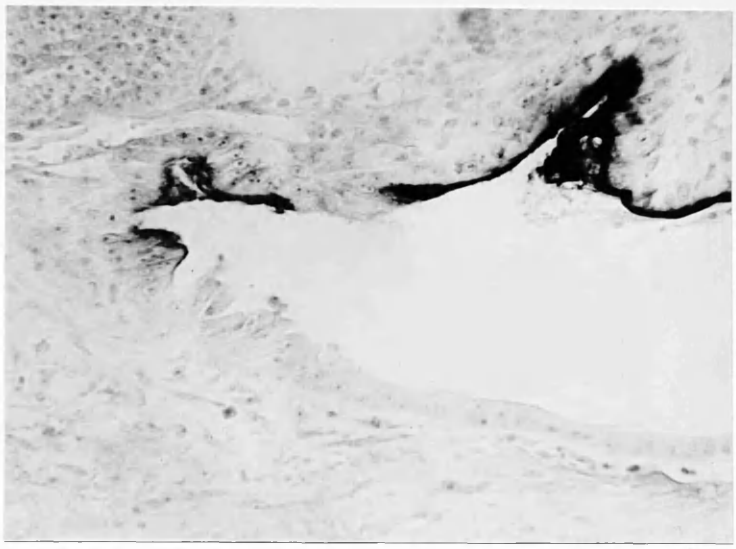

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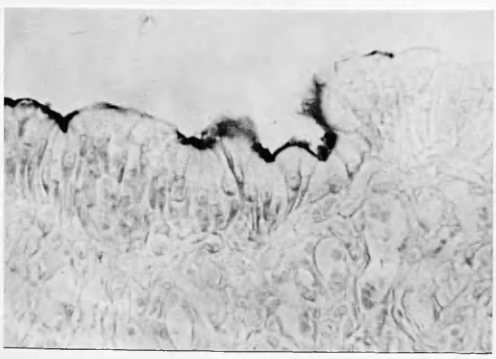

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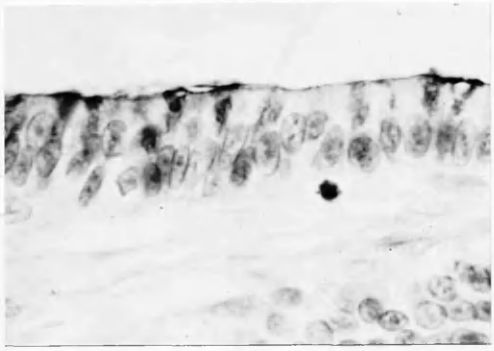

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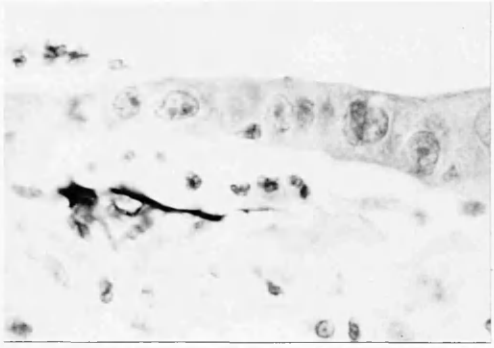

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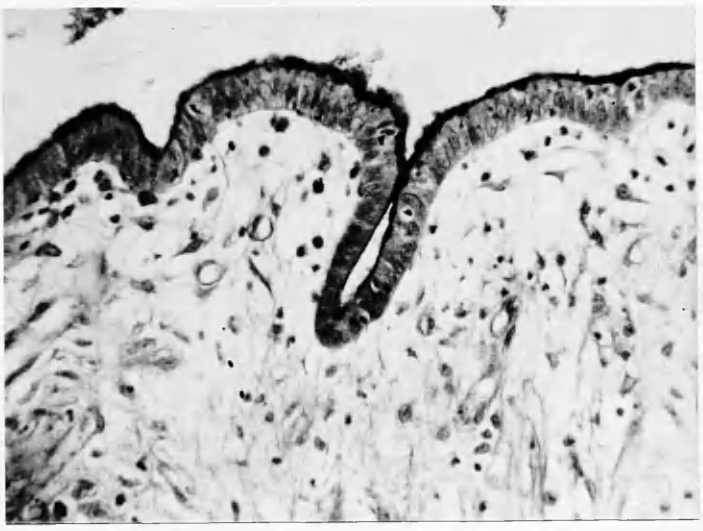

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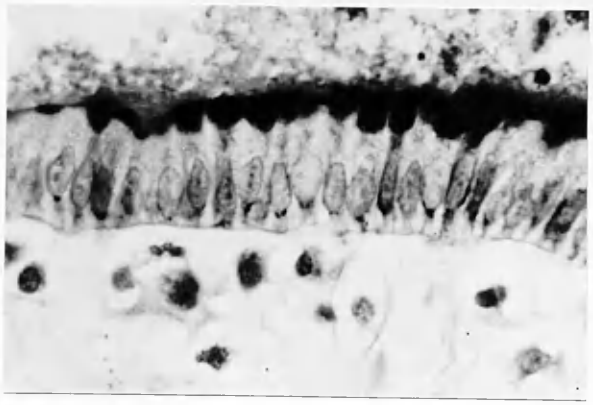

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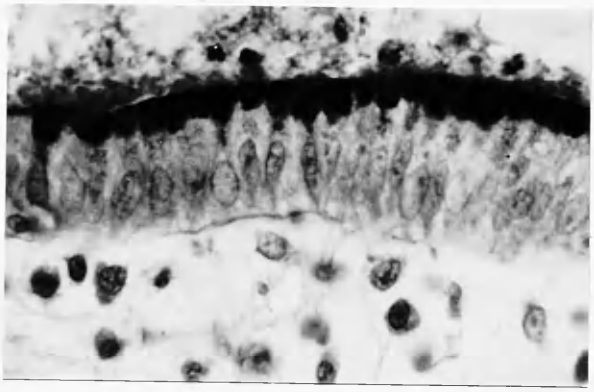

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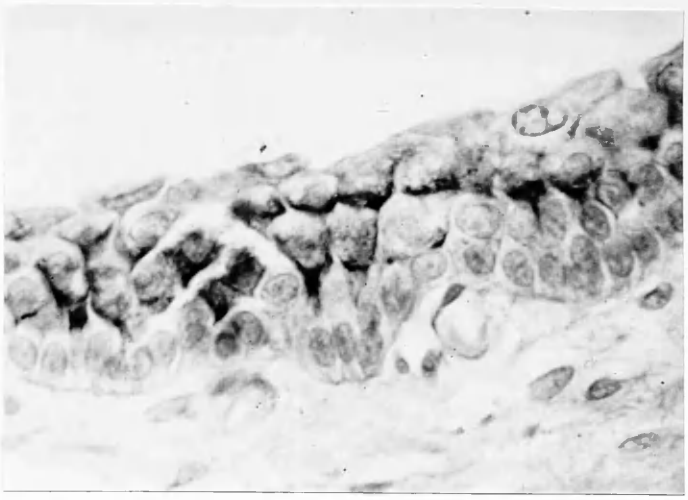

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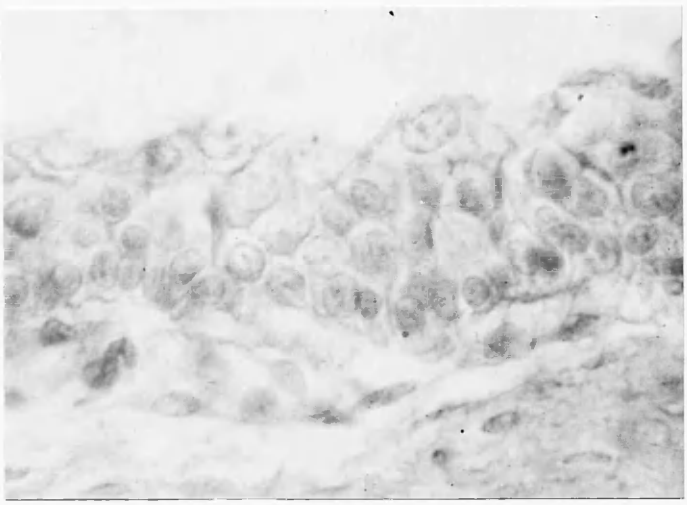

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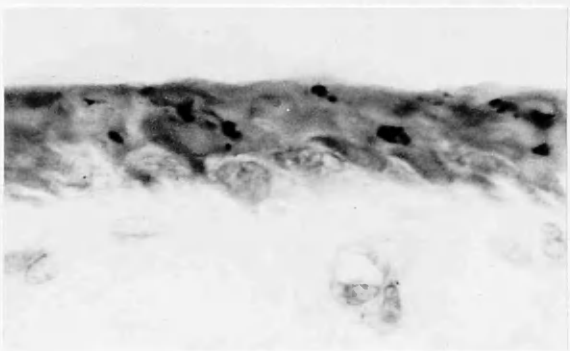

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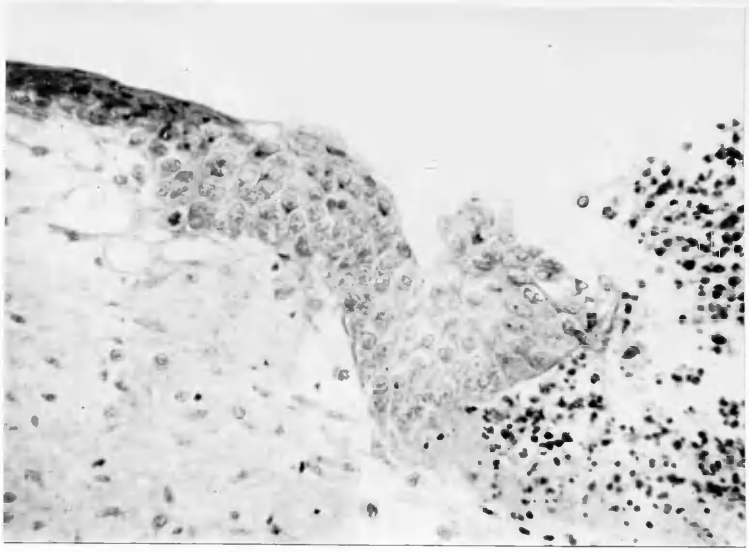

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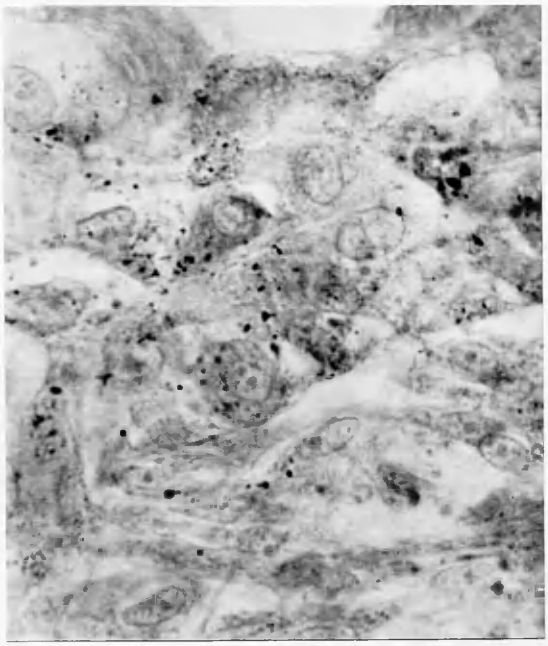

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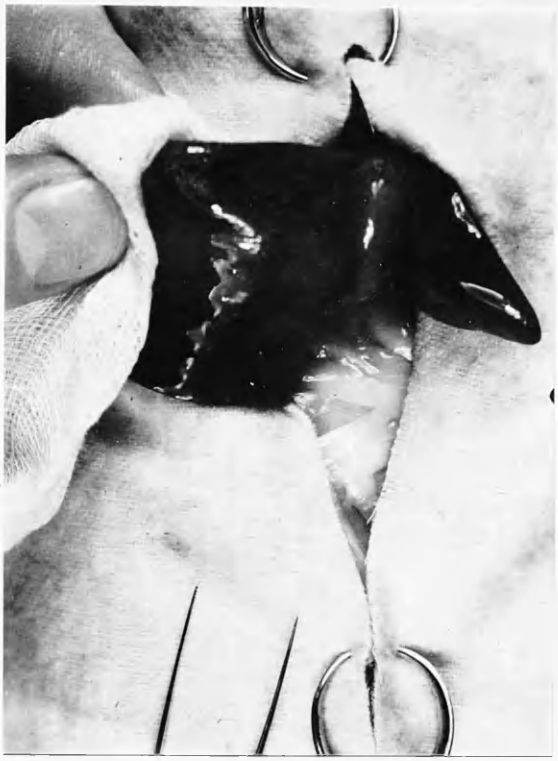

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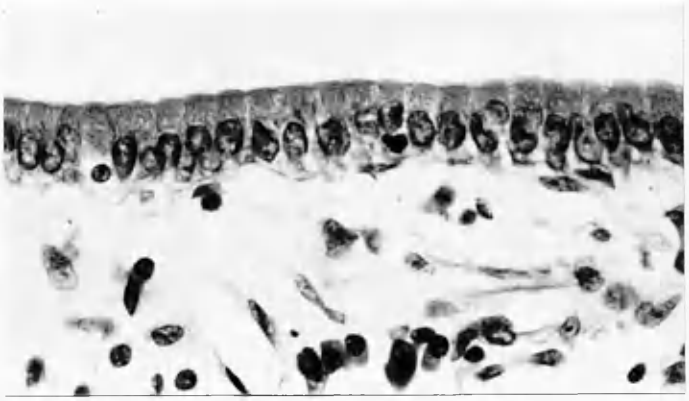

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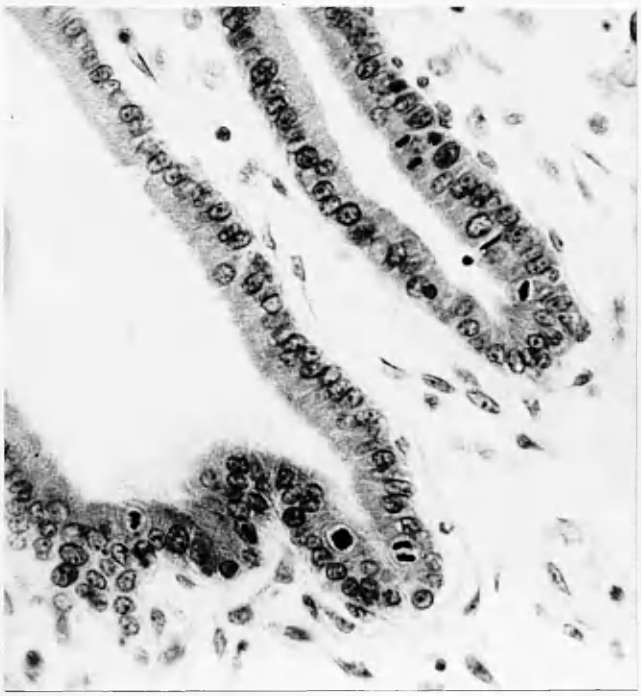

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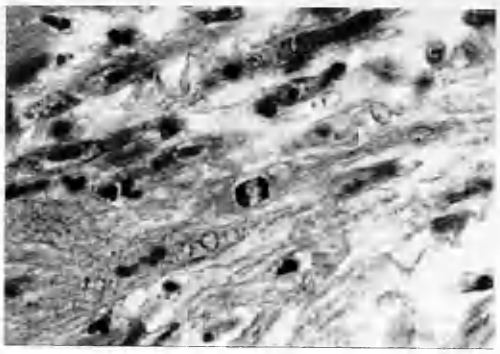

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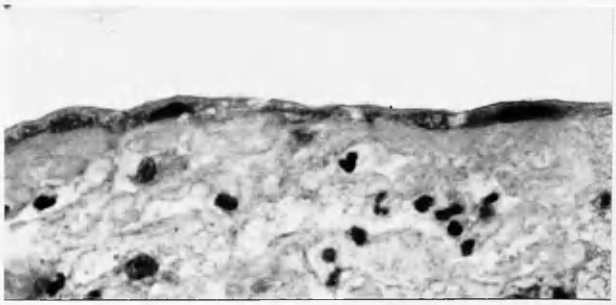

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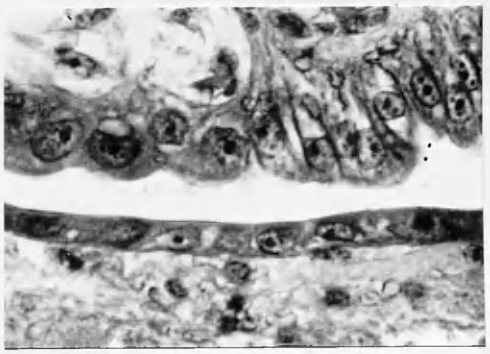

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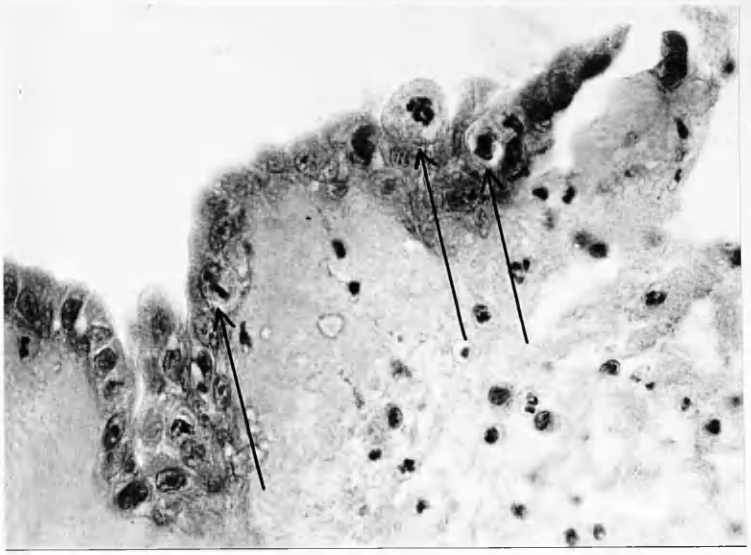

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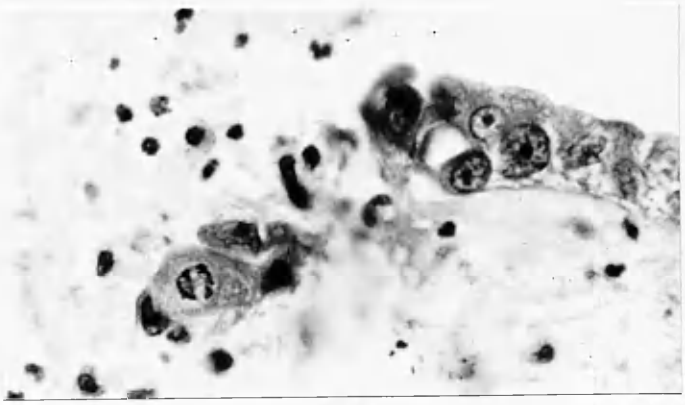

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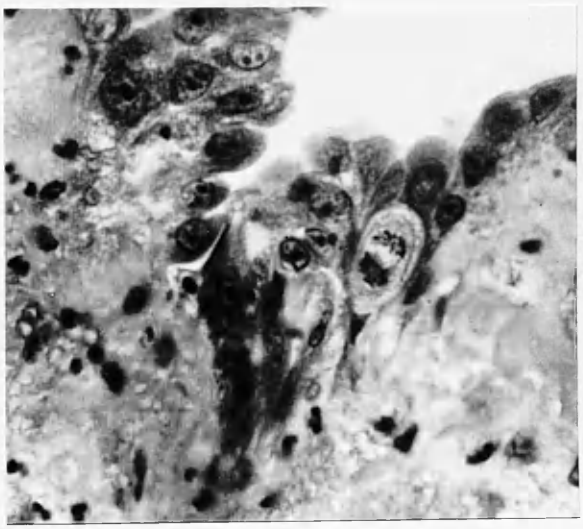

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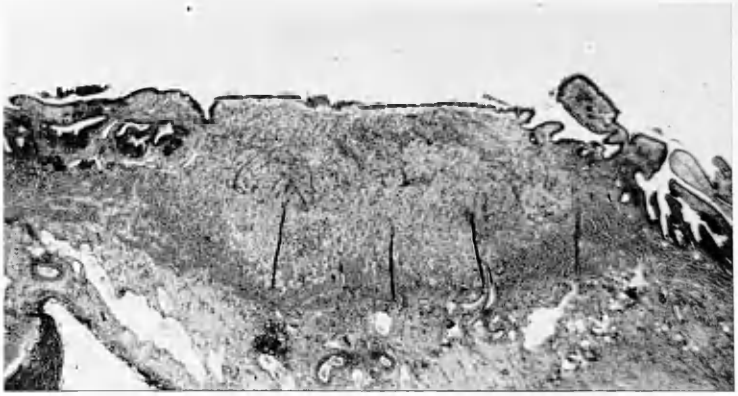

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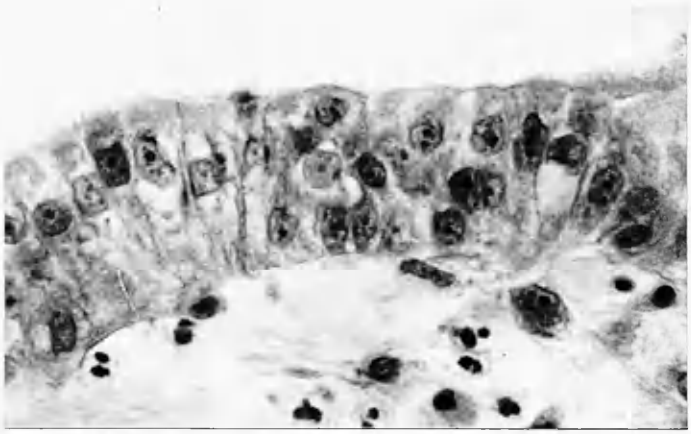

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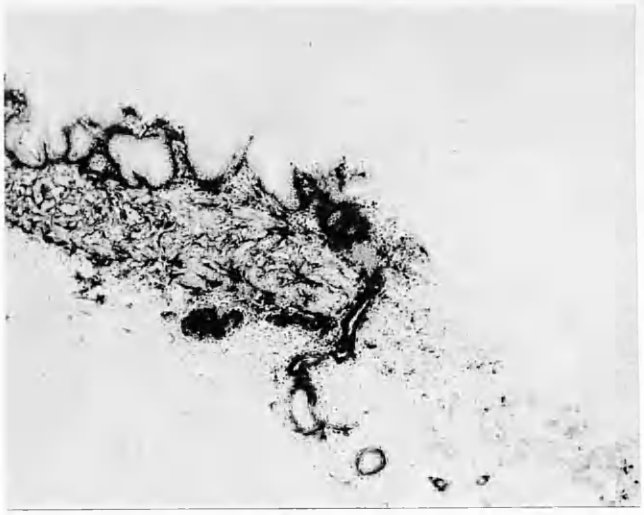

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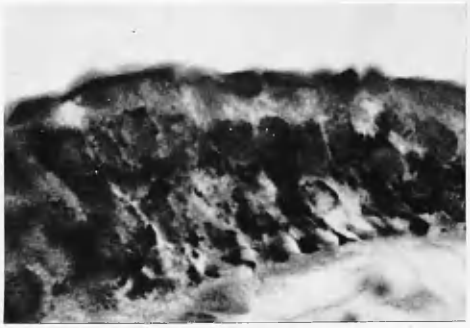

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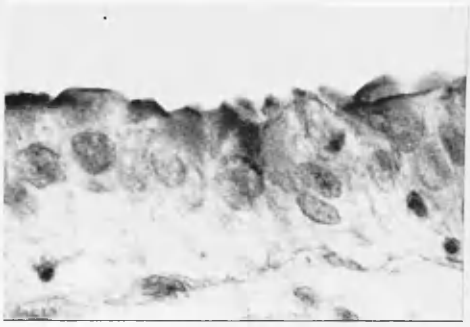

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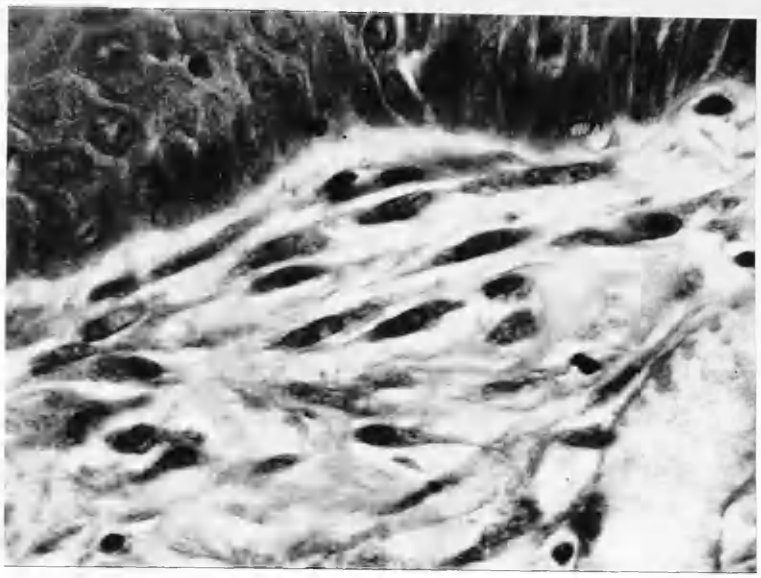

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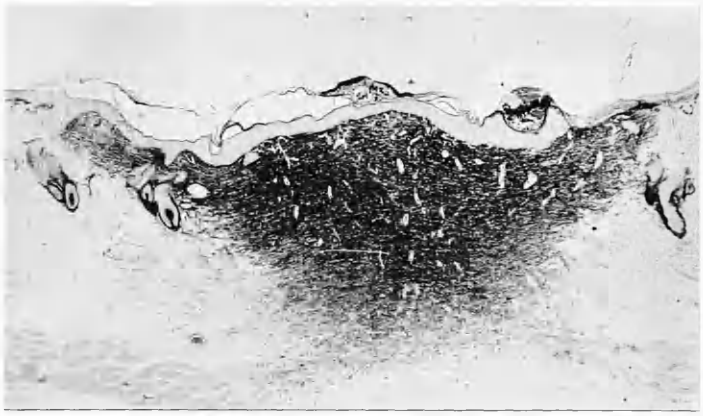

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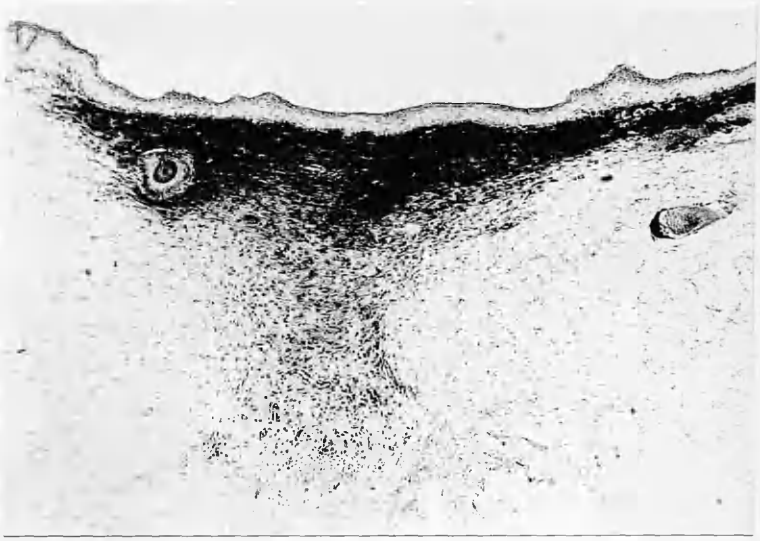

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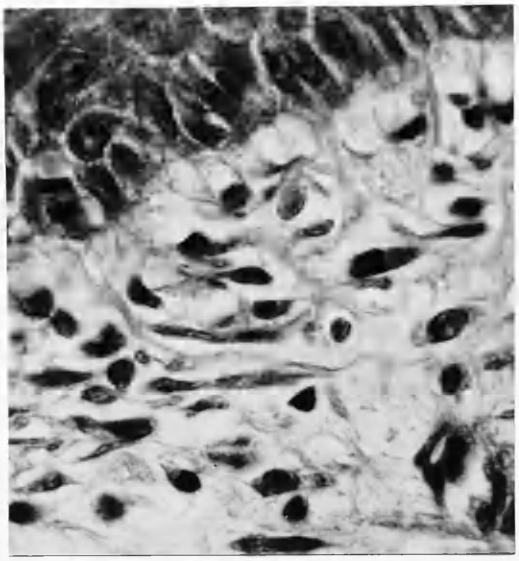

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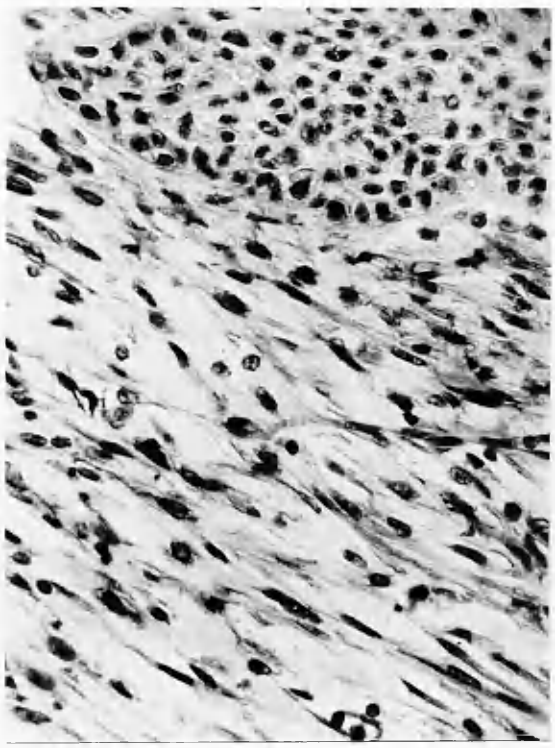

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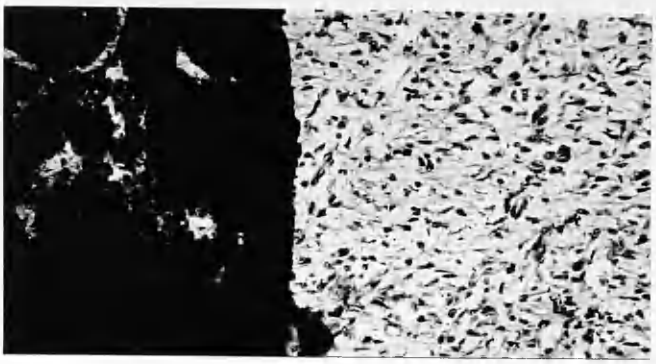

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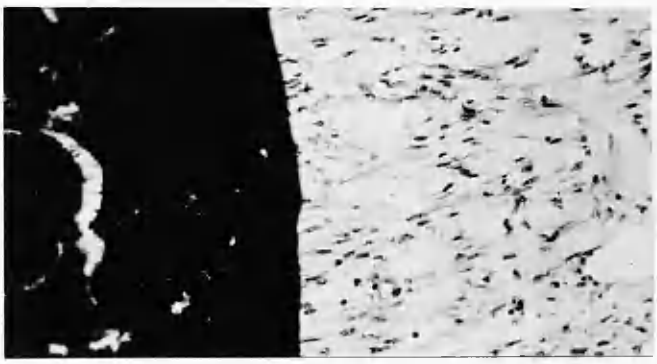

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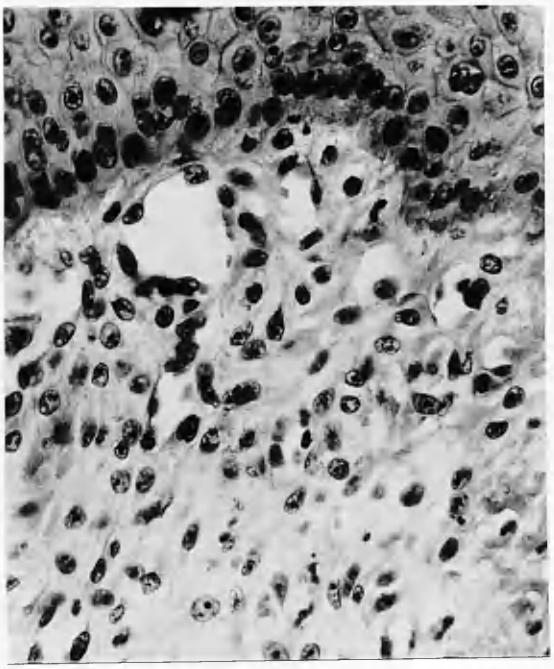

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