A HISTOCHEMICAL STUDY OF ALKALINE PHOSPHATASE, DOPA OXIDASE AND GLYCOGEN DURING THE PROGRESSIVE STAGES OF WOUND HEALING IN THE ALBINO RAT

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A THESIS

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INTRODUCTION

The presence of phosphatases, oxidases and glycogen have been demonstrated in the tissues of various organisms. The phosphatases as dehydrogenases remove hydrogen from substrates, leaving it free to unite with activated oxygen which, in the presence of an oxidase, serves as an acceptor of hydrogen. Glycogen, following amination and degradation processes, acts as the building stone in tissue growth and repair or as a source of energy.

The knowledge of their presence in both plant and animal tissues and of their role in cellular metabolism has led us to investigate their role during certain metabolic conditions. Because injuries accelerate metabolic activity for repair in localized areas, the investigator has approached the problem through a study of the activities of alkaline phosphatase, dopa oxidase and glycogen during the process of wound healing in the skin of albino rats. By demonstrating their presence during progressive stages of healing, it was hoped that evidence would be obtained concerning the time they became active during healing, the time of maximum activity, and their role in the healing of experimentally induced wounds in the Sherman strain of albino rats.

REVIEW OF LITERATURE

Studies of the phosphatases began with the discovery of their presence in plants. Later, other studies were made on animal tissue as well. Robinson ('22) suggested that phosphatases act upon hexose monophosphate in the presence of soluble calcium salts forming a precipitate of calcium triphosphate. Robinson and Soames ('24) advanced the now generally accepted theory that alkaline phosphatase activity is a causative factor in the deposition of bone salts. Annersten ('40) was able to detect an increase in the callus tissue of healing bones. Moog ('44) noted its presence more generally in the nuclei than the cytoplasm in the early embryogeny of the chick. It appeared in abundance in the primitive streak and associated structures and made its appearance in embryonic structures as soon as they were set down.

Moog was of the opinion that phosphatase activity was due to the synthesis of proteins under the influence of the phosphate-bearing nucleic acids. Dean ('45) observed alkaline phosphatase in the Golgi region of the cells of the intestinal villi and in the epithelial cells of the uterus and epididymis in the rat, mouse, hamster, guinea pig, bat and deer. Johnson ('45) observed it in the developing hair follicles in rats and found that its activity decreased as the hair elaborated. Danielli ('45) found that a vitamin C deficiency would reduce its activity and in addition would cause retarded wound healing. Mayson ('49) observed an increase in its activity during morphogenesis and a decrease when cyto-differentiation occurred. An increase mainly in the nuclei observed by Mayson, confirms Moog's idea that the enzymes play an important role in the phenomenon of

organogenesis. Further studies on the activities of the phosphatases were those of Elliott ('51) who observed in Tetrahymena phosphatases capable of hydrolyzing nucleic acids and their components at a rapid rate, and the experiments of Leduc ('52) who demonstrated alkaline phosphatase in the central nervous system in studies of hematoencephalic barriers.

The name dopa oxidase was given to an enzyme which was found to oxidize ℓ-3,4 dioxyphenylalanine (dopa) to melanin. Melanoblasts and leucocytes react positively and specifically with dopa, while pigment cells of the malpighian layer and the dermal chromatophores do not. Laedlow ('32) found that the number and complexity of dopa-positive cells correspond to the rate of pigment formation and suggested that the dopa-positive cells are probably melanin, makers of human skin and the ectodermal mucous membrane.

Herman and Boss ('45) obtained evidence that cytochrome oxidase and succinic dehydrogenase are associated with dopa oxidase during the oxidation of dopa in crude suspensions of the bovine ciliary body. Holtz and Credner ('48), by injecting the d- or d,ℓ-dopa into rats, collecting the urine, and determining its hydroxytyromine content by the increase in blood pressure, found that after the administration of d,ℓ-dopa, more hydroxytyromine was formed and excreted than could be formed from the ℓ-component alone. They concluded that in the body of the rats the d-dopa must be converted into ℓ-dopa, which then undergoes decarboxylation. Similar experiments on the guinea pig indicate that they can only convert the ℓ-dopa compound into hydroxytyromine. The difference in metabolism of the d,ℓ-dopa by these two animals is ascribed to differences in concentration

of d-amino acid oxidase in the tissues. Barov ('42) was unable to demonstrate the presence of dopa oxidase in hydrolyzates of proteins.

Glycogen has been demonstrated in various tissues. Its presence has been demonstrated during regeneration and in embryonic development, at which time the active regions give intense reactions. During development glycogen serves to furnish the energy necessary for morphogenetic movement growth and differentiation.

Harvet ('42) demonstrated its importance in the connective tissues of the theca interna of the ovarian follicles, in the cells of Sertoli of the testis, in the cells of Kupffer in the liver, and the connective tissue fibrils of the placenta. It is involved in the process of transfer and storage. In all cases studied the connective tissue fibrils were shown to form a network around both the capillaries and the organ tissue and was present in the form of granules.

Johnson, et. al. ('45) observed both glycogen and alkaline phosphatase in the developing hair bud. Glycogen remained abundant while the phosphatase became less active with elongation of the hair. Mancini ('48) made a comparative study of tissue fixed by the chemical fixation method, the frozen-dried method and smears. He reported that glycogen could be observed in greater amounts and was more regularly distributed in the frozen-dried sections and in the smears than it was in the chemically fixed tissue. He observed during skin regeneration an increased amount of glycogen which was more widely distributed at that time.

These investigations have established evidence for the possibility of determining the role and activity of alkaline phosphatase, dopa oxidase and glycogen during wound healing.

MATERIALS AND METHODS

Forty-five adult albino rats of the Sherman strain were employed in these experiments. The rats were secured from the Rockland Farms, Rockland County, New York. All animals were kept in cages in the laboratory and fed a diet of Gaines Krunchons, carrots and lettuce. The rats were anesthetized with ether, shaved across the area of the shoulders and a one-inch long wound was made between the shoulders. Five series were run, each series consisting of nine rats. The stages used in this investigation were wounds which had healed for one, two, three, four, five, fix, twelve, twenty-four and forty-eight hours.

Three pieces of tissues were taken from each wound and individually fixed for alkaline phosphatase, dopa oxidase and glycogen as outlined in the methods given below.

Alkaline phosphatase:(By the Gomori Revised Method for Alkaline Phostase)--

1. Fresh tissues under two millimeters thick were placed in chilled absolute acetone and fixed for 12-24 hours in a refrigerator. The fixative was drained off.

2. To prevent the tissue from breaking up when floated on warm water, it was impregnated in a five per cent solution of acetylcellulose in acetone for 24 hours.

3. The fluid was rapidly drained off and the tissue cleared in two changes of benzol for 30 minutes each.

4. The tissue was embedded in three changes of paraffin for twenty minutes each. The second change was carried out *in vacuo*.

5. The tissue was blocked and sectioned at 12 microns and floated on the slides.

6. The slides were allowed to dry for 10 minutes, run through xylol and the alcohols to distilled water.

7. Incubated tissue for 4-6 hours at 37° C. in substrate medium, pH 9.4 (25 ml. 2% sodium glycerophosphate, 25 ml. 2% sodium barbitol, 50 ml. distilled water, 5 ml. 2%calcium chloride, 2 ml. 2% magnesium sulfate and a few drops of chloroform).

8. Slides were washed in water, immersed in 2% cobalt solution for 5 minutes, rinsed in three changes of distilled water.

9. Placed in dilute ammonium sulfide for 1-2 minutes.

10. Washed in water, counterstained with hematoxylin, dehydrated and mounted in balsam.

Controls were treated as above except for the absence of glycerophosphate from the substrate. Sites of phosphatase activity appeared brown or black.

<u>Dopa Oxidase Fixation</u>: (By the Laidlaw Method for Dopa Oxidase)—

1. Fix tissue in 5% formalin 2-3 hours.

2. Carry through 70%, 50% and 35% alcohols to water, wash in running water 12 hours.

3. Place in gum-syrup moss 24 hours.

4. Wipe off gum-syrup, place in gum acacia on freezing microtome and freeze. Section at 50 microns.

5. Rinse sections in distilled water for a few seconds, transfer to buffered dopa solution, pH 7.4 (2 ml. potassium dihydrogen phosphate,

6 ml. disodium hydrogen phosphate, 25 ml. dopa stock solution--0.3 gm. dopa in 300 ml. cold water). Sections incubated at 37° C. until solution became sepia, 3-4 hours.

6. Clear, mount in balsam.

Controls were treated as above, but incubated in Buffered Solution for Controls (25 ml. distilled water replaced stock dopa). Dopa oxidase was indicated by blackening in the sections.

Glycogen Fixation: (By Bensley's Bauer--Feulgen Stain for Glycogen)--

1. Small pieces of tissues fixed for 24 hours in ice cold alcohol-formol solution (9 vol. absolute alcohol, 1 vol. neutral formalin saturated with picric acid).

2. Wash tissue with absolute alcohol, clear with benzol, embed in paraffin, section at 12 microns. Deparaffinize, run through alcohols to water.

3. Place in 4% chromic acid for one hour or in 1% overnight.

4. Wash in running water for 5 minutes, place in Feulgen reagent 10-15 minutes.

5. Rinse 1.5 minutes each in three changes of bisulfite solution, wash in running water for 10 minutes.

6. Counterstain with hematoxylin, dehydrate, clear, and mount in balsam.

Negative controls were made by digesting glycogen from the tissue with saliva. The saliva was then removed by washing in water at 37° C. Tissue was stained as previously indicated. Glycogen appeared deep red-violet.

EXPERIMENTAL OBSERVATIONS

Alkaline Phosphatase. Within the first hour of wound healing, cells containing granules of alkaline phosphatase could be seen migrating from the derma toward the site of the wound. Even at this early stage of healing, phosphatase activity could be observed within the wound. Hair follicles and capillaries gave an intense phosphatase response. Throughout the entire section, some phosphatase activity could be observed.

During the first three hours cells exhibiting phosphatase activity continued to move toward the wound (figs. 1-3). In the fourth through the sixth hour wounds, migration was less intense or could not be detected at all, and the granules were concentrated within the wound (figs. 4-6).

Wounds which were allowed to heal for twelve, twenty-four and forty-eight hours gave evidence of a possible second migrating stage. The granules observed were smaller than those of the first migration. The cells of the first migration continued to move toward the wound, and the granules reached their greatest degree of concentration within the wound at the forty-eighth hour.

Dopa Oxidase. The leucocytes and melanoblasts reacted positively in in the presence of dopa. As wound healing advanced through the first three hours, particles of black pigment indicating dopa oxidase activity could be observed from the surrounding regions in the derma toward the wounded area. There was very little concentration of pigment in the wound during the first hour but a gradual increase could be observed as healing continued (figs. 11-13). No pigmentation was observed in the hair follicles and capillaries.

Four, five and six hour sections gave evidence of only a slight degree

of migration (figs. 14-16). A second stage of migration similar to that observed with the phosphatases was evident at the twelfth hour.

Pigment concentration within the wound continued through the forty-eighth hour of healing and was at its maximum activity within the wound at this hour (figs. 17-19).

The controls for dopa oxidase presented no evidence of its presence (fig. 20).

Glycogen. In sections stained for glycogen by the Bauer Feulgen method, sites of glycogen activity were stained red violet. Glycogen was found to be distributed throughout the tissue. Except for the hair follicles and capillaries, which stained an intense red-violet, the remainder of the section stained with much less intensity. This indicated less glycogen than is found within the hair follicles and the capillaries. In the wounded area of the first through sixth hour wounds, there could be detected a gradual increase in the intensity of staining in the area of the wound and an increased distribution of the stain in that region (figs. 21-26).

At the twelfth hour and through the forty-eighth hour, glycogen distribution in the area of the wound became more concentrated in the immediate wounded area. Intensity of staining became progressively increased and reached its maximum at the forty-eighth hour (figs. 27-29), at which time it was possible to suggest that the degree of staining had reached that observed in the hair follicles and capillaries.

Controls for glycogen presented very weak indications of its presence. Where the slightest degree of stain was seen, it could be credited to incomplete digestion of glycogen from the tissue with saliva.

DISCUSSION

The presence of alkaline phosphatase and dopa oxidase, identified as lack pigmented granules, was established in the tissues of healing wounds. vidence of the presence of glycogen, which stained a red violet, was also stablished.

Alkaline Phosphatase. Alkaline phosphatase appeared in the sites of apid growth and activity. Its presence within the nuclei of cells during egeneration and morphogenesis has been attributed to its role in the synhesis of proteins under the influence of the phosphate-bearing nucleic cids. Alkaline phosphatase in the migrating cells during wound healing s probably due to the same influence. Alkaline phosphatase activity could e seen within the first hour of healing and gradually increased. At the welfth, twenty-fourth and forty-eighth hour it was at its highest rate of ctivity, being present in an amount greater than at any other stage. rey ('32) and Herrick ('32) described a migratory response of the epihelial and connective tissue cells during wound healing. The increased ctivity of the cells at wound healing is probably the source of the inreased phosphatase observed, since, during increased activity, there is n increase in the metabolic rate of the cells.

Dopa Oxidase. Evidence of dopa oxidase activity was observed at the irst hour of healing and became progressively more active through the orty-eighth hour. The activity of dopa oxidase observed during these xperiments was due to the positive reaction of the migratory cells to he dopa which was added to the substrate and probably has no direct earing on the healing of the wound in albino rats. As in the case of lbino rabbits, the albino rats, having no pigmentation, do not synthesize

dopa, which is necessary for pigmentation. This may be attributed to the absence of a gene which may be necessary for the synthesis of an enzyme somewhere in the chain of reactions from phenylalanine to tyrosine to dopa which blocks the reaction before reaching the point of dopa formation. This position is taken in the light of the fact that the reaction proceeds to melanin formation on the addition of dopa to the substrate, and does not when distilled water is substituted for dopa as was observed in the case of the controls.

That albinism represents a case of genetically determined inability to convert precursor substances into melanin is well substantiated by the conclusions of Beadle and Tatum ('41) who found that the ability to synthesize any particular enzyme is dependent upon the presence of a particular gene. The investigator is of the opinion that due to the lack of a gene for the synthesis of dopa in albino rats, the oxidase activity observed was due to the dopa added to the substrate to which the migrating cells gave a positive reaction, and the acceleration of activity was due to the increased metabolic rate of the cells during the wound healing process.

Glycogen. The glycogen response during wound healing was also accelerated. The acceleration began during the first hour and became progressively greater through the forty-eighth hour at which time it reached its peak. Glycogen acceleration in these experiments is in keeping with the generally accepted theory that there is an acceleration in carbohydrate metabolism during regeneration and growth.

In the light of the fact that glycogen can be converted into glucose

and serve as a source of energy or be converted to amino acids and serve as building stones during growth and repair of tissue, it is possible to assume during the healing of a wound, when the metabolic rate of the cells is accelerated, that the need for glycogen as energy and as amino acids for building materials by the cells would be greater than that of normal tissue. Therefore, glycogen would be present within the tissues in an increased amount at the time of wound healing.

SUMMARY AND CONCLUSIONS

1. Phosphatase activity is accelerated during wound healing, beginning at the first hour and reaches its height of activity at the forty-eighth hour. Its synthesis is probably due to the synthesis of proteins under the influence of the phosphate-bearing nucleic acid during the increased metabolic rate occurring during wound healing.

2. The activity of dopa oxidase in wound healing begins within the first hour and reaches its maximum at the forty-eighth hour. The dopa oxidase activity observed was due to the positive reaction of the migrating cells, in the area of the wound, to the dopa added to the substrate, the metabolic rate of the cells being increased.

3. Dopa oxidase has no direct bearing on wound healing in albino rats, since these rats do not synthesize dopa.

4. Glycogen acceleration began during the first hour of healing and became gradually more pronounced up to the forty-eighth hour when it was at its greatest height. Its activity was due to the increased metabolic rate of the cells at the time of healing and served as a source of energy and of building material for tissue repair.

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PLATE 1

(Explanation of Figures)

PLATE 1

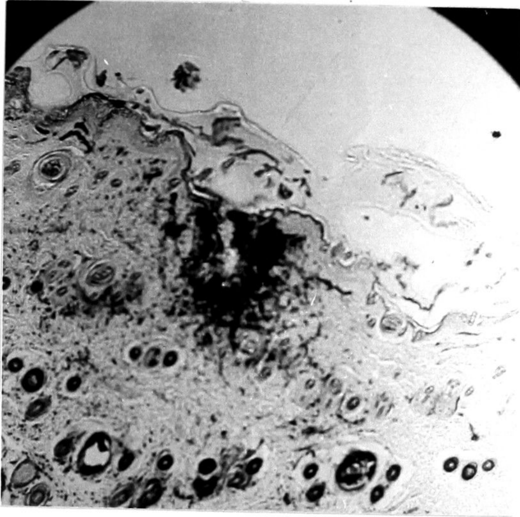
(Explanation of Figures)

Fig. 1 Photomicrograph of a one hour wound. Dense aggregate of dye granules, indicating alkaline phosphatase in wounded areas may be noted. Pigment granules in derma are in positive migrating cells. X 80.

Fig. 2 Photomicrograph of a two hour wound. Note dense aggregates in wounded area. Pigmented granules are in positive migrating cells. X 80.

Fig. 3 Photomicrograph of a three hour wound. Note pigmented granules on edges of wound and at the base. Others may be seen migrating toward the wound. X 80.

Fig. 4 Photomicrograph of a four hour wound. Note alkaline phosphatase within the wound. Cells which are alkaline phosphatase positive have declined in their rate of migration. X 80.



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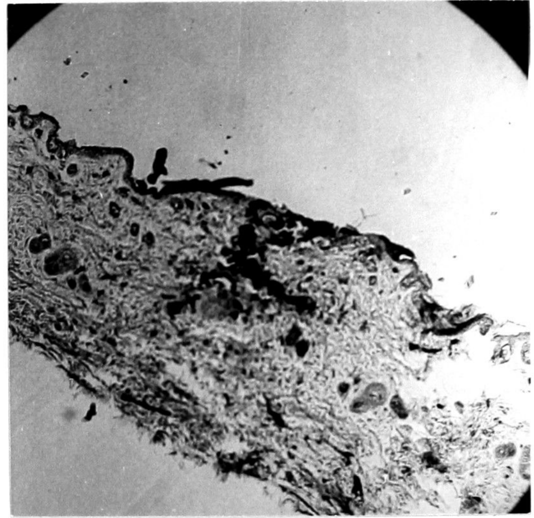

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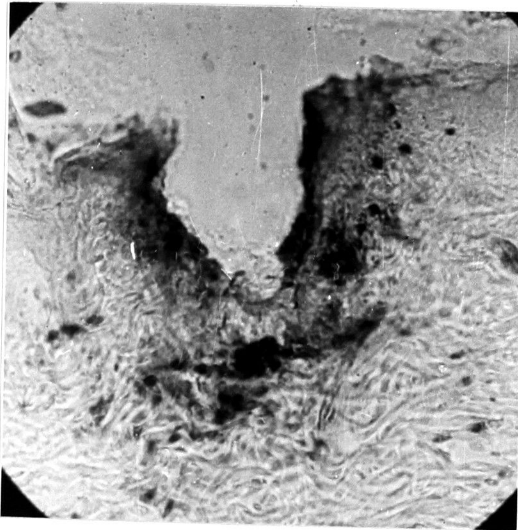

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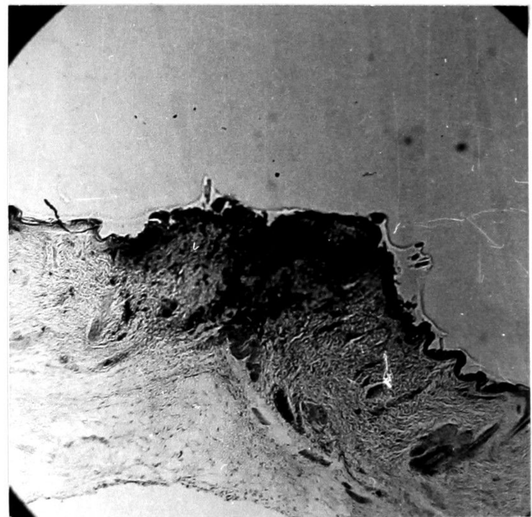

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PLATE 2

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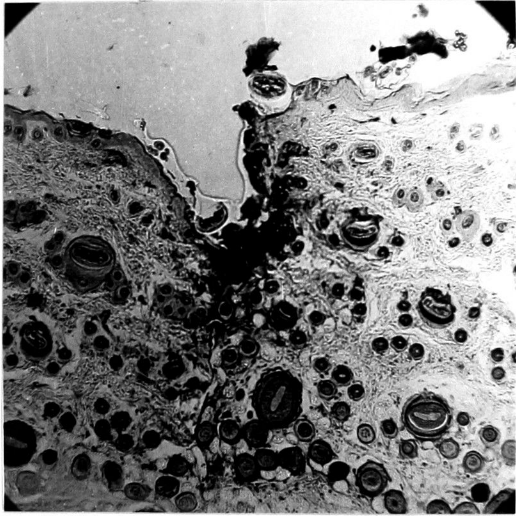
Fig. 5 Photomicrograph of a five hour wound showing alkaline phosphatase within the wound and surrounding structures. X 80.

Fig. 6 Photomicrograph of a six hour wound showing alkaline phosphatase within the wound. Activity has become progressively stronger. Note activity within the hair follicles and capillaries. X 80.

Fig. 7 Photomicrograph of a twelve hour wound showing alkaline phosphatase. The intensity of reaction has become greater. Note the migration of small granules within the cells. X 80.

Fig. 8 Photomicrograph of a twenty-four hour wound showing alkaline phosphatase activity. Note density of pigmentation. The migration of cells with small pigmented granules continues from the derma. X 80.



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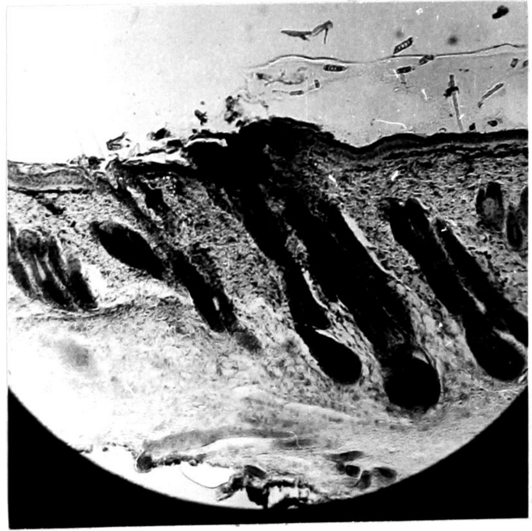

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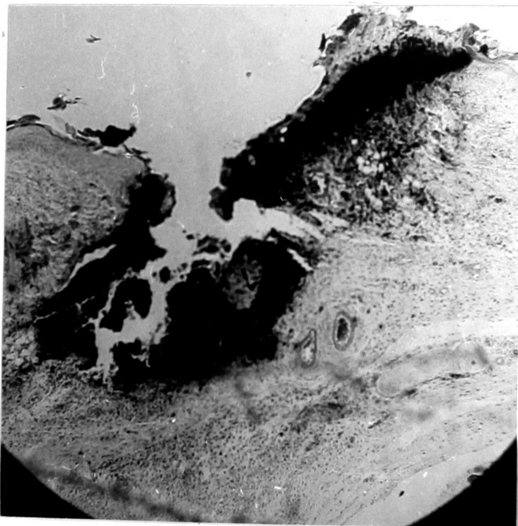

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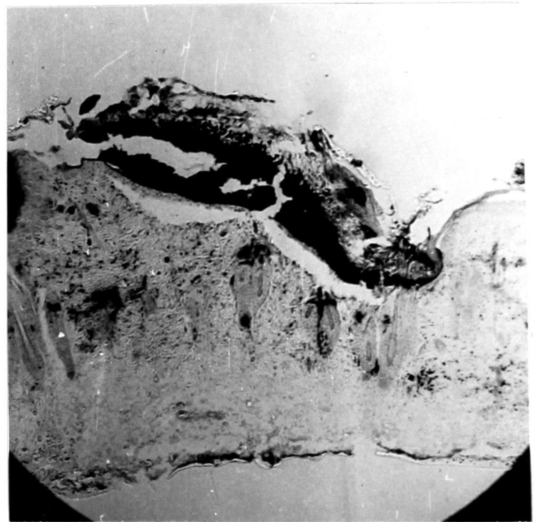

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PLATE 3

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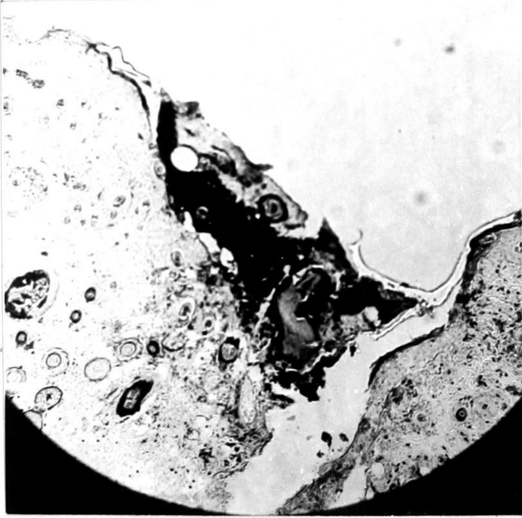
Fig. 9 Photomicrograph of a forty-eight hour wound showing alkaline phosphatase activity. Concentration of pigmented granules within the wound is at its highest peak. Migration of granules has declined. X 80.

Fig. 10 Photomicrograph of a control wound for alkaline phosphatase showing no alkaline phosphatase in wounded tissues and surrounding areas. X 80.



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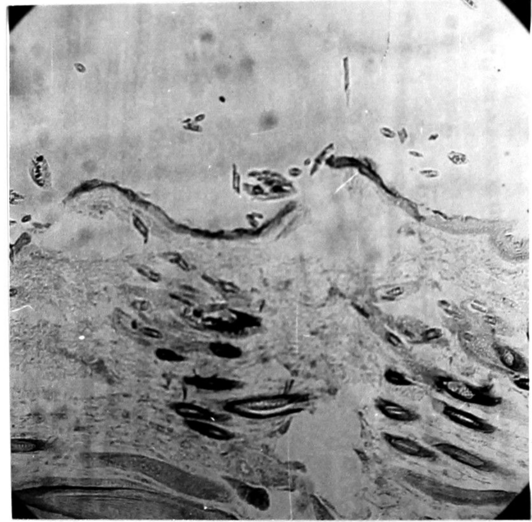

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PLATE 4

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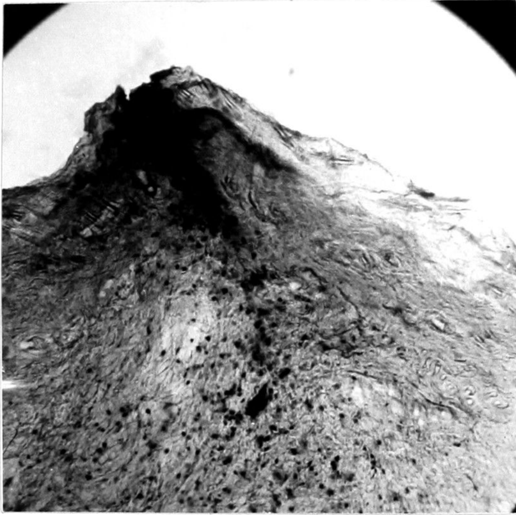
Fig. 11 Photomicrograph of a one hour wound showing dopa oxidase activity. Note migration of dopa-positive cells from the derma to the wounded area. X 80.

Fig. 12 Photomicrograph of a two hour wound showing dopa oxidase activity. Dopa-positive cells continue to migrate toward the wound. Note concentration of pigment on the periphery of wound. X 80.

Fig. 13 Photomicrograph of a three hour wound showing dopa oxidase activity. Migration of dopa positive cells is very slight. Note increased concentration of pigment in the wounds. X 80.

Fig. 14 Photomicrograph of a four hour wound showing dopa oxidase activity. Migration of dopa positive-cells toward the wound has ceased. X 80.



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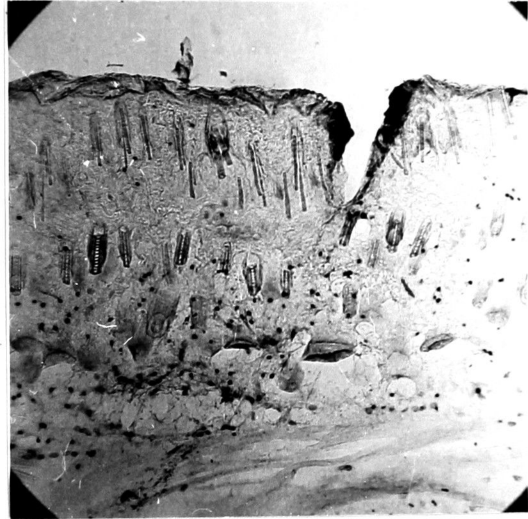

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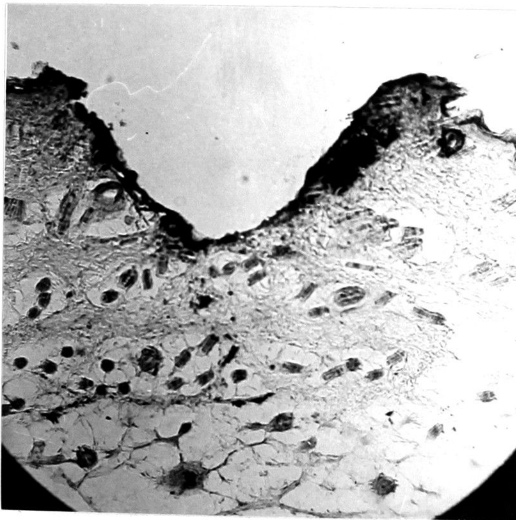

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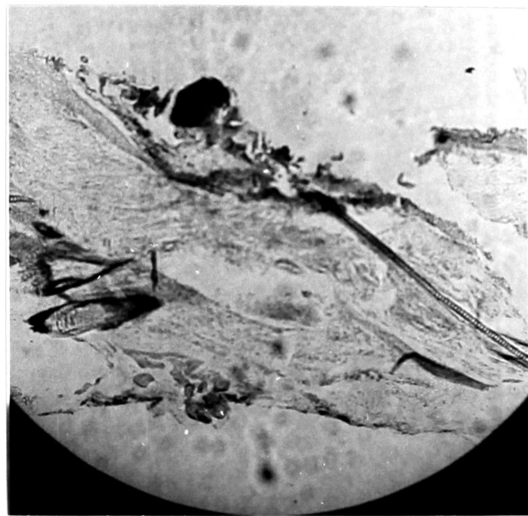

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PLATE 5

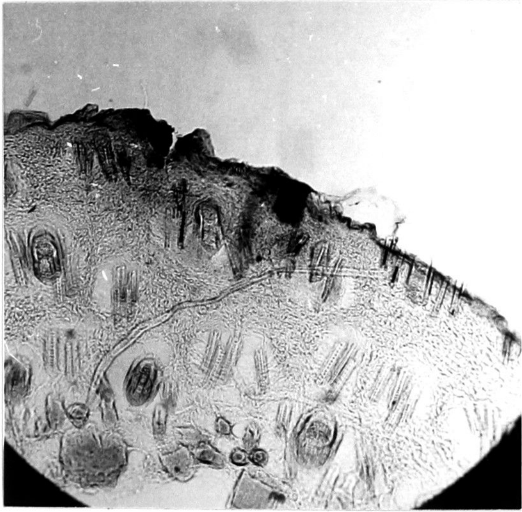
(Explanation of Figures)

Fig. 15 Photomicrograph of a five hour wound showing dopa oxidase activity. This activity can be seen only within the wound. X 80.

Fig. 16 Photomicrograph of a six hour wound showing dopa oxidase activity. Surrounding tissue shows no dopa oxidase activity. Wounded area is strongly dopa oxidase positive. X 80.

Fig. 17 Photomicrograph of a twelve hour wound showing dopa oxidase activity. Note migration of dopa oxidase-positive cells, second migrating stage. The wound is very strongly positive. X 80.

Fig. 18 Photomicrograph of a twenty-four hour wound showing dopa oxidase activity. Dopa oxidase positive cells of the second stage migration continue to migrate toward wound. Note the increased activity within the wound. X 80.



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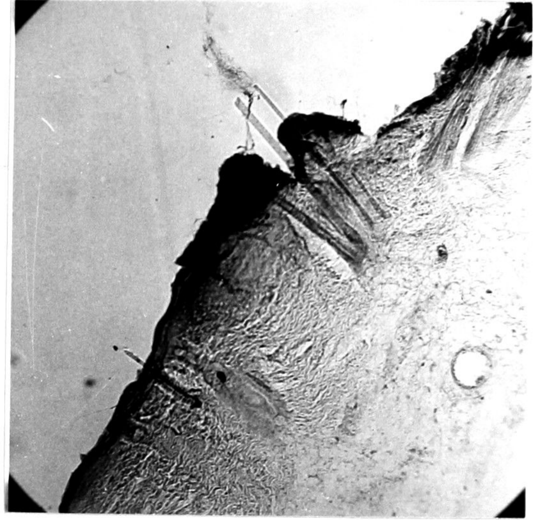

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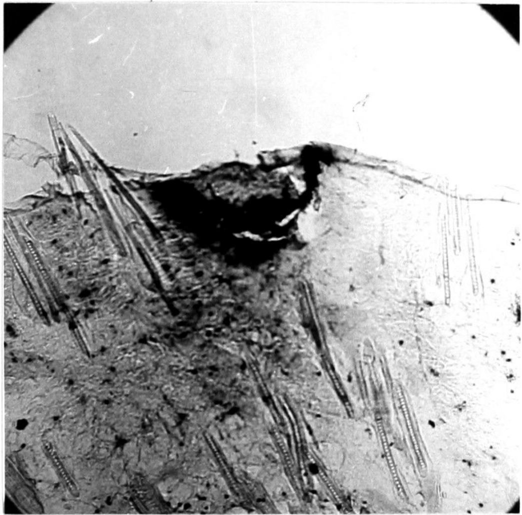

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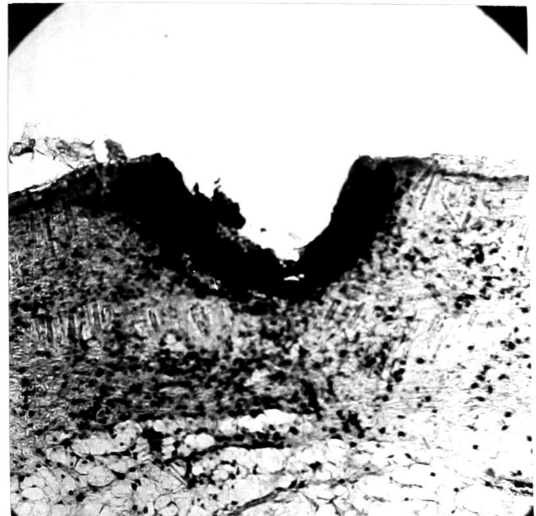

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PLATE 6

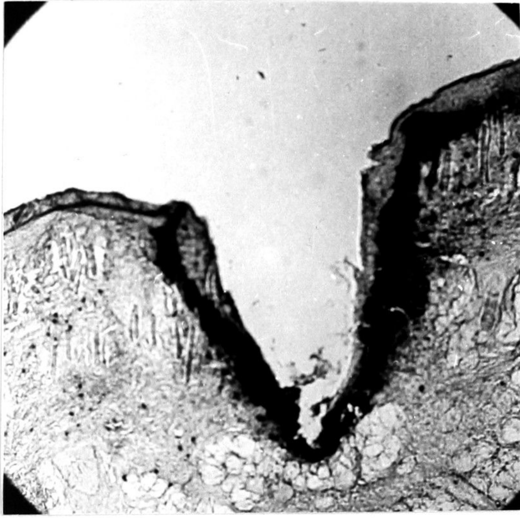
(Explanation of Figures)

PLATE 6

(Explanation of Figures)

Fig. 19 Photomicrograph of a forty-eight hour wound showing dopa activity. The second stage migration has nearly ceased. Concentration of pigment within the wound is at a high degree. X 80.

Fig. 20 Photomicrograph of a control wound for dopa oxidase showing the absence of dopa oxidase. X 80.



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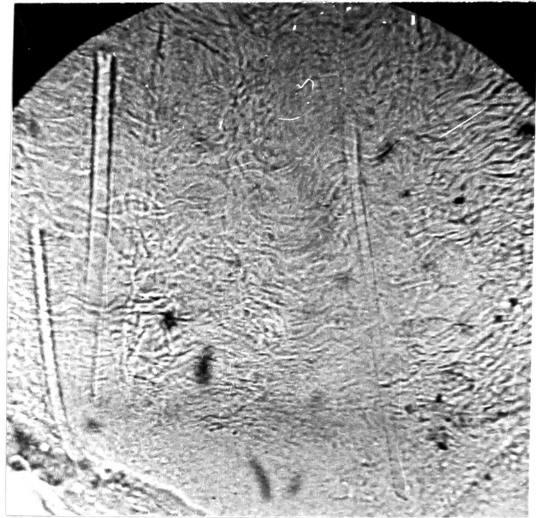

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PLATE 7

(Explanation of Figures)

PLATE 7

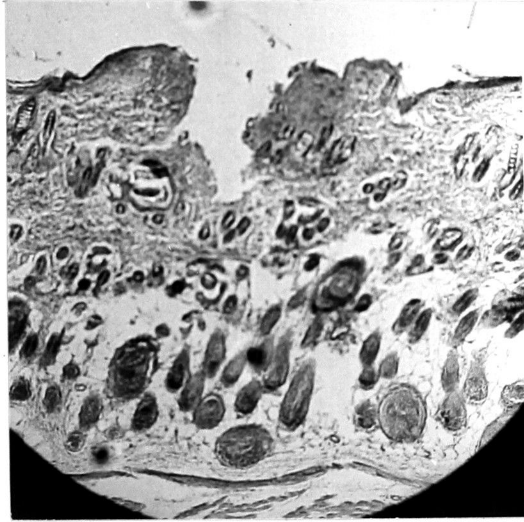
(Explanation of Figures)

Fig. 21 Photomicrograph of a one hour wound showing glycogen activity. Hair follicles and capillaries give strong reaction. The area of the wound gives a stronger reaction than the surrounding tissues. X 80.

Fig. 22 Photomicrograph of a two hour wound showing glycogen activity. Note the intensity of the stain in the edges of the wound. X 80.

Fig. 23 Photomicrograph of a three hour wound showing glycogen activity. Edges of wound and scab stain intensively. X 80.

Fig. 24 Photomicrograph of a four hour wound showing glycogen activity. Edges of wound show a gradual increase in the intensity of the stain. X 80.



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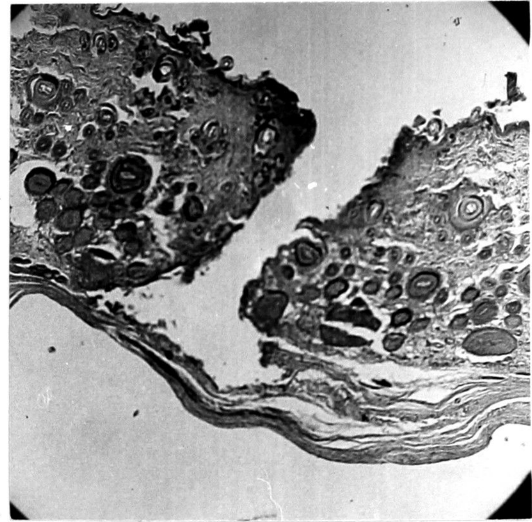

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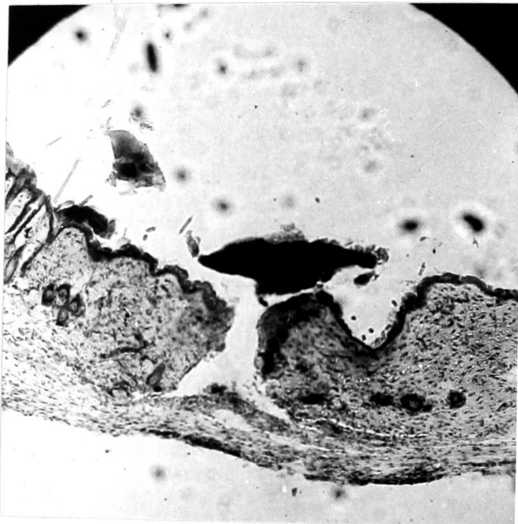

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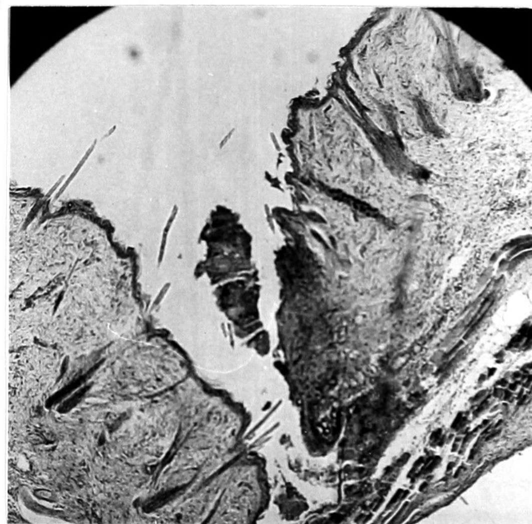

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PLATE 8

(Explanation of Figures)

PLATE 8

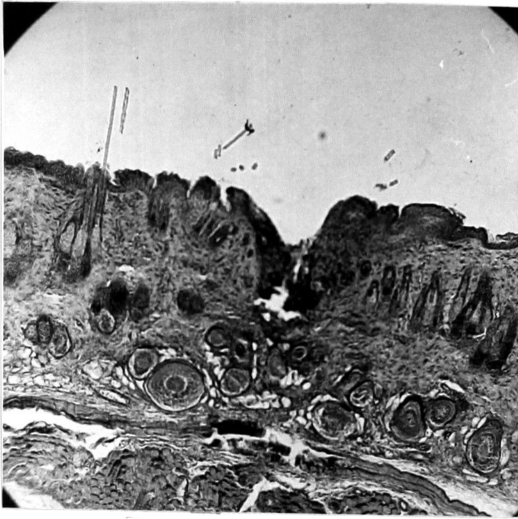
(Explanation of Figures)

Fig. 25 Photomicrograph of a five hour wound showing glycogen activity. Note the increased intensity of stain within the wound. X 80.

Fig. 26 Photomicrograph of a six hour wound showing glycogen activity. Note the intensity of the stain in the wound. X 80.

Fig. 27 Photomicrograph of a twelve hour wound showing glycogen activity. Note the increased activity in the wounded area over the six hour wound. X 80.

Fig. 28 Photomicrograph of a twenty-four hour wound showing glycogen activity. Note the intensity of the stain within the wound over that of the hair follicles. X 80.



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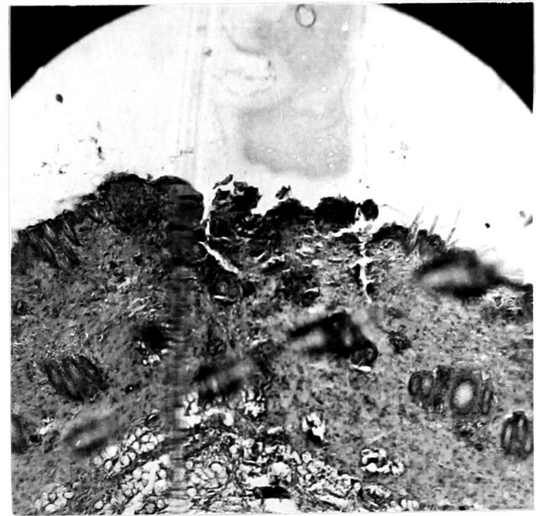

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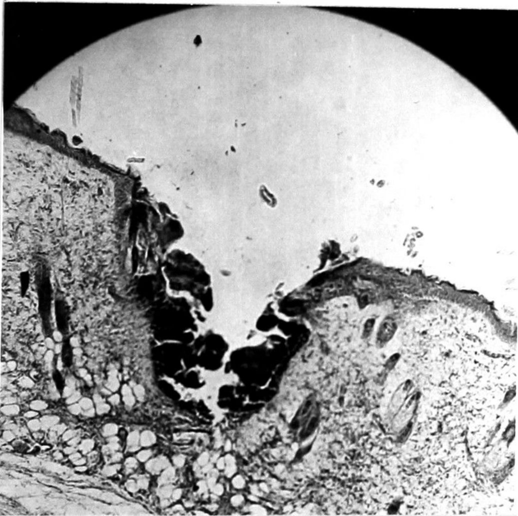

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PLATE 9

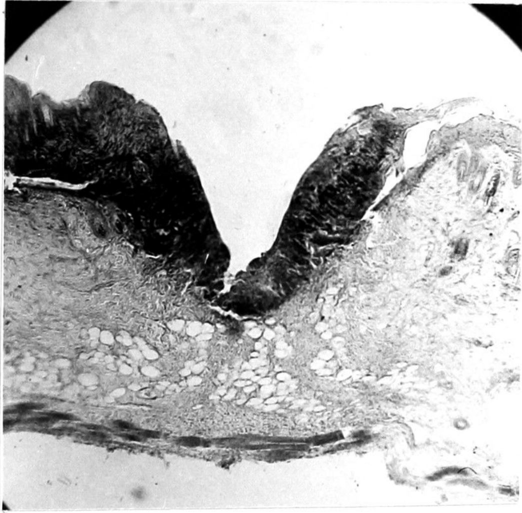
(Explanation of Figures)

PLATE 9

(Explanation of Figures)

Fig. 29 Photomicrograph of a forty-eight hour wound showing glycogen activity. The intensity of the stain within the wound is at its highest peak. X 80.

Fig. 30 Photomicrograph of a control wound for glycogen showing the absence of glycogen activity in the wounded area. X 80.



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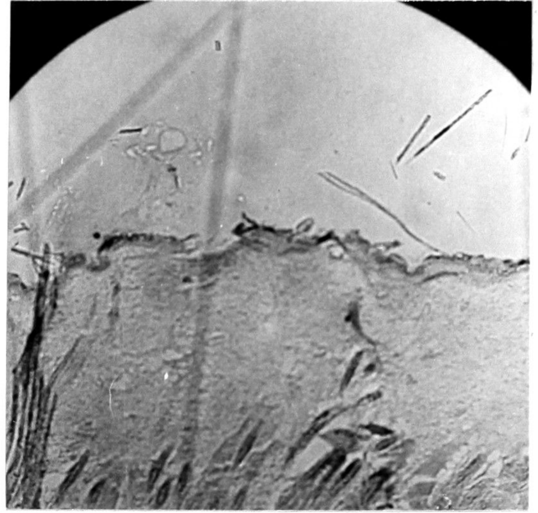

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