THE CARBOHYDRATE COMPONENTS OF THE VAGINA OF THE NORMAL AND OVARIECTOMIZED MOUSE DURING OESTROGENIC STIMULATION

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The earliest description of the histology of the vagina of the mouse is that of Morau (1889), who was concerned mainly with changes occurring in the vaginal epithelium at the beginning of pregnancy. Since the sexual cycles of animals had not been recognized at that time no correlation was noted between the appearance of the vaginal epithelium and the stages of the oestrous cycle. Morau noted, however, that cornification occurred at the time when mating normally takes place (i.e. oestrus); that the epithelium reverts to a low stratified form if pregnancy does not ensue, and that five days later the cornification reappears. Several other French histologists made similar observations with rodents but were not specifically concerned with the mouse.

The first important paper on the vaginal epithelium of the mouse is that of Allen (1922). Prior to this, Stockard & Papanicolaou (1917) showed that in the guinea-pig the vaginal epithelium undergoes cyclical morphological changes which may be correlated with the phases of the ovarian cycle, and that these stages may be easily diagnosed by the microscopical examination of vaginal smears. Also, in 1922, Long & Evans showed that similar changes occur in the vaginal epithelium and smears of the rat.

Allen & Doisy (1923) and Allen, Francis, Robertson, Colgate, Johnston, Doisy, Kountz & Gibson (1924) described the effect of double ovariectomy in the mouse. They showed that 4–6 days after the operation the vaginal epithelium is in the resting (dioestrous) state consisting of from two to four layers of cells. This state persists for about 6 weeks, after which slow progressive atrophy occurs. However, they noted that even after 15 weeks the vaginal epithelium will respond to an injection of oestrogen. Following a subcutaneous injection of oestrogen the basal cells of the stratum germinativum proliferate, and at 36 hr. after an injection there are 12–15 layers of cells. An outer 'vacuolar' layer represents the remnants of the resting vaginal epithelium, and beneath it cornification occurs by transformation of the stratum granulosum. Although cornification occurring below the superficial layer of cells was first described by Retterer (1892) in the guinea-pig, it was Long & Evans (1922) who drew attention to the singular position in which it occurs in the epithelium. The cornified and superficial layers are subsequently sloughed off and are destroyed together with the outer layers of the epithelium by a heavy infiltration of leucocytes.

Many workers have shown that the local application of oestrogens to the vagina results in cornification of the epithelium. The earliest study is that of Loewe & Voss (1926) in the guinea-pig, and since then many workers have confirmed the effect in

both the rat and mouse (see Emmens (1950), for a review of the literature). However, histological studies in the rat (Freud, 1939) and in the mouse (Biggers, 1952) have failed to detect any differences between the cornified epithelium produced by intravaginal or subcutaneous administration of oestrogen. The only difference detected in the mouse is that the average time of onset of cornification is slightly less with the intravaginal method.

Few detailed histochemical studies have been made of the vagina. In the mouse the only work seems to be that of Jeener (1948) and Kamell & Atkinson (1948), who followed the changes in alkaline phosphatase, desoxyribonucleic acid, and ribonucleic acid during the process of cornification. Studies of the carbohydrate components are lacking, except in the monkey and in woman where cyclical changes in the glycogen content of the vagina during the menstrual cycle and pregnancy have been examined (for the literature see Lison & Vokaer, 1949; and Foraker & Brawner, 1951). In order to gain further insight into the cellular processes which occur in the cornifying vaginal epithelium of mice, the changes in the carbohydrate components have been studied both in ovariectomized animals under the influence of oestrone and in intact mice undergoing their normal cycles.

MATERIALS AND METHODS

Forty ovariectomized mice have been used in this study. Twenty were given 0·5 μg. oestrone subcutaneously in nut oil, and the other twenty 0·002 μg. oestrone intravaginally in water. The oestrogen solutions were prepared according to methods described elsewhere (Biggers, 1951). In both cases the oestrogen was injected in two doses, separated by an interval of 24 hr., the total dose being large enough to ensure cornification in all animals. At intervals, groups of mice were killed and the vaginae prepared for histological examination. Since the time of onset of cornification is independent of dose, provided the doses are maximal (Biggers, 1952), the results obtained by both routes of administration should be comparable.

In addition, eight intact mice were examined to see if the changes which occur naturally are similar to those produced by artificial oestrogenic stimulation. Pairs were killed at each of the four main stages of the oestrous cycle as judged by the vaginal smear. Daily examination of the vaginal smears for some days before the mice were used showed that they were undergoing normal oestrous cycles.

The vaginae were fixed in either Zenker-formol, picric-alcohol or 4 % basic lead acetate followed by neutral buffered formalin. Sections were cut at 5–7 μ.

The sections were stained with (i) haematoxylin and eosin, (ii) the Lillie Allochrome method (Lillie, 1951*a*), (iii) the periodic acid Schiff (PAS) technique (McManus, 1946) and (iv) 0·5 % toluidine blue.

RESULTS

Histological and cytological aspects of the vaginal response

The histological changes in the vaginal epithelium of ovariectomized mice following the systemic and local administration of oestrone are illustrated in Pl. 1. No difference can be seen in the response for either method of administration. In both cases keratinization is seen to occur beneath a superficial layer of cells representing the former vaginal epithelium.

The resting epithelium is two to three cells thick, the cells being small with densely staining nuclei (Pl. 1, fig. 1), the basal cells forming the stratum germinativum. Following the administration of oestrone the cells of the stratum germinativum divide and the daughter cells migrate outwards pushing the old superficial cells before them and giving rise to a new layer of cells having distinct cell outlines, a large amount of cytoplasm, and large vesicular nuclei with up to three prominent nucleoli (Pl. 1, figs. 2, 3). These daughter cells appear to have intercellular bridges between them and collectively constitute the stratum spinosum (or prickle cell layer). As cornification proceeds they continue to move outwards, and in so doing become flattened and develop granular material in the cytoplasm and form the stratum granulosum. Peripheral to the stratum granulosum, fully cornified cells are found, comprising the stratum corneum (Pl. 1, figs. 4, 5). Outside this layer are the swollen cells of the former vaginal wall. Layers of cells with a similar appearance have been found in the epidermis of the mouse by Hanson (1947), who points out that these various layers are not always distinct; for example, dividing cells may be found in the stratum spinosum for a short period after its formation, and before the cell cytoplasm has increased in quantity. Similar cells may be found in the vaginal epithelium.

Changes of a similar type are seen in the normal intact animal.

Periodic acid-Schiff technique (PAS)

It has been generally accepted that periodic acid oxidation forms aldehyde from 1,2-glycol linkages (—CHOH—CHOH—). However, —CHOH—$CHNH_2$— and —CHOH—CHNHR— groupings are also oxidized with the formation of aldehydes (Meyer, Odier & Siegrist, 1948; Jeanloz & Forchielli, 1951; Lillie, 1950, 1951*b*). In tissue sections these aldehydes can be demonstrated with Schiff's reagent, and the method is now widely used as a histochemical test for polysaccharides, whether they are free or in combination with proteins. Recently, McManus & Cason (1950) have introduced an acetylation technique in which treatment of sections with acetic anhydride in pyridine blocks the glycol groups and prevents the production of aldehydes; the acetylation can be reversed by subsequent treatment with potassium hydroxide. They claim that the prevention of the PAS reaction by acetylation provides additional proof that a carbohydrate is present in the sections.

In using this technique for the demonstration of carbohydrates two control procedures must be adopted. Free aldehydes may be found in the sections, and their presence can be detected by omitting the periodic acid oxidation step in the PAS method. Also, Hack (personal communication to Gersh, 1949) and Wolman (1950) have shown that many free unsaturated lipids and sphingolipids may recolorize Schiff's reagent after periodic acid oxidation. These free lipids can be extracted from sections with hot 1:1 methanol-chloroform (Gersh, 1949).

Four regions react with the PAS technique in the vaginal wall of both ovariectomized and normal animals (Pl. 1, figs. 6–9):

(1) the former vaginal epithelium beneath which keratinization occurs,

(2) the cytoplasm of the cells forming the stratum spinosum,

(3) the basement membrane,

(4) the intercellular ground substance.

Table 1 gives a summary of the results obtained after the treatment of sections with various reagents and enzymes.

Table 1

	Former epithelium	Stratum spinosum	Basement membrane	Intercellular ground substance
No periodate treatment	—	—	—	—
Acetic anhydride + pyridine	—	—	—	—
Acetic anhydride + pyridine followed by KOH	+	+	+	+
Methanol:chloroform (1:1), 16 hr.	+	+	+	+
Saliva (1:4), 1 hr.	+	—	+	+
Diastase (1% in acetate buffer pH5), 1 hr.	+	—	+	+
Pepsin (0·1 mg./ml. in 0·01N-HCl), 5 min.	+	+	+	+
Pepsin (5 mg./ml. in 0·01N-HCl), 1 hr.	+	+	—	—

+ indicates a positive PAS reaction.

(1) *Former vaginal wall*

The cells of this layer become very swollen and stain intensely with the PAS technique. The results shown in Table 1 demonstrate the carbohydrate nature of the mucinous material present in these cells. The fact that the strong pepsin solution does not destroy this material, while it does destroy the staining of the intercellular ground substance, indicates that the material is not linked to any appreciable amount of protein. A similar substance has been demonstrated in the acrosome of the sperm of the Hemipteran insect, *Arvelius albopunctatus*, by Schrader & Leuchtenberger (1951), who concluded that the substance was a mucopolysaccharide.

(2) *Stratum spinosum*

The results shown in Table 1 demonstrate that the PAS-positive material in this layer is glycogen. Table 2 shows the results from material obtained at various stages after the injection of oestrone.

Table 2. *The time of deposition of glycogen in the stratum spinosum*

Time after injection (hr.)	Intravaginal	Subcutaneous
36	—	—
48	Trace*	Trace*
60	Trace	Trace
72	++†	++†
84	++	++

* Commencement of keratinization. † Fully keratinized.

In intact animals glycogen deposition occurs at the end of oestrus. It is seen that in both artificially stimulated and normal animals glycogen is deposited in the cells of the stratum spinosum when full keratinization of the vagina is attained (Pl. 1, figs. 7, 9). The glycogen is in a granular state and in places forms caps on the poles of the nuclei. However, the observed form of the glycogen is probably an artefact, since recent freeze-drying techniques indicate that glycogen is distributed diffusely throughout the cytoplasm (Mancini, 1948; Lison & Vokaer, 1949).

(3) *Basement membrane*

At all stages a prominent basement membrane is demonstrated by the PAS technique, although Allen (1922) reported the absence of a basement membrane in the dioestrous and ovariectomized animal. No difference in its structure could be demonstrated during the cornification process. Its destruction by strong pepsin solution suggests it contains a glycoprotein.

(4) *Intercellular ground substance*

The PAS reaction was observed in all sections examined, although its intensity was not as pronounced as in the former vaginal wall or the glycogen of the stratum spinosum. Oestrogens seemed to have no effect upon the staining reaction. Its destruction by strong pepsin solution suggests that the ground substance contains a glycoprotein.

Metachromasia with toluidine blue

Many papers have been published on the metachromatic staining of various tissue components with basic dyes (the relevant literature has been reviewed by Dempsey & Wislocki, 1946; Dempsey, Bunting, Singer & Wislocki, 1947; Wislocki, Bunting & Dempsey, 1947; and Bunting, 1949). Aspects of the chemical basis of the reaction have been discussed by Michaelis & Granick (1945), Michaelis (1947) and Landsmeer (1951). At present it is impossible to list all substances which stain metachromatically. Lison (1936) was the first to recognize the main group; he states that they are substances which are esters of sulphuric acid with carbohydrate of a high molecular weight, sometimes combined with a protein and sometimes not, e.g. chondroitin sulphuric acid and mucoitin sulphuric acid. Lison's statement is not the whole truth, since phosphoric acid or carbonic acid can be substituted for sulphuric acid above (Bank & de Jong, 1939).

While all workers are unanimous in the belief that all sulphate-containing compounds are strongly metachromatic there is a controversy as to whether or not hyaluronic acid produces metachromasia. Several authors maintain that hyaluronic acid does not produce metachromasia (see Lillie, Emmart & Laskey, 1951, for a review); Dempsey & Wislocki and their co-workers, however, have maintained that it does. The main difficulty in this work is that no pure preparations of the various mucopolysaccharides are available; this is well illustrated in a recent paper (Rapport, Meyer & Linker, 1951) where the 'pure' hyaluronic acid preparation had about 12 % of sulphated polysaccharide impurity. The conclusion must be reached that the metachromatic test is not molecule specific. However, the test is useful in detecting regions of acid mucopolysaccharides and mucoproteins and assists in the interpretation of other histochemical tests.

In the present work a 0·5 % aqueous solution of toluidine blue has been used.

Only one region of the vaginal wall stains metachromatically with toluidine blue. This is the former vaginal wall which, as noted earlier, stains with the PAS technique. The metachromatic staining, however, is not alcohol-resistant. The intercellular ground substance fails to stain metachromatically, although metachromatic mast cells are distributed throughout. In these cells the staining reaction is alcohol-resistant.

DISCUSSION

Epithelial mucin

The PAS-positive nature of the vaginal mucin of rodents has been mentioned by Leblond (1950) and Lillie (1951*b*). In the present work, two main histochemical properties of this material have been demonstrated. First, the material stains with the PAS technique and is not destroyed by strong solutions of pepsin, and secondly, the material is weakly metachromatic. The first property indicates that the substance is a mucopolysaccharide. Lillie (1951*b*) has suggested that substances which react strongly with the PAS technique and which are weakly metachromatic may be relatively short-chained polysaccharides.

Preliminary studies on the mucinous substances found in the vagina of the mouse during pregnancy show that the material is the same as that described above. Further studies on the nature of these mucinous substances are in progress.

Keratinization occurs in the flattened cells which lie immediately below the mucified layer. Although this fact was described by the pioneer workers in the field, in recent times it seems to have passed unnoticed. The phenomenon demonstrates that oestrogens cause a unique and rapid differentiation of cells of the vaginal epithelium, the main feature of the differentiation being the ability to produce keratin. Early views on keratinization were to the effect that it is a drying up process caused by the removal of cells from their source of nutriment, full keratinization being attained upon death of the cell. This theory is quite untenable in the case of the vagina where the superficial cells do not keratinize. While undoubtedly the fully keratinized cells are dead the cause of death is not desiccation but the excessive synthesis of keratin, i.e. the cells die as the result of their own activity. This view of keratinization as an active anabolic process is consistent with the demonstration of increased amounts of ribonucleic acid in the keratinizing vagina (Jeener, 1948), skin (Leuchtenberger & Lund, 1951), and hair follicles (Hardy, 1952).

Glycogen

The presence of glycogen in the vaginal mucosa is well known. Robertson, Maddux & Allen (1930) showed in the monkey that the glycogen content increases as cornification proceeds. Van Dyke & Ch'en (1936) in biochemical studies on the macaque showed similar changes. They also showed that ovariectomy causes a large reduction in the glycogen content of the mucosa, and that it is deposited in large amounts following injections of oestrone. Bullough & Eisa (unpublished observations quoted by Bullough & Van Oordt, 1950) have also shown the deposition of glycogen in the vagina of the mouse during the oestrous cycle. The studies reported in the present paper have shown that glycogen is deposited after the attainment of full keratinization, which is subsequent to the peak of mitotic activity reported by Allen, Smith & Gardner (1937). This is of interest in view of the importance of carbohydrates in the mechanisms involved in mitosis (Bullough, 1952). Glycogen is also a prominent constituent of growing stratified epithelium in other parts of the body, e.g. embryonic skin (Bernard, 1859), healing wounds (Bradfield, 1951) and skin grafts (Scothorne & Scothorne, 1953). In addition to its possible role in mitosis,

glycogen may also play an important function in the keratin synthesis which occurs in all the above tissues. Bradfield (1951) has recently discussed this latter problem.

Intercellular ground substance

Two main histochemical properties characterize this material. First the substance is PAS-positive and can be destroyed by strong pepsin solution, and secondly, it fails to stain metachromatically with toluidine blue. Recently, Lillie *et al.* (1951) have suggested that regions which are PAS-positive and fail to stain metachromatically contain predominantly hyaluronic acid. A further example of this is provided by the vitreous body. Such an interpretation, however, is not consistent with the recent observation of Davies (1952), who has shown that a sulphate-free preparation of hyaluronic acid does not give a PAS-positive reaction following its deposition on microscopic slides. At the moment, therefore, the staining reactions cannot be attributed to any specific substance.

Two other regions of connective tissue lying beneath stratified squamous epithelium, and highly sensitive to oestrogenic stimulation, have been studied by histochemical techniques.

(1) *Sex skin of the monkey*

Duran-Reynals, Bunting & Van Wagenen (1950), following the earlier work of Chain & Duthie (1940), have studied the changes which occur in the ground substance of the sex skin of *Macaca mulatta*; by means of testicular and streptococcal hyaluronidases they claim to have demonstrated changes in the hyaluronic acid content of the skin. Their arguments depend on (i) changes in the metachromatic staining reaction, and (ii) the destruction of most of the metachromasia by streptococcal hyaluronidase; the bacterial enzyme is believed to be exclusively a hyaluronidase, while the testicular one is believed to contain in addition a chondroitinase. This work cannot be regarded as conclusive, other than that it demonstrates changes in the degree of metachromasia. There is no general agreement that hyaluronic acid can cause metachromasia, and recent reports indicate that streptococcal hyaluronidase does not consist exclusively of that enzyme (Lillie *et al.* 1951; Meyer & Rapport, 1952).

(2) *Vagina of the rat*

Recently, Bostrom & Odeblad (1952) have shown by autoradiographic means that in the rat vagina there is an accumulation of ^{32}S following oestrogenic stimulation. They believe that this represents an increased formation of chondroitin sulphate. As yet neither metachromasia nor the PAS reaction have been specifically studied in the rat vagina. If chondroitin sulphate is formed, metachromasia of the intercellular ground substance should be observed, in contrast to the observations reported in the mouse.

The above results show that connective tissues which are highly sensitive to oestrogens have different histochemical properties. Until all the various techniques have been applied to at least one species of animal interpretation of results at the chemical level is impossible. Further studies on this problem are in progress.

SUMMARY

1. Four regions of the vagina of the mouse react positively to the periodic acid oxidation Schiff procedure: (i) the former vagina wall, (ii) the cells of the stratum spinosum, (iii) the basement membrane, and (iv) the intercellular ground substance. The only region of metachromasia is the former vaginal wall. The metachromasia is not alcohol-resistant.

2. The mucinous material of the former vaginal epithelium is not lipoidal in nature, and is resistant to salivary amylase, diastase and strong pepsin solutions. The material only forms during cornification and beneath it keratinization occurs. Following completion of cornification the layer is sloughed off. Non-keratinization of these superficial cells is strong evidence that keratinization is an active anabolic process.

3. The PAS-positive material in the stratum spinosum is glycogen and is deposited at the end of cornification.

4. No differences have been shown between the carbohydrate components of the vagina during cornification in animals undergoing normal cycles and in ovariectomized animals stimulated by either the subcutaneous or intravaginal administration of oestrone.

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EXPLANATION OF PLATE

Fig. 1. Vaginal wall of an untreated ovariectomized mouse. Haematoxylin and eosin. ×280.

Fig. 2. Vaginal wall of an ovariectomized mouse 24 hr. after the subcutaneous injection of 0·5 μg. oestrone. Haematoxylin and eosin. ×280. The inner large 'mucified' cells represent the former vaginal wall; the cells beneath are newly formed cells.

Fig. 3. Vaginal wall of an ovariectomized mouse 24 hr. after the intravaginal injection of 0·002 μg. oestrone. Haematoxylin and eosin. ×280. The cellular pattern of the epithelium is similar to that of fig. 2.

Fig. 4. Vaginal wall of an ovariectomized mouse 48 hr. after the subcutaneous injection of 0·5 μg. oestrone. Haematoxylin and eosin. ×280. Keratinization can be seen in the layer of cells immediately below the superficial mucified cells.

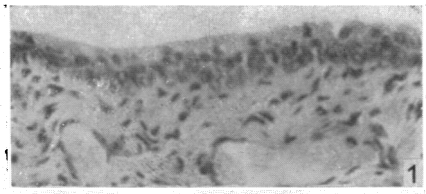
Fig. 5. Vaginal wall of an ovariectomized mouse 48 hr. after the intravaginal injection of 0·002 μg. oestrone. Haematoxylin and eosin. ×280. The cellular pattern of the epithelium is similar to that of fig. 4, although at a somewhat earlier stage.

Fig. 6. Vaginal wall of an ovariectomized mouse 36 hr. after the intravaginal injection of 0·002 μg. oestrone. PAS technique. ×665. The mucified cells of the former vaginal wall and the basement membrane have reacted strongly (dark regions), while the intercellular ground substance has reacted to a lesser degree. No PAS-positive material is seen in the stratum spinosum.

Fig. 7. Vaginal wall of an ovariectomized mouse 72 hr. after the intravaginal injection of 0·002 μg. oestrone. PAS technique. ×665. The positive reaction is seen in the basement membrane and intercellular ground substance. In addition, PAS-positive material is seen in the stratum spinosum. Enzymatic tests have shown this material to be glycogen.

Fig. 8. Vaginal wall of an ovariectomized mouse 48 hr. after the subcutaneous injection of 0·5 μg. oestrone. PAS technique. ×665. The mucified cells, the basement membrane and intercellular ground substance has reacted strongly. Traces of PAS-positive material (glycogen) are seen in the stratum spinosum.

Fig. 9. Vaginal wall of an ovariectomized mouse 84 hr. after the subcutaneous injection of 0·5 μg. oestrone. PAS technique. ×665. Positive reactions are seen in the basement membrane and intercellular ground substance. Also a large deposition of glycogen is seen in the stratum spinosum.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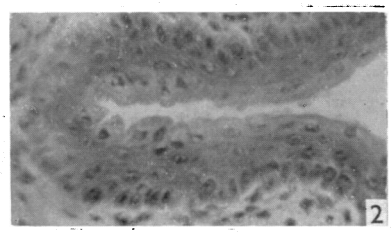
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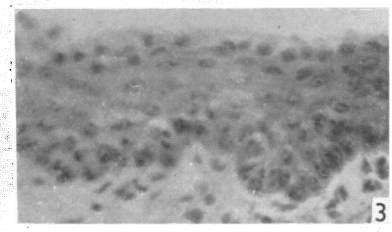
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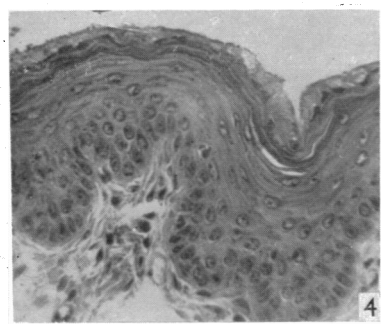
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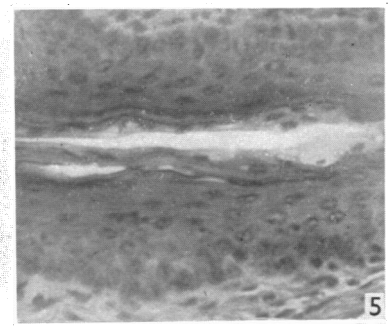
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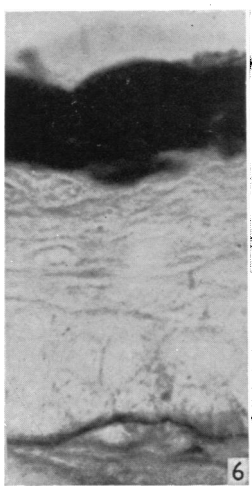
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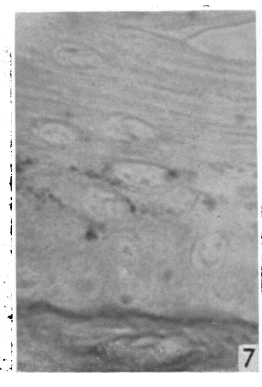
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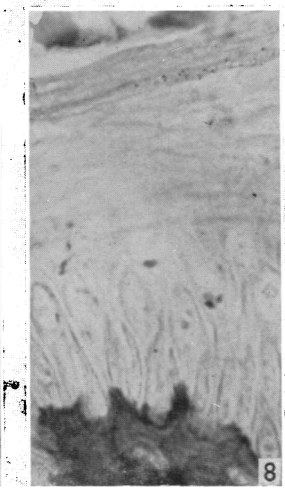
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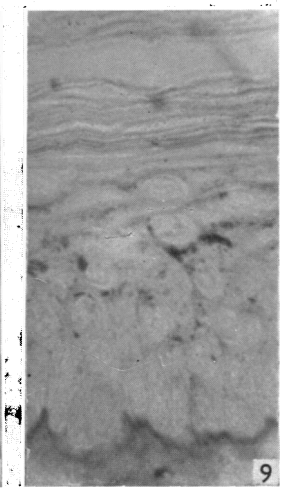
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