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Mucous Glands in the Skin of *Ichthyophis glutinosus* (Amphibia: Gymnophiona)

by

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(With eight plates)

INTRODUCTION

Integumentary mucous glands and secretions have been studied histochemically in both vertebrate and invertebrate species e. g. *Megascolex mauritii* (Krishnan and Sundara Rajulu, 1969), *Pheretima elongata* (Varute and Nalawade, 1970), *Sabella penicillium* Kryvi (1971), *Amadillidium vulgare* (Stevenson and Murphy, 1967) *Rhodnius prolixus* and *Calpodes ethlius*. (Lai-Fook, 1972), *Lymnaea stagnalis* and *Biomphalaria pfeifferi* (Zylstra, 1972), *Asterina stellifera* (de Sousa Santos and da Silva Sasso, 1968), *Harrimania kupfferi* (NΦrrevang, 1965), hagfish species (Leppi, 1968), teleost fish (Mittal and Datta Munshi, 1971), and *Rana pipiens* (Dapson, 1970). *Ichthyophis glutinosus* is a limbless burrowing amphibian (also called an apodan or caecilian) whose distribution is confined to the tropics and the skin of the adult, unlike that of a toad for example, is not rough and warty but smooth and somewhat slimy. A preliminary communication of our study of the mucous glands in the skin of this amphibian has been presented earlier (Breckenridge and Murugapillai, 1971) and we now report our observations in some detail.

MATERIALS AND METHODS

Small pieces of skin from different regions of the body of a healthy adult female were fixed in Bouin's, susa, cold (0.4°) Rossman's, Carnoy and formol-calcium (formula according to Baker, as given in Pearse, 1968). Paraffin sections were cut at 6 microns and stained with azan and haematoxylin-eosin for general histology. The histochemical procedures used were as follows:

The periodic acid Schiff sequence (PAS) with its appropriate controls—acetylation with acetic anhydride in pyridine, acetylation and subsequent saponification with alkali, and the omission of periodate oxidation — was used to detect carbohydrates, (details in Pearse, 1968). Saliva and malt diastase digestions followed by the PAS test were used for the detection of glycogen.

Mucosubstances were visualised using the battery of tests outlined by Spicer et al. 1967. The rationale for the use of these tests and their histochemical basis are dealt with in the excellent accounts of Spicer et al. (1967,) Spicer and Henson (1967) and Leppi (1971). Sections fixed in formol calcium were stained with 1 % alcian blue (AB) 8GX at pH 2.5 and 1, AB with graded concentrations of magnesium chloride from 0.1M to 1M, colloidal iron, toludine blue—the slides being examined wet before dehydration, and aldehyde fuchsin (AF). The combined AF/AB stain and high iron diamine-alcian blue stain were also used for the detection of sulphated and non-sulphated acid groups. Methylation with acid methanol for 4 hours at 37 (mild methylation) and 4 hours at 60 (active methylation) followed by staining with AB and AF/AB were employed further to characterise the nature of the acid groups. Methylation followed by saponification with KOH (procedure according to Sorvari and Stoward, 1971) and subsequent staining with AB and AF/AB. For the simultaneous detection of acid and vicinal glycol moieties the combined AB-PAS test proved useful. The AB-PAS test was also used following an active methylation. The proximity of acid groups to vic glycols was determined by subjecting slides to treatment with the phenylhydrazine Schiff procedure and the periodate-meta diamine - alcian blue sequence. Enzyme lability of the mucus was tested with Neuraminidase (Calbiochem AG, Lucerne, Switzerland) and Hyaluronidase (BDH, England.)

Tests for proteins included mercuric bromphenol blue, Biebrich scarlet, DMAB nitrite for tryptophan, performic acid alcian blue for protein disulphide groups, DDD for SH groups, ninhydrin schiff for NH_2 groups, and the Sakaguchi test for arginine (all as outlined in Pearse, 1968). Millon's test for tyrosine was carried out by pipetting a few drops of Millon's reagent (BDH) onto dewaxed hydrated sections which were then observed at 5 minute intervals for half an hour.

RESULTS AND DISCUSSION

The skin of *Ichthyophis* presents a histological picture which is basically similar to that of anurans except for the presence of rows of scales in the dermis. Two prominent kinds of glands are present— the large so-called poison or granular glands and the smaller, more superficially located mucous glands. The mucous gland is flask-shaped with a spherical alveolus joined to a thin long neck by a short bulb-like collar (figs. 3,4). The alveolus is lined by cuboidal secretory cells lying on a thin basement membrane. These cells elaborate the mucus which is in the form of small granules within the cell and a mixture of granules (large and small) and mucous threads, and in many instances a dense mass filling the entire lumen of the alveolus. Although the mucus may be clearly demonstrated within the glands it is not visible on the surface of the skin in stained sections. This may be due to its removal during processing of the material. The mucus stains blue with azan and red-brown with haematoxylin-eosin.

The PAS positive nature of the mucus clearly shows the presence of vic glycols of a carbohydrate component. PAS reactivity is abolished on acetylation and restored on saponification. Saliva and diastase do not diminish or abolish PAS staining thereby eliminating the presence of glycogen.

Equally convincing are the results obtained with the tests for acid mucosubstances. The mucus shows purple beta metachromasia with toluidine blue and stains intensely (deep prussian blue) with colloidal iron, which results are considered to be indicative of acid groups. This finding is further substantiated with the tests using alcian blue, aldehyde fuchsin and the diamines. Mucus is stained turquoise with alcian blue at pH 1 and 2.5 indicating the presence of both sulphated and non-sulphated acid groups. Whereas both sulphated and non-sulphated groups stain at the higher pH—at the low pH (1) only the sulphated groups remain dissociated and free to combine with the dye. Positive results with aldehyde fuchsin (purple) and the high iron diamine stain (purple-black) further confirm the presence of sulphated groups: sulphated compounds are known to stain selectively with these two staining procedures. Combined staining sequences are also useful in the identification of the acid groups in the secretion: with the aldehyde fuchsin-alcian blue sequence mucus stains predominantly purple with flecks of blue, and the high iron diamine-alcian blue stain resulted in the mucus staining an intense purple-black with characteristic alcianophilia showing as well. These results are interpreted as indicating the presence of both sulphated and non-sulphated (carboxylic) groups in the mucus.

Methylation procedures are also helpful in characterising the acid groups. A mild methylation (4 hours at 37°) blocks basophilia through esterification of carboxyl groups leaving the sulphated groups unaffected, whereas an active methylation (4 hours at 60°) hydrolyses sulphate esters blocking alicianophilia. Saponification with KOH after methylation deesterifies carboxyls restoring alcianophilia due to these groups only. The mucus shows alcianophilia after a mild methylation, no alcianophilia after active methylation and alcianophilia, though much diminished, is restored following active methylation and saponification. These results further substantiate the view that sulphated and carboxyl groups are present. Methylation procedures with the combined AF/AB stain lead to a similar conclusion; the mucus staining purple without methylation, purplish-blue on mild methylation, unstained after an active methylation and light turquoise after active methylation followed by saponification.

Staining with alcian blue with different concentrations of magnesium chloride (critical electrolyte concentration) may also be used to identify acid groups: generally carboxy mucins stain at or below concentrations of 0.1M $MgCl_2$ whereas sulphomucins stain selectively at 0.2M $MgCl_2$ and lose alcianophilia with increasing concentrations of $MgCl_2$. The mucous glands showed strong alcianophilia at 0.1M and 0. 2M $MgCl_2$, reduced alcianophilia at 0.5M and 0.6M concentration and faint or no alcianophilia at higher $MgCl_2$ concentrations.

The combined AB-PAS procedure helps to visualise both carbohydrate and acid moieties in a single section. Periodate reative mucosubstances will stain in shades of blue purple to blue magenta whereas neutral mucosubstances would stain pink to magenta. The mucous glands stain deep blue with the above sequence showing the presence of periodate reactive vic glycols and acid groups.

The proximity of acid groups to vic glycols may be "estimated" using the phenylhydrazine - Schiff sequence and the periodate-meta diamine-alcian blue sequence. Phenylhydrazine will normally prevent the reaction between aldehydes and Schiff's reagent giving a negative PAS reaction, except when acid groups sufficiently near the periodate engendered aldehyde, block the action of phenylhydrazine. A negative result in this study shows that periodate reactive groups and acid groups are not close to each other. The mucous glands are alcianophilic after the periodate-meta diamine-alcian blue sequence which supports the above conclusion. Diamine normally condenses on the aldehydes produced by periodate oxidation, and acid groups, if located adjacent to these groups would not react with alcian blue due to steric hindrance by the diamine.

Alcianophilia was unaffected after the digestion with hyaluronidase, neuraminidase, and neuraminidase following the exposure of the sections to 1% KOH in 70% ethanol for five minutes.

Mucus does not stain with any of the tests for proteins and this may be attributable to the protein being masked by other substances or present in too low a concentration for histochemical detection. To ascertain whether protein was masked by the acidic moiety, sections were subjected to an active methylation and then stained for protein. Negative results indicate that acid groups may not be responsible for masking any protein present. The poison glands on the contrary react positively with many of the stains for proteins. The poison glands are stained positively with Millon's reagent, Biebrich scarlet, DMAB nitrite for tryptophan, the Sakaguchi method for Arginine and the DDD procedure for protein sulphydryl groups. A sequence of Biebrich scarlet-alcian blue strikingly shows the contrast between the two kinds of glands — the mucous glands stain turquoise and the poison glands an orange red.

The above histochemical tests indicate that mucus in the skin of *Ichthyophis* is acidic (including carboxyl and sulphate groups) with vicinal glycol groups, no demonstrable protein, and resistant to digestion with hyaluronidase and neuraminidase. The vicinal hydroxyl groups and acid groups are not in close proximity to each other. And following the classification of mucosubstances as suggested by Spicer et al. (1965) mucus in the skin glands of *Ichthyophis* would be typed as CSG-mucin B 1.0 (0.6M $MgCl_2$). In these histochemical characteristics it is similar to the mucus in the integumentary glands of the leopard frog *Rana pipiens* (Dapson 1970.) Although protein could not be histochemically detected in the secretion of mucous glands, this does not exclude the possibility that this mucosubstance is a glycoprotein. It should also be borne in mind that there are certain limitations imposed by the techniques used: thus we cannot say whether the acidic groups and vic glycols are widely spread on the same macromolecule or located in different molecules, and also whether the sulphate esters and carboxyl groups, shown to be present, are constituents of a single macromolecule or are on different molecules. Such questions may be resolved by biochemical studies.

This study also incidentally presents another criterion for distinguishing between mucous glands and poison glands: not only are they morphologically different, but also they differ in the histochemical nature of the secretions they elaborate. The secretion of the poison glands has a PAS positive component, lacks acid mucosubstances and is predominantly proteinaceous.

The precise physiological significance of the acidic mucous secretion is difficult to state. Mucus on the skin surface could serve many functions: it could bind water thereby maintaining a moist surface which would facilitate cutaneous respiration as well as preventing desiccation. It is believed that mucoid substances are able to bind water (Jeanloz, 1963, and other references in Elkan, 1968, page 57) and the importance of this property cannot be over-emphasised in animal species that are subject to desiccation. Elkan (1968) in his study of the anuran skin emphasises the possible importance of the Eberth-Kastschenko layer or G layer, which contains acid mucopolysaccharide and calcium, as a line of defence against desiccation. This layer seems to be absent in *Ichthyophis** and in this connection it is interesting to note that Elkan (1968) could find "only small amounts of G in fossorial species like *Cyclorana* or *Rhinophrynus*". The mucus is probably responsible for the slipperiness of the body which could be used to escape from predators and avoid capture. It could also function as a lubricant in locomotion and tunneling, in reducing friction during such activity, and affording some measure of protection from mechanical wear and tear especially since the skin has a very thin keratinised stratum corneum.

The pH at the frog skin surface is alkaline (vide Dapson 1970) and according to this author it is "difficult to understand how this acidic mucus alone can maintain an alkaline pH at the surface of the skin" (Dapson 1970). No data is available as to the pH at the skin surface of *Ichthyophis*. It has been generally held that the acidic nature of the human skin surface has a protective function in preventing or inhibiting the establishment of pathogenic bacteria and fungi. But it appears that this case has been somewhat overstated (Marples, 1969), and caution is essential in attributing any bactericidal or fungicidal action to the skin mucus in *Ichthyophis*. Enzyme studies on the mucous glands may help to clarify this question. It has been suggested that the sulphated acid mucopolysaccharide secreted by the large mucous gland cells in the integument of *Megascolex mauritii* (Krishnan and Sundara Rajulu, 1969) may serve as an alarm pheromone. The mucus in *Lumbricus terrestris* (Ratner and Boice, 1971) is believed to function as an alarm pheromone as well as in repelling predators. The possibility that mucus in *Ichthyophis* could function as a pheromone cannot be dismissed since no evidence is available. The poison glands on the contrary are more likely to produce the predator repellant.

Table 1 is based on some selected examples and indicates that integumentary mucus secretions, performing a variety of functions, in animals from different phyla, in different habitats and having different modes of life so to speak, nevertheless appear to show certain common histochemical characteristics. An appreciable number of procedures are now

*Isolated aggregates of metachromatic material may be seen above the stratum compactum, but we are not certain whether this represents the so-called G-layer.

TABLE I

A Survey of Some Investigations of Integumentary Mucous Glands of Animals Belonging to Different Phyla.

Name	*Phylum*	*Habitat*	*Gland Types*	*Histochemical Nature of Secretion*	*Reference*
Megascolex mauritii	Annelida	Terrestrial burrowing in soil	1. Small mucous gland	Acid mucopolysaccharide probably hyaluronic acid, some protein	Krishnan and Sundara Rajulu 1969
			2. Larger mucous gland	Sulphated mucopolysaccharide	
Armadillidium vulgare	Arthropoda Crustacea	Terrestrial, in soil and dirt	Rosette glands in head	Carbohydrate including acid mucopolysaccharide, no glycogen very little protein or lipid	Stevenson and Murphy 1967
Rhodnius prolixus (4th and 5th) instar larva)	Arthropoda Insecta	Terrestrial	Type 'B' dermal glands	Acidic sulphated mucosubstance with hyaluronic acid as main carbohydrate component. S-mucin B 1.5 A 0.2 (0.6M $MgCl_2$) T$\pm$	Lai-Fook 1972
Calpodes ethlius 4th and 5th) instar larva)	Arthropoda Insecta	Terrestrial	Verson's glands	Acidic sulphated mucosubstance with sialic acid as main carbohydrate component SG-mucin B 3.0 A1.0 (0.2M $MgCl_2$) S$\pm$	
[1]*Lymnaea stagnalis*	Mollusca	Freshwater	1. Muciparous gland cell (ubiquitous distribution)	Sulphated mucopolysaccharide	Zylstra 1972
			2. Lip gland cell type B	Neutral mucopolysaccharide	
			3. Anterior pedal gland cell (dorsal surface of fore-part of foot)	Sulphated mucopolysaccharide	
Asterina stellifera	Echinodermata	Marine	1. Type A gland cell	Acid and neutral mucopoly-saccharide predominantly acid	de Souza Santos and
			2. Type B gland cell	Acid and neutral mucopoly-saccharide predominantly neutral	da Silva Sasso 1968
			3. Type C gland cell (All three gland cell types in tube feet)	Sulphated mucopolysaccharide	

[1]In *Lymnaea* as many as 14 differernt gland cell types are recognized, of which 3 were selected at random for inclusion in the above Table.

Harrimania kupferi	Hemichordata	Marine, bottom dwelling	1. Acid mucous cell	Acid mucopolysaccharide	Nørrevang 1962
			2. Goblet cell (Both gland types in proboscis epidermis)	PAS reactive, not metachromatic, some protein	
Bdellostoma stouti *B. deani* *Myxine circifrons*	Chordata Agnatha	Marine	Mucous cells in skin and slime glands	Probably glycoprotein containing sulphate esters (sulphomucin) and in addition a sialomucin in large epidermal mucous cells of *M. circifrons*	Leppi 1968
Heteropneustes fossilis	Chordata Pisces	Drying pools and ponds	1. Flask-shaped glands	Weakly acidic sulphated mucopolysaccharide	Mittal
			2. Spherical glands	" "	and
Amphinous cuchia	Chordata Pisces	Pond and mud semiterrestrial	Elongate mucous glands	Strongly acidic sulphated mucopolysaccharides	Datta Munshi 1971
Mastacembelus pancalus	Chordata Pisces	Mud and mud holes	Flask-shaped glands	Strongly acidic sulphated mucopolysaccharides	
Rana pipiens	Chordata Amphibia	Land and freshwater ponds	Mucous glands	Mucosubstance with vic glycols, carboxyl and sulphate groups, and no protein. Categorised as CSG-mucin B 1.0 A 0.4 (0.6M $MgCl_2$)	Dapson 1970

available for histochemists to identify and define mucosubstances, and it would therefore be very useful if the mucosubstance(s) under investigation can be classified following certain precise and generally accepted standards. Such a classification is that of Spicer et al. (1965) and the classification given in Pearse (1968) which is closely based on that of the above authors. Further biochemical and physiological data would increase our understanding of the chemistry of integumentary mucous secretions and their diverse functions.

SUMMARY

1. Mucous glands in the skin of *Ichthyophis glutinosus*, a limbless, burrowing amphibian have been studied histologically and histochemically.

2. The glands are flask shaped and produce a copious mucus which is transported through the neck of the gland to the surface of the skin.

3. Histochemical tests show that the mucus is acidic with carboxyl and sulphate groups, with vicinal glycols and no detectable protein. The secretion is resistant to digestion with hyaluronidase and neuraminidase. The mucus is classified as CSG B 1.0 (0. 6M $MgCl_2$).

4. The mucous glands can be readily distinguished from the poison glands both morphologically as well as histochemically. The poison glands are larger, elongate-oval in shape with a predominantly proteinaceous secretion.

5. The possible functions of the mucus are discussed.

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EXPLANATION OF PLATES

Plate I

Fig. 1 Section of skin showing a mucous gland (m) with granular secretion. sc. stratum corneum; e, epidermis; sm, stratum malpighii; c, chromatophores; ss, stratum spongiosum. Bouin's, Haematoxylin and Eosin, x 725.

Fig. 2. PAS. Mucous glands (m) with scanty secretion in lumen. Note the intense PAS positive material in the lumen of the poison gland (p). Formol calcium, x 475.

Plate II

Fig. 3. Toluidine blue. Mucous gland (m) with mucus purple. Formol calcium, x 375.

Fig. 4. Alcian blue (AB), pH 2.5 Mucus turquoise. Note unstained poison glands (p). s, scales. Formol-calcium, x 400.

Plate III

Fig. 5. Aldehyde fuchsin. Dense purple secretion in mucous glands (m) and unstained poison glands (p). Formol-calcium, x 430.

Fig. 6. High Iron diamine-Alcian blue. Mucus stained purple-black. Compare with fig. 5. Formol-calcium, x 375

Plate IV

Fig. 7. Alcian blue-PAS. Mucus deep blue. Formol-calcium, x 325.

Fig. 8. Phenylhydrazine - PAS. Mucus unstained. Formol calcium, x 375.

Plate V

Fig. 9. Metadiamine-Alcian blue, mucus turquoise. Formol calcium, x 375.

Fig. 10. Periodic acid - metadiamine - Alcian blue. Mucus turquoise. Formol-calcium, x 375.

Plate VI

Fig. 11. Hyaluronidase - Alcian blue pH 2.5 Mucus turquoise. Formol-calcium, x 400

Fig. 12. Neuraminidase after alcoholic KOH. Mucus turquoise. Formol-calcium, x 400.

Plate VII

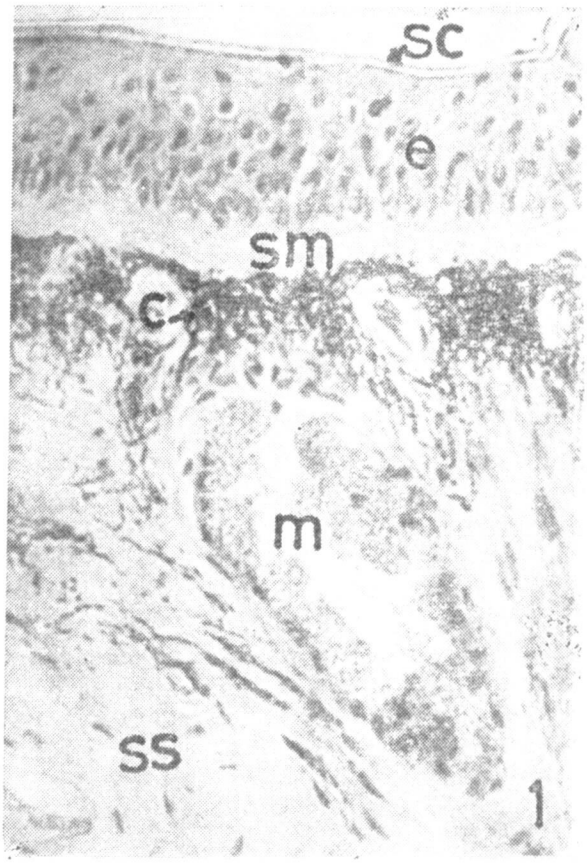
Fig. 13. DMAB-Nitrite. Poison glands (P) stained blue, mucous glands (m) unstained, Formol-calcium, x 375.

Fig. 14. DDD. Poison glands (P) stained blue, mucous glands (m) unstained. Formol calcium, x 400.

Plate VIII

Fig. 15. Biebrich scarlet pH. 9.5 - Alcian blue. Orange-red secretion granules in poison gland (P), and mucus glands (m) greenish. Carnoy, x 400.

(MS. received 22.11.73)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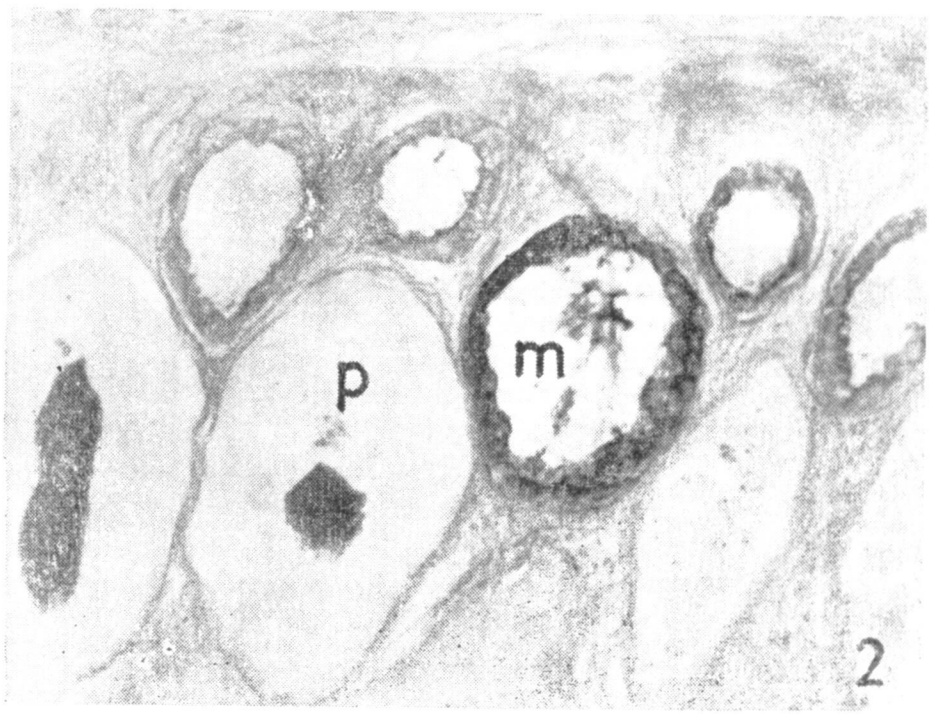
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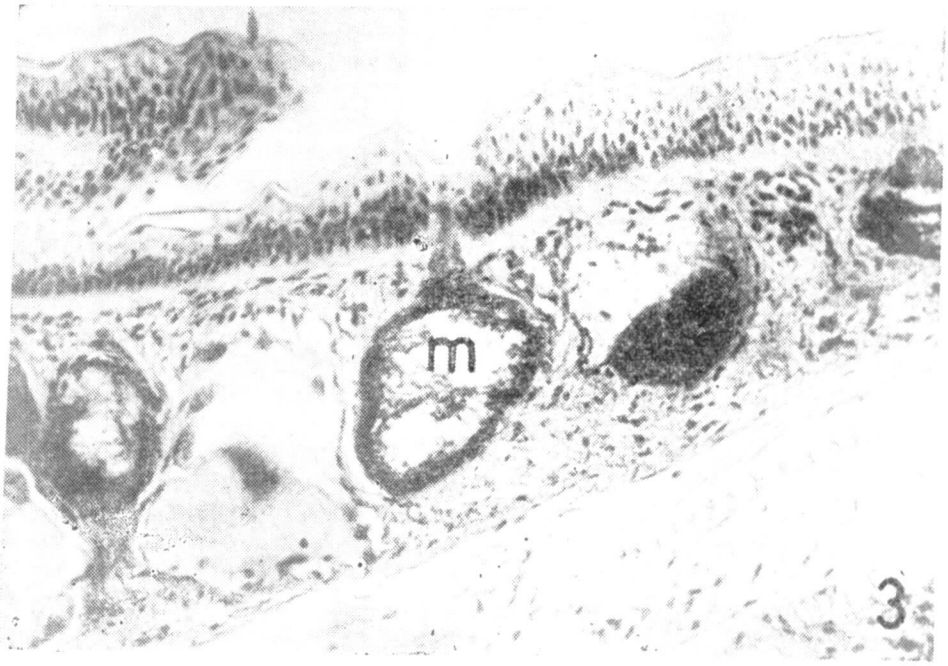
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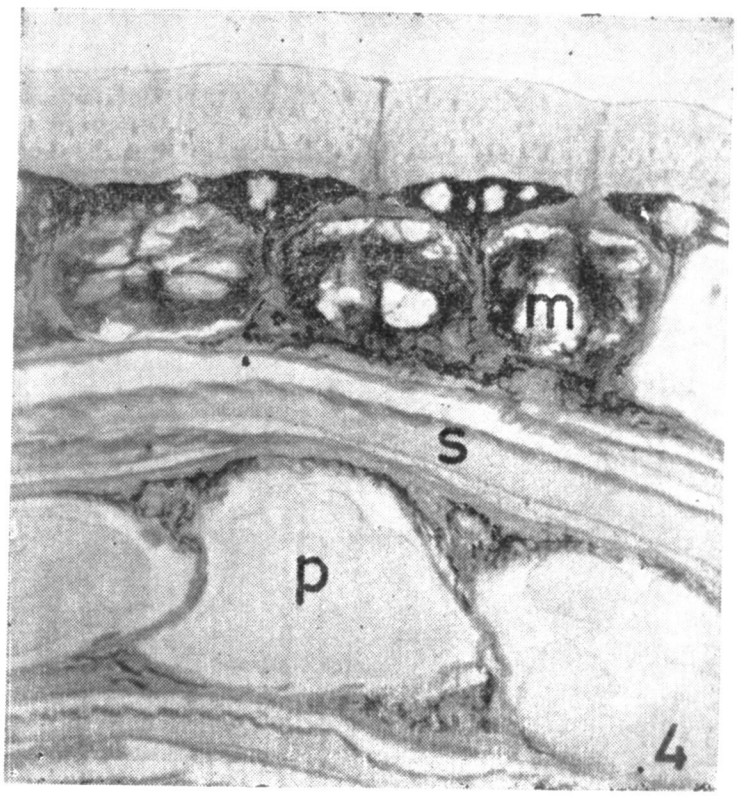
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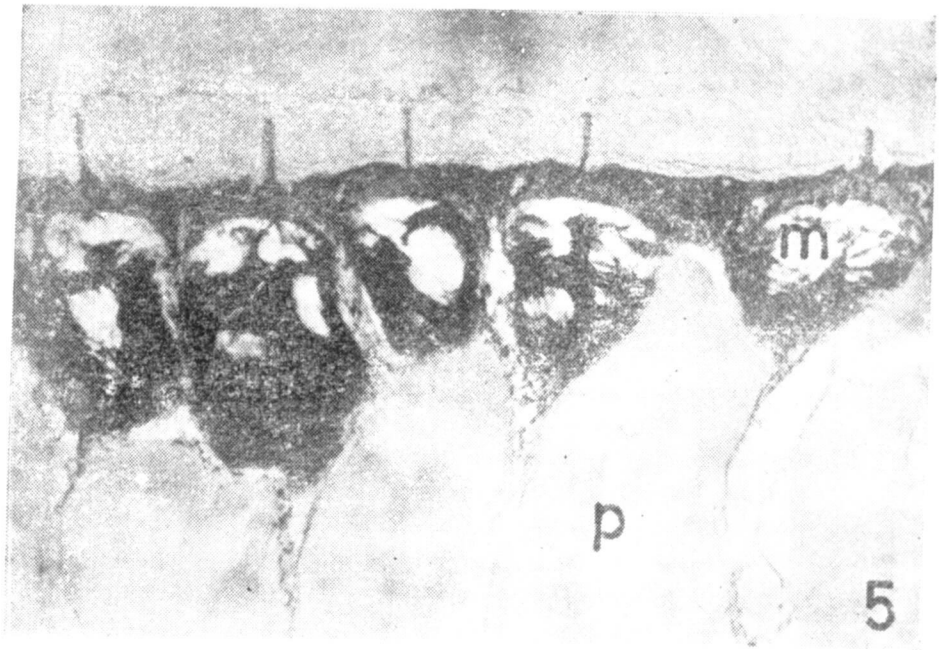
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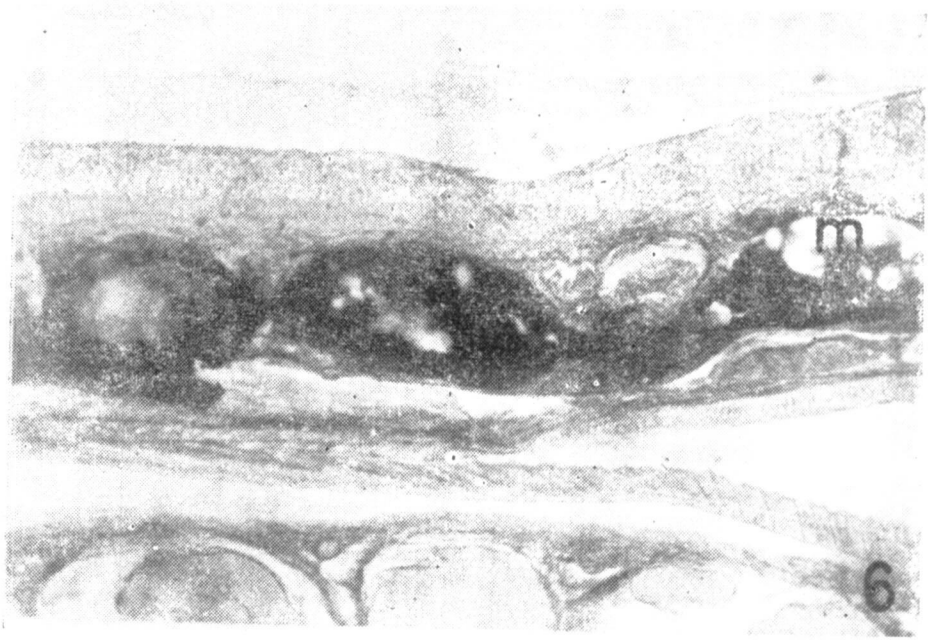
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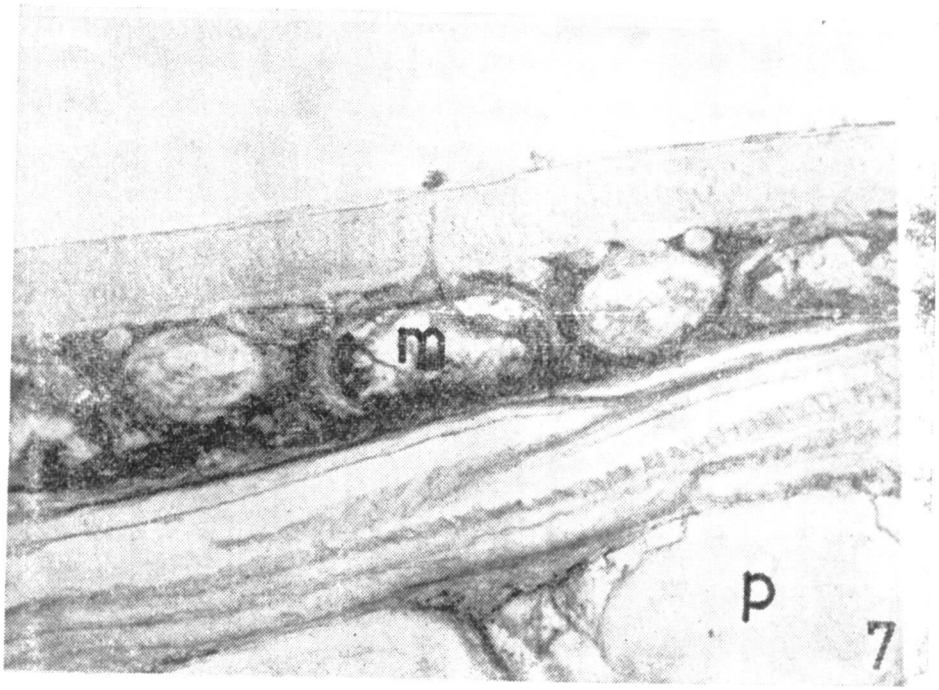
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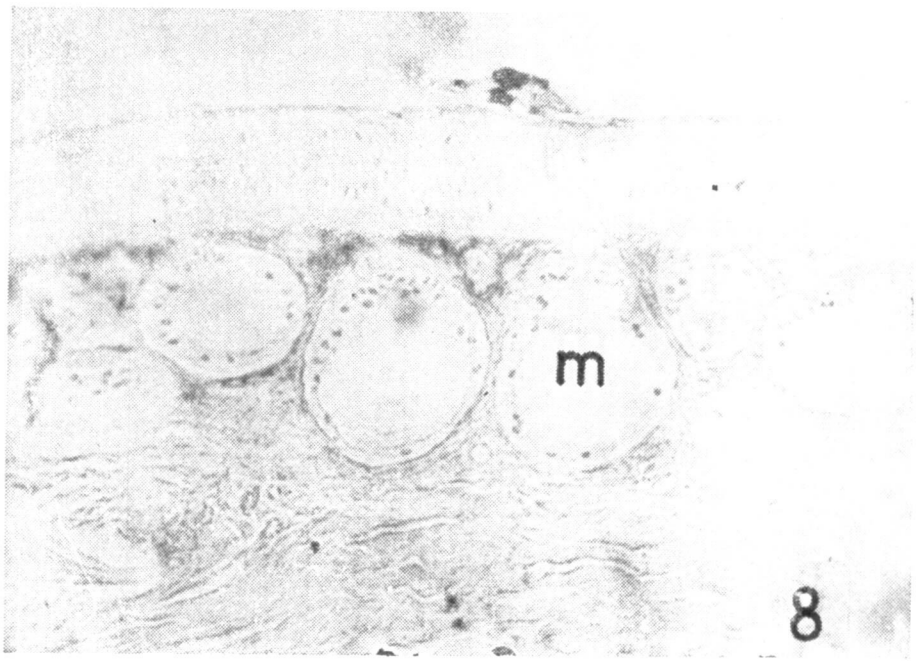
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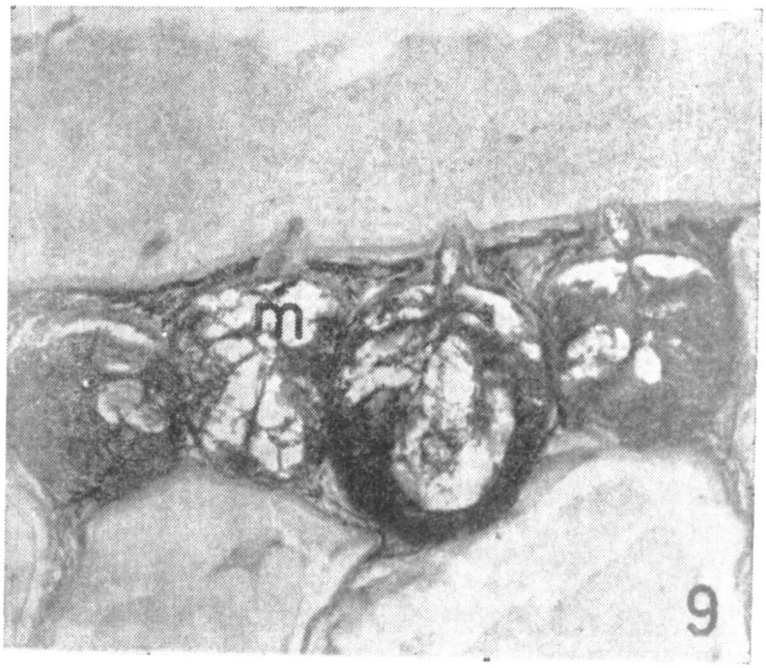
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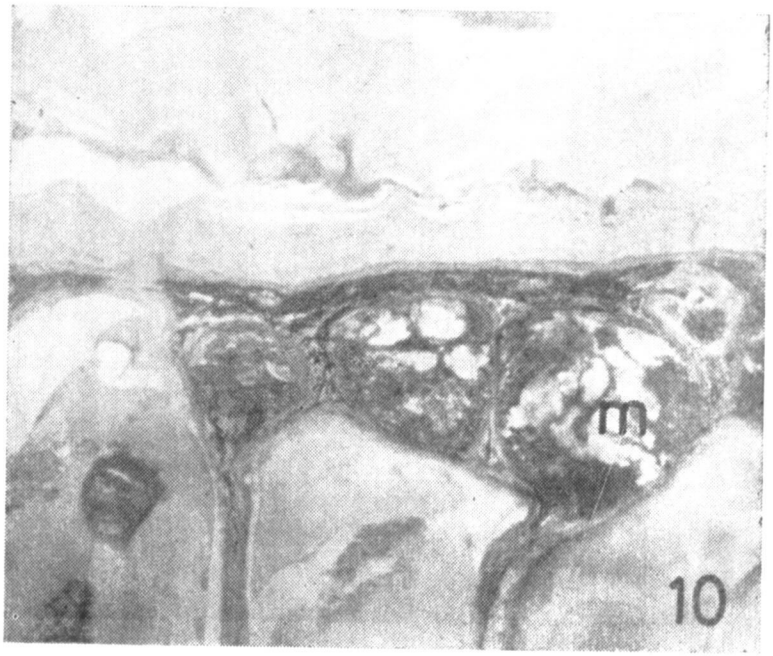
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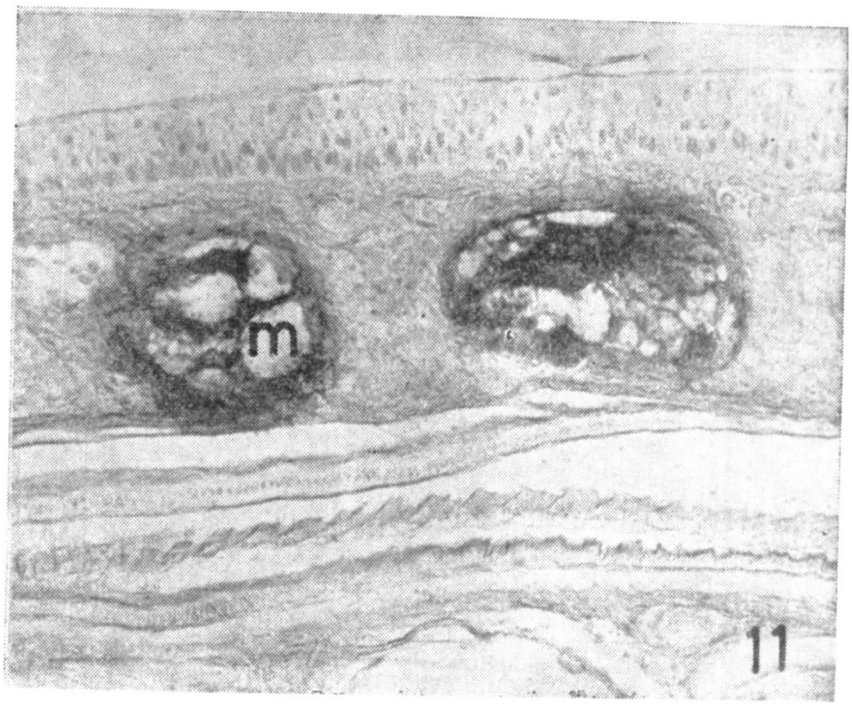
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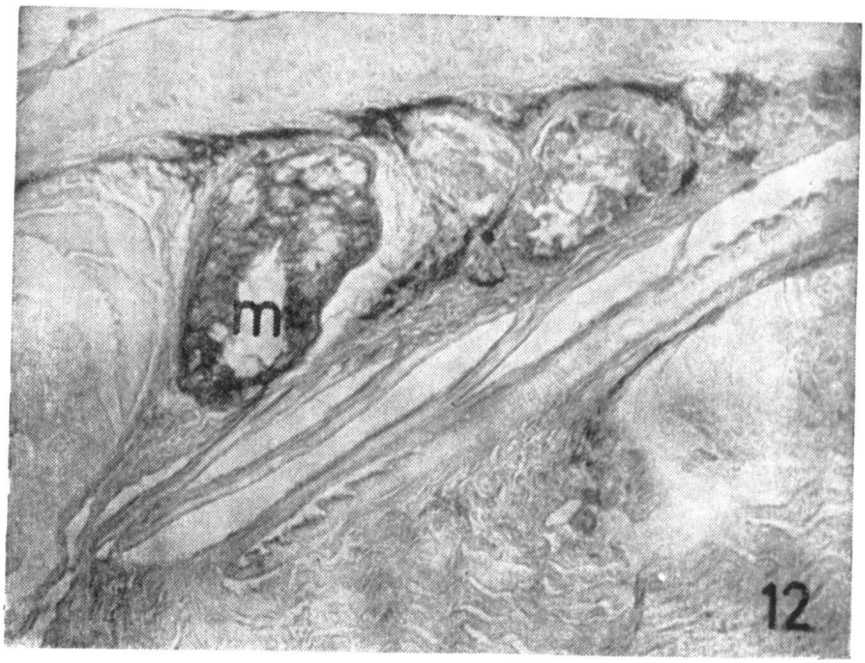
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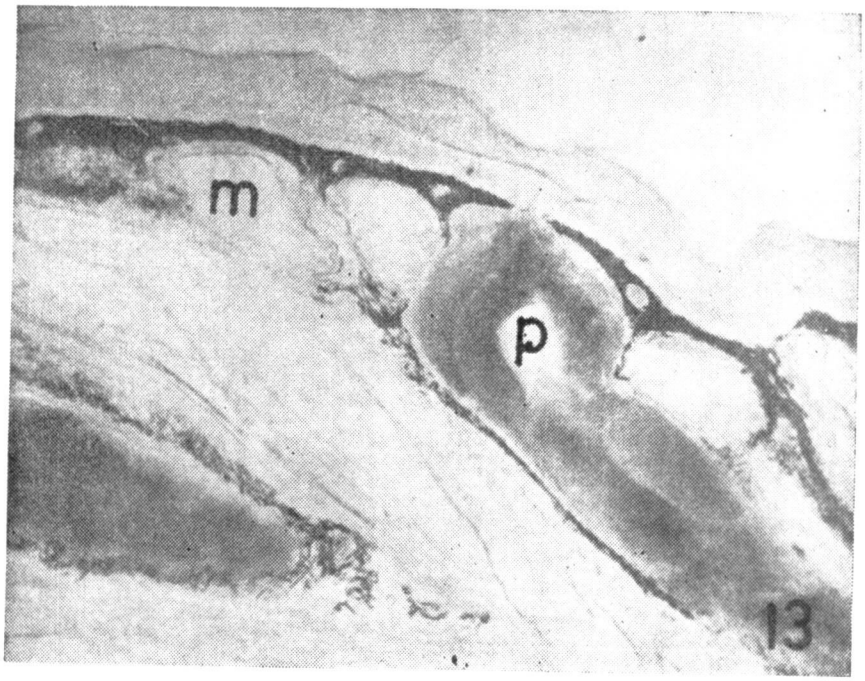
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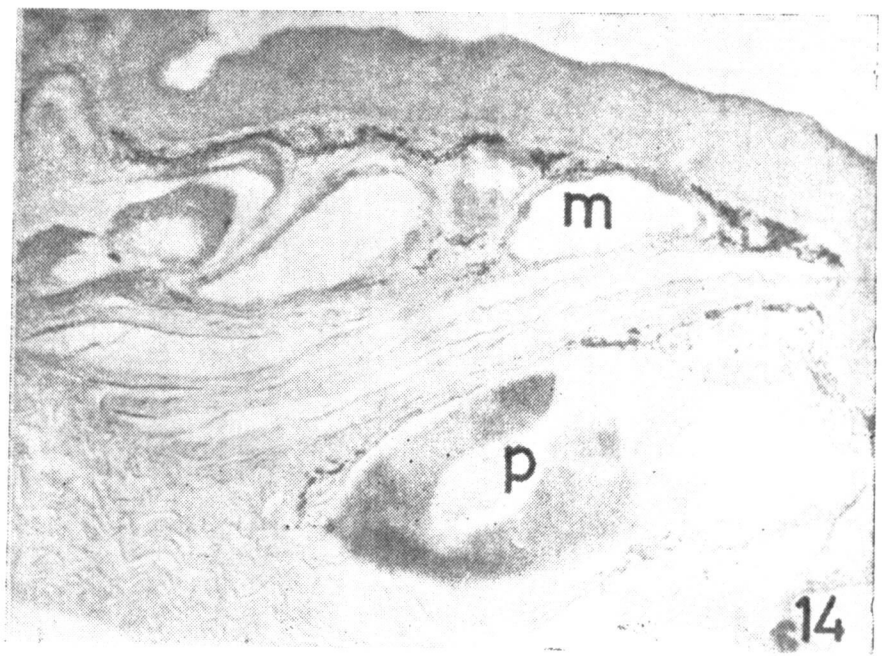
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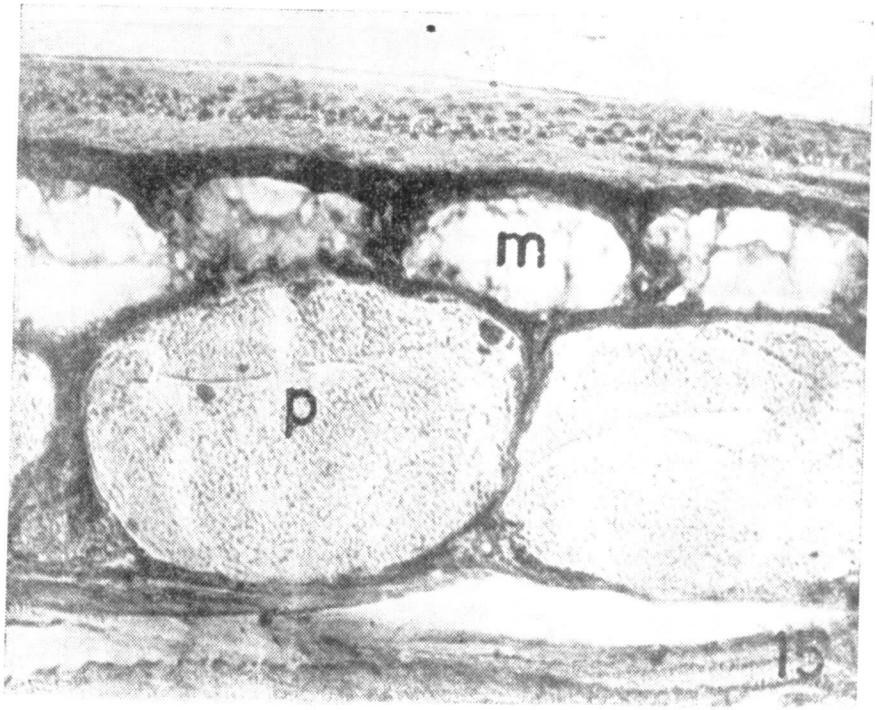
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