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Studies on organ cultures of slug reproductive tracts

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STUDIES ON ORGAN CULTURES OF SLUG REPRODUCTIVE TRACTS.

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Contents.

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ABSTRACT.

An anaesthetic technique was established for slugs which allowed 100% recovery.

Preliminary studies were carried out on the haemolymph of Agriolimax reticulatus. The depression of the freezing point of the haemolymph was determined in freshly captured and laboratory reared animals. The pH of the haemolymph in individuals in vivo was 7.72, while in pooled samples it was 8.86. Qualitative and simple quantitative studies were made on the haemolymph proteins. After the removal of haemocyanin, eight factors were found to be constant. In addition two factors were apparently related to the sexual state of the animal.

Experiments were undertaken to establish a suitable physiological saline for the maintainance of the heartbeat of the excised heart of A.reticulatus. This information, together with that obtained from the studies on the haemolymph, was used to adapt the medium of Burch and Cuadros (1965) for the culture of organs of A.reticulatus. This organ culture technique is described in detail.

A study of the histology, cytology and development of the organs used in the culture experiments was necessary in order to understand any changes resulting from the culture of the organs. Such studies of the hermaphrodite gland, hermaphrodite

ductules and constituent glands of the common duct are described and compared to those published for related animals.

The results of experiments on the culture of isolated organs, and associations of various organs, are described. The effects of culturing the tissues were assessed using the electron microscope. The preliminary results obtained are discussed in the light of previous work.

GENERAL INTRODUCTION.

Agriolimax reticulatus, the grey field slug, is a member of the stylomatophoran family Limacidae. It is reputedly the commonest slug in this country and may be found in most moist habitats. It is most abundant on or near cultivated land where it causes considerable damage, especially to young crops.

Agriolimax reticulatus was chosen as the study animals for the following reasons:-

1. It is common and may be found in all stages of sexual development throughout the year since it is not restricted to a breeding season.
2. It is widely used in this laboratory.
3. Laboratory reared cultures of this animal are readily available.
4. It is of great commercial importance.

A knowledge of reproduction in this and other slugs is mainly limited to descriptions of the anatomy and histology of the organs. Descriptions of the development of the reproductive organs in the maturing individual are very limited (Runham & Laryea 1968). Information on the control of their development and functioning has been obtained from naturally occurring phenomena, e.g. multiple sexual cycles, and from the results of the surgical experiments of Laviolette and others.

Work on pulmonates, prosobranchs and cephalopods, using

organ culture techniques, has produced useful information about their hormones. It therefore appeared probable that such a technique applied to a study of reproductive endocrinology in slugs would yield useful information.

Hormones have been shown to be carried in the haemolymph of molluscs (Laviolette, Streiff etc.). It was therefore logical to attempt to detect hormones in the haemolymph of A.reticulatus, and if possible isolate them. By a combination of these two approaches, it was hoped that our knowledge of the hormonal control of reproduction would be extended.

In this present work the results are presented as papers. Section One, published as a short note in Experientia, describes the anaesthetic technique developed for use in this study. This is followed by two sections on the analysis of the haemolymph of A.reticulatus, the first concerned with the physical properties of the haemolymph, and the second with a study of the haemolymph proteins. The results obtained in Section Two were used to develop the organ culture technique, and this is described in Section Four. Section Five is a detailed account of the structure and development of some of the organs which were cultured, while the final section is an account of the results obtained using this organ culture technique.

Studies on the haemolymph of the slug Agriolimax reticulatus

1. Osmotic pressure and pH measurements of the haemolymph and of the culture media and saline used in subsequent in vitro organ culture studies

Introduction.

While developing a medium for the *in vitro* culture of organs of the slug *Agriolimax reticulatus* (Bailey 1970), it became essential to know the values of the pH and osmotic pressure of its haemolymph and their variation. With this information, the saline and culture media used in such work may be adjusted to correspond with the values found in the animal.

One satisfactory method of determining the osmotic pressure of a complex solution such as haemolymph, is by measuring its freezing point depression. Great variation has been found in the freezing point depression of the haemolymph of pulmonates. It has been shown to vary from -0.18 to -0.43°C in *Arion ater* (Roach 1963), while that of *Helix pomatia* varies from -0.37 to -0.43°C when hibernating and from -0.30 to -0.40°C when active (Duval 1930, Kamanda 1933). The haemolymph of *Agriolimax reticulatus* varies from -0.20 to -0.80°C (Roach 1963 - calculated from Hughes & Kerkut 1956).

The pH of pulmonate haemolymph is high when compared with

the blood and tissue fluids of other animals. Roach (1963) records the high value of pH 8.83 for Arion ater, while values of pH 8.4 have been recorded for Helix pomatia (Lockwood 1961); pH 7.8 for Achatina (Michon & Alaphilippe 1958) and pH 7.86 for Pila globosa (Saxena 1957). Culture media with pH values as high as those recorded in the literature do not maintain organs of A.reticulatus in a healthy condition. Thus an accurate pH determination of the haemolymph must be carried out in vivo.

Materials and Methods.

Haemolymph was collected in two ways for the determination of the freezing point depression. Firstly, unanaesthetised animals were pinned out on wax and covered with liquid paraffin. Then the body wall was opened and the haemolymph collected directly from between the viscera. Care was taken to ensure that there was no contamination of the sample by mucus from the cut edges of the body wall. Animals which had damaged viscera were not used as a source of haemolymph. In this way alternate droplets of liquid paraffin and haemolymph could be collected in the fine capillary used for the determination of the freezing point depression.

In the second method, haemolymph was collected from animals anaesthetised in carbon dioxide (Bailey 1969). After about 15 minutes in anaesthetic the completely relaxed animal was moved so that it was in a normal crawling position. A 2 mm incision was made

in the dorsal body wall at the base of the optic tentacle. A small perforated aluminium cup about 1 mm in diameter (made by folding aluminium foil), was passed into the animal through the incision. A 30 gauge hypodermic needle was inserted into the cup and the cup, with the tip of the needle inside it, was moved to a position just posterior to the buccal mass. Up to 0.025 ml of haemolymph could be collected from this cup without any danger of the needle becoming blocked by the viscera. The small incision in the base of the optic tentacle healed after a short time without any need for suturing, and even with the aluminium foil cup inside, the animal appeared unharmed. Longevity and egg production did not seem to differ from those of unoperated animals. This method of haemolymph collection allows repeated sampling of one individual over long periods if necessary.

The microcryoscopic method of Ramsay & Brown (1955) was used to determine the freezing point depression (Δ). The sample of haemolymph or other liquid to be tested, was placed under liquid paraffin. Alternate droplets of liquid paraffin and sample were then drawn into the silica glass capillary, mounted on the carrier, frozen, and placed in the apparatus. When the first method of haemolymph collection was used, the alternate droplets of liquid paraffin and sample were already in the capillary. Three or four sample droplets were taken up in each capillary. The end points

(temperature at which the last ice crystal dissolved) of the samples that could be recorded simultaneously under the microscope, were noted. Each specimen was then re-frozen and the end points re-determined. Occasionally, the same number of samples could not be observed simultaneously under the microscope due to movement or coalescence of the sample droplets on re-freezing. Such cases account for the occurrence of differing numbers of first and second readings. (In a few cases third readings were made).

Measurements were carried out on four animals in Spring (April), four in late Summer (September), and four which had been kept in the relatively constant conditions of the laboratory for more than 30 days. Animals 6, 7, 8, 9 and 10 were anaesthetised before haemolymph collection, the remainder being unanaesthetised. Due to the small size of A.reticulatus and the relatively small amounts of haemolymph that can be collected from individuals, a pooled sample from 30 animals was collected. Determinations were made on this sample. The haemolymph was collected from anaesthetised animals and stored in a small glass container immersed in a freezing mixture at approximately -10°C. The resulting sample was allowed to stand, open to the air, in a refrigerator at 20°C for 12 hours so that dissolved carbon dioxide in the haemolymph could equilibriate with the air. Four separate determinations were made on this sample using a capillary micro-electrode (Pye Ltd).

In situ pH measurements were made using a micro-spear combination glass/reference electrode (type No. MS22DE Activion Glass Ltd). Large animals, which were freshly collected from the wild, were placed upside down on dry cellulose wadding. The animals tended to stick to this wadding and were thus partially immobilised. An incision was made in the mid-region of the foot, along the mid-line, and for about $\frac{1}{4}$ of the length of the animal. The pH sensitive glass of the electrode was carefully placed between the viscera inside the body cavity, via the pedal incision. The flexible reference junction was placed on the viscera which protruded from the incision. Great care was taken to ensure that none of the viscera was damaged, and this was checked both before and after the determinations were made. Initially measurements were made by placing the electrode either posteriorly between the lobes of the digestive gland or anteriorly between the reproductive organs. No difference was noticed between these sites. All subsequent measurements were therefore taken from the region of the reproductive organs since these are less easily damaged than the delicate organs of the digestive system.

Formulae for the saline and culture media used in the freezing point determinations are shown in table I.

TABLE I.

Saline I. after Hedon-Fleig. (Lockwood 1961)

NaCl	7.0 g/l
KCl	0.3 g/l
$CaCl_2$	0.1 g/l
$NaHCO_3$	1.5 g/l
Na_2HPO_4	0.5 g/l
$MgSO_4$	0.3 g/l
Glucose	1.0 g/l

Saline II. Saline I with the following additions:-

10,000 I.U. Penicillin per ml

10,000 g Streptomycin sulphate per ml.

Saline III. Saline II diluted 3:1 with double distilled water.

Culture medium I. Bailey (1970) - derived from Burch & Cuadros (1965).

Culture medium II. Medium I with the inorganic salt concentrations adjusted to the value of Saline I.

Culture medium III. Medium II diluted 3:1 with double distilled water.

Culture medium IV. Medium I containing 40% mature Helix aspersa haemolymph.

Results and discussion.

Ramsay & Brown (1955) made determinations on a 0.487N aqueous

solution of sodium chloride using their original technique. Their results varied from -1.678°C ± 0.003°C to -1.678°C ± 0.014°C, the different variations being due to varying sample sizes. Such results give an accuracy better than 1% of the mean. When studying complex solutions, other factors, such as the instability of proteins, will affect this accuracy. As only complex solutions such as haemolymph are measured in this present study, the accuracy of the technique will probably be less than that quoted by Ramsay & Brown.

The results for the determinations of the salines and culture media are shown in table II. The standard errors in these and following results are corrected to a value of P = 0.1% or 99.9% probability.

TABLE II

Sample.	Values of - °C.	$\bar{x} \pm$ S.E.	S.E.as % $\bar{x}$
Saline 1.	0.855 0.875 0.875	0.8694 ± 0.0126	± 1.5%
	0.875 0.870 0.875		
	0.865 0.865		
Saline 2.	0.835 0.835 0.830	0.8278 ± 0.0158	± 1.9%
	0.835 0.835 0.815		
	0.820 0.835 0.810		
Saline 3.	0.750 0.750 0.745	0.745	
	0.750 0.740 0.735		
Culture	0.685 0.685 0.690		
medium 1.	0.685 0.700 0.700		
	0.695 0.695 0.685	0.6891 ± 0.0082	± 1.2%
	0.685 0.685		
Culture			
medium 2.	0.930 0.895 0.920		
Culture			
medium 3.	0.765 0.755 0.750		
Culture			
medium 4.	0.740 0.740 0.740		
	0.745 0.720		

An estimate of the experimental error of the technique, as used here, was obtained from the results of the determinations of

salines 1 and 2 and culture medium 1. The maximum standard error, that of saline 2 is 0.8278°C ± 0.0158°C or 1.9% of the mean. This value of ± 0.0158°C is similar to the value of 0.014°C obtained by Ramsay & Brown. It would therefore seem that the accuracy of this technique as used here is almost as good as that obtained by these workers. The experimental error of this technique would thus seem to be less than ± 0.0158°C, an acceptable error in this present study.

The results for the determinations of Agriolimax reticulatus haemolymph are shown in table III.

TABLE III

Specimen No.	Value of 1st readings °C	Value of 2nd readings °C
Animal 1	0.690 0.690	0.680 0655
" 2	0.655	0.655
" 3	0.715	0.720
" 4	0.635	-
" 5	0.695	-
" 6	0.630 0.610 0.605	0.700 0.625 0.605
" 7	0.665 0.665	0.640 0.615
" 8	0.585 0.600	0.545 0.665
" 9	0.815 0.795 0.785	0.815 0.770 0.770 0.785
	0.750 0.755	0.770 0.765
" 10	0.735 0.725 0.765 0.755	0.720 0.690 0.740 0.675
	0.750 0.780 0.750 0.760	0.655 0.815 0.760 0.815
		0.745
" 11	0.800 0.815 0.820 0.815	0.860 0.855 0.835 0.835
" 12	0.750 0.740 0.705 0.705	0.720 0.720 0.750 0.745
	0.705	0.745

Sample.	$\bar{x} \pm$ S.E.	S.E. as $\bar{x}$
Animal 10 1st readings	0.7525 ± 0.0305	4.1%
" 10 2nd readings	0.7350 ± 0.0899	12.2%
Lab reared Ans 9-12 inclusive 1st readings	0.7625 ± 0.0291	3.8%
" Ans 9-12 2nd readings	0.7648 ± 0.0412	5.4%
Wild Ans 1-8 inc 1st readings	0.6492 ± 0.0481	7.4%
" " 2nd readings	0.6459 ± 0.0638	0.9%

It is evident from these results that the variation in the values of for A.reticulatus haemolymph are not as great as expected. The values of $-0.7625 \pm 0.0291^{\circ}C$ for laboratory reared animals and $-0.6492 \pm 0.0481^{\circ}C$ for wild ones are similar to the lower values calculated from the data of Hughes & Kerkut (Roach 1963). The calculated upper value of $-0.20^{\circ}C$ was never attained. However a greater variation might have been found had a greater number of animals been studied from widely varying habitats.

The standard error calculated for the first readings on wild animals is $\pm 0.0481^{\circ}C$ or $\pm 7.4\%$ of the mean. This value is greater than that which could be attributed to experimental error and must be due to a true variation in the osmotic pressure of the haemolymph. There was no obvious difference between the values obtained from animals 1-4, collected in the Spring, and animals 5-8 collected in the late Summer.

Measurements on animals kept in the laboratory for upwards of one month were rather lower than those of wild animals. The standard error for these first readings on laboratory reared animals is 0.7525 ± 0.0291 or ± 3.8% of the mean. This reduced standard error is probably due to the stabilising effect of the relatively constant, but rather dry conditions of the laboratory. However, it is also in part due to the increased number of determinations made for each animal and in part to the small numbers of animals used.

Second and third determinations were made on saline and medium samples with no variation in results. However, second and third measurements of haemolymph samples showed great variation. With animal 10, the standard error is ± 0.0305°C or 4.1% of the mean on the first determinations and ± 0.0899°C or 12.2% of the mean on the second determinations. Such a variation cannot be accounted for by experimental error.

All haemolymph determinations showed this increased variation after being frozen more than once. The repeated freezing and thawing of the haemolymph sample is probably affecting the stability of the blood proteins. It is well known that haemocyanin is easily broken into sub-units. This would explain any lowering of the . Higher values might be due to the coagulation or precipitation out of solution of some of the proteins, although no precipitate was ever noticed in the samples.

It is evident from this data that the true value for the osmotic pressure of A.reticulatus haemolymph can only be obtained from samples which have not yet previously been frozen. The variation in the osmotic pressure of the haemolymph sample is increased by freezing, but the mean value remains relatively constant. The osmotic pressure of Salines I and II and Medium II are outside the range found for A.reticulatus haemolymph. A dilution of 3 parts of solution to 1 part of double distilled water reduced their osmotic pressures to acceptable levels. The value of approximately 0.7500°C was chosen as the value to which all culture media and saline were adjusted.

The results of pH measurements on the pooled haemolymph sample are shown in Table IV. These readings have a mean of pH 8.86. This high value is similar to that recorded by Roach (1963) for Arion ater. Measurements of the in vivo pH of individual animals are also shown in table IV. These readings have a mean and standard error of pH 7.72 ± 0.04.

TABLE IV

pH determination

from pooled samples 8.85 8.85 8.90 8.85 mean = 8.86

in vivo pH determinations 7.70 7.65 7.70 7.70 7.75 7.75 7.75 7.70

7.70 7.65 7.70 7.72 7.75 7.65 7.75 7.75 7.80

pH = 7.72 ± 0.04 (p = 0.1 or 0.1%).

The pH of the haemolymph of pulmonates has been shown to be high. However there is great variation in the actual values recorded. Using these two methods, the results obtained for the pH of the haemolymph of Agriolimax reticulatus are very distinct. Recent work by Speeg and Campbell (1968) has shown that loss of carbon dioxide from the haemolymph of Otala lactea quickly causes the pH to rise from "around 7.4 - 7.6" to a value of 8.6 or higher. This value is similar to that recorded for the pooled sample of A.reticulatus haemolymph and similar to the high values recorded in the literature. Thus an estimate of the true pH of the haemolymph of terrestrial pulmonates must be made very rapidly, in vivo if possible, to reduce this pH rise due to the loss of carbon dioxide. Therefore, the pH of the haemolymph as determined in vivo was chosen as the required pH for all salines and media used for the culture of Agriolimax reticulatus organs.

Summary.

1. Two methods of haemolymph collection are described.
2. The freezing point depression (Δ) of the media and salines used for culture and the haemolymph of Agriolimax reticulatus were measured using the micro-cryoscopic method of Ramsay & Brown.
3. Values of $0.6492 \pm 0.0481^{\circ}C$ for animals freshly collected from the wild and $0.7625 \pm 0.0291^{\circ}C$ for laboratory reared animals were recorded for the Δ of the haemolymph of A.reticulatus.
4. The pH of pooled sample of haemolymph was measured using a

capillary micro-electrode and was found to be pH 8.86.

5. The *in vivo* pH of haemolymph was measured using a micro-spear combination glass/reference electrode and was found to be pH 7.72 ± 0.04.

Bibliography.

BAILEY, T.G., 1969 A new anaesthetic technique for slugs.
Experientia 25:1225.

BURCH, J.B., & CUADROS, C. 1965 A culture medium for snail cells and tissues. Nature. Lond. 206: 637-638.

DUVAL, M. 1930 Quoted in Kamada (1933).

HUGHES, G.M., & KERKUT, G.A. 1956 Electrical activity in a slug ganglion in relation to the concentration of Locke solution. J. Exp. Biol., 33, 282-294.

KAMADA, T. 1933 The vapour pressure of the blood of the edible snail. J. Exp. Biol., 10, 75-78.

LOCKWOOD, A.P.M. 1961 'Ringer' solutions and some notes on the physiological basis of their ionic composition. Comp. Biochem. Physiol., 2, 241-289.

MICHON, J. & ALAPHILIPPE, F. 1958 Comparison de quelques constantes biologiques de l'haemolymphe d'individus normaux de Achatina marginata (Gastéropode, Pulmoné) et d'individus présentant une anomalie de croissance. C.R. Soc. Biol. Paris. 152, 1349-1352.

RAMSAY, J.A. & BROWN, R.H.J. 1955 Simplified apparatus and procedure for freezing point determinations upon small samples of liquid. J. Sci. Instrum. 32, 372-375.

ROACH, D.K. 1963 Analysis of the haemolymph of Arion ater L. (Gastropoda, Pulmonata). J. Exp. Biol., 40, 613-623.

SAXENA, B.B. 1957 Inorganic ions in the blood of Pila globosa (Swainson). Physiol. Zool., 30: 161-164.

SPEEG, K.V. & CAMPBELL, J.W. 1968 Formation and volatilization of ammonia gas by terrestrial snails. Am.J.Physiol., 214: 1392: 1402.

Studies on the haemolymph of Agriolimax reticulatus

II. Studies on the haemolymph proteins

Introduction.

Observations by many workers (Streiff, 1966, 1967a, 1967b; Laviolette, 1954a, 1954b; Choquet, 1965; Pelluet & Lane, 1961; Pelluet, 1964; Runham, Bailey & Laryea, unpublished; Gomot & Guyard, 1967, 1969) have shown that hormonal substances are present in the haemolymph of gastropods. It was decided to fractionate the haemolymph proteins of Agriolimax reticulatus and attempt to observe any variable fraction which might be correlated with reproductive development. It was thus preferable that the method adopted be sensitive enough for the fractionation of haemolymph samples from individual animals.

Materials and Methods.

All haemolymph samples were obtained from animals anaesthetised in carbon dioxide (Bailey, 1969). Untreated haemolymph was obtained directly from anaesthetised animals, and was termed "whole haemolymph". In some cases it was found necessary to remove the haemocyanin as this protein masked the results. This was effected by the ultracentrifugation of whole haemolymph at 50,000 G for 90 minutes. Whole haemolymph was placed in a perspex container machined from solid rod. Six small holes, whose volumes were 0.015ml, 0.020ml or 0.025ml, were drilled in the top

of this piece of perspex. This container exactly fitted the bucket of a Swinging Bucket Rotor (type SW 39L) of a Spinco Ultracentrifuge model L. After ultracentrifugation haemocyanin was visible as a dark blue pellet at the bottom of the drilled holes. The supernatant of haemocyanin-free haemolymph was termed "centrifuged haemolymph".

1. Analysis of whole haemolymph by electrophoresis in a fluid film of agar.

The fluid film agar electrophoresis technique was modified from that of Ressler & Zak (1956) as described by Wild (1963). The sample was adsorbed onto 2.5 x 0.25cm Whatman No.3 filter paper strips by dipping the end of the strip into a small drop of the liquid. This was placed on the agar and a current of 300 V was applied, giving an initial current of 50 m/A, rising to 75 m/A after 15 minutes. After 2½ hours, the plates were dried and stained in a saturated solution of Amido Black 10B (Gurr) in methanol: distilled water: glacial acetic acid(50:50:10 v/v). Excess stain was removed by washing in the same solvent, and the plate then allowed to dry in air.

2. Analysis of whole and centrifuged haemolymph by starch gel electrophoresis.

Hydrolysed starch (Connaught Medical Research Laboratories, Toronto, Canada) was used to prepare gels for vertical

Fig 1 Polyacrylamide gel electrophoresis apparatus.

Fig 2 Gel holder, enabling gel to be scanned in microdensitometer.

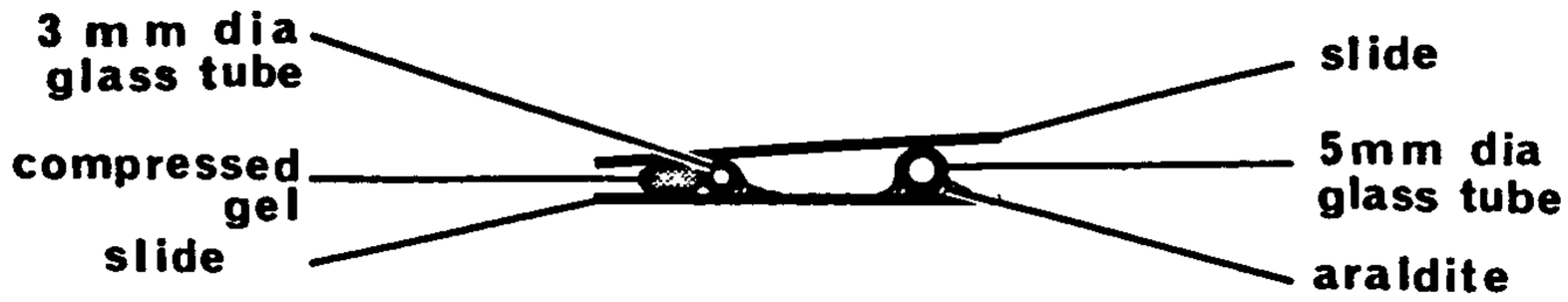

3 mm dia
glass tube
compressed
gel
slide
slide
5mm dia
glass tube
araldite

Fig 2

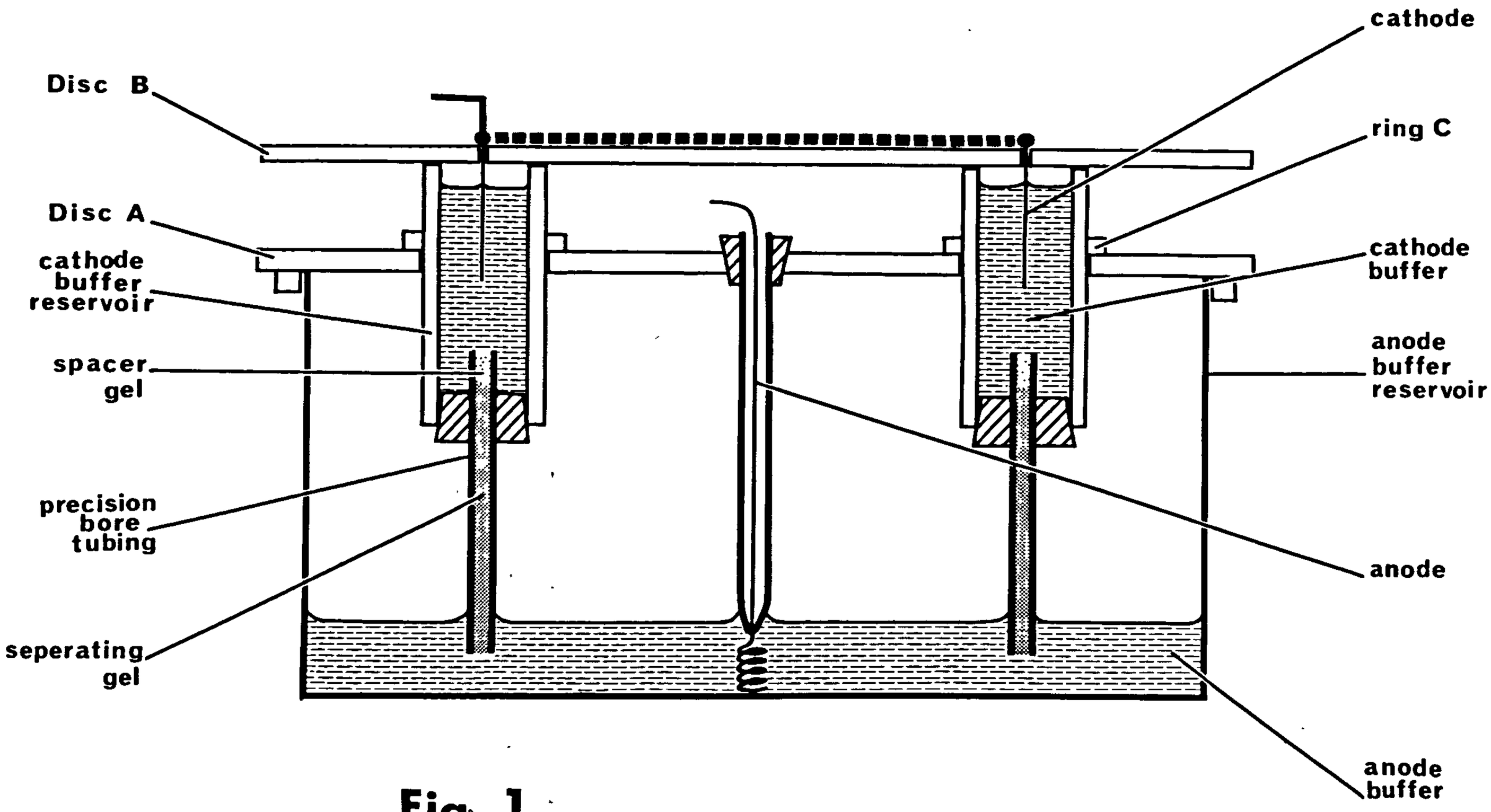

Disc B
Disc A
cathode
buffer
reservoir
spacer
gel
precision
bore
tubing
seperating
gel
cathode
ring C
cathode
buffer
anode
buffer
reservoir
anode
anode
buffer

Fig 1

electrophoresis which was carried out at room temperature for 16 hours under a voltage gradient of 4.5 volts per centimeter using a 0.026 M borate buffer of pH 9 in the electrode reservoirs (Smithies, 1959). Evaporation was prevented during the run by coating the surface of the gel with petroleum jelly. After electrophoresis, the gel was sliced with a thin bladed knife. The two slices were stained by the same method as that described for fluid film agar electrophoresis.

3. Analysis of whole and centrifuged haemolymph by Isoelectric focussing on 5% polyacrylamide gel.

The technique of isoelectric focussing of Awdeh, Williamson & Askonas (1968) was adopted. Samples of both centrifuged and whole haemolymph were placed on the gel. After the run, extensive washing of the gel was carried out in 10% T.C.A. prior to staining, in order to remove the carrier ampholytes. The gel was stained with Coomassie blue in 10% T.C.A. (Chrambach, Reisfeld, Wycoff & Zaccari, 1967), followed by extensive washing in 10% T.C.A.

4. Analysis of centrifuged haemolymph by disc gel electrophoresis.

Disc gel electrophoresis (Ornstein, 1962, 1964; Davis, 1964) was carried out in an apparatus (Fig.1) similar to that used by Davis & Lindsay (1967).

A large glass evaporating dish, 18.5cm diameter, was used

as the common anodal buffer reservoir and as a support for the remainder of the apparatus. An 8mm thick perspex disc A, with locating lugs around its rim, was made to fit the rim of the glass anodal buffer reservoir. A central 2cm diameter hole and 10 equally spaced 3cm diameter holes were drilled in this disc. The anode electrode, a coil of platinum wire mounted at the end of a glass tube fitted with a rubber bung, was placed in the central 2cm hole in disc A. 5mm internal diameter precision bore tubing (Chance) 7.5cm in length, was used to support the gel rods. These lengths of glass tubing were held in bored rubber bungs which fitted into 7.5cm lengths of 2.5cm internal diameter perspex tubing. These lengths of perspex tubing served as individual cathodal buffer reservoirs which were held in the 3mm diameter holes in disc A with interference fit perspex rings. These movable rings allowed the height of the cathodal buffer assemblies to be adjusted. The cathode assembly consisted of a ring of silver wire, of the same diameter as the centers of the 10 cathodal assemblies and was mounted on a 3mm thick perspex disc B. Ten 5cm lengths of platinum wire were soldered to this ring of silver wire so that they projected through holes in disc B into the cathode assemblies. This arrangement allowed any number of gels, up to a maximum of 10, to be run simultaneously.

The glass tubes, used to support the gel rods, were washed

in Calgon/sodium metasilicate solution, then in 1/100 N hydrochloric acid, and finally several times in distilled water (Paul, 1959) prior to filling with gel. A small rubber bung was placed in the end of each glass tube, projecting only 1-2mm into the lumen of the tube, and then the tube was placed in a stand so that it was held vertically. The required amount of Cyanogum 41 mixture (B.D.H.) was dissolved in 24.5ml of buffer (4.575 g tris + 6ml 1 N hydrochloric acid made up to 100ml with water - pH 8.9). 20 l of TEMED (N.N.N'N' Tetramethylmethylenediamine) was added to this solution which was then filtered. Polymerisation was initiated by adding 0.5ml of freshly prepared 7% w/v ammonium persulphate solution. The rate of polymerisation can be varied by altering the volume of ammonium persulphate added.

One ml of the above complete solution, made with 5% Cyanogum, was added to each tube and layered with distilled water. Polymerisation was judged to be complete when the Cyanogum solution became opaque. This first layer forms the separating gel. The spacer gel was formed in a similar manner. The distilled water used to layer the separating gel solution was removed with filter paper. 0.15ml of a 3% Cyanogum solution was then placed over the separating gel and layered as before. When polymerised, this solution produced the spacer gel.

The apparatus was assembled as shown in fig 1. The cathode and anode electrode reservoirs were filled with buffer at pH 8.3, prepared by dissolving 2.88 g glycine and 0.6 g tris in 1000ml water. Enough bromophenol blue solution was added to the cathode buffer reservoirs to colour the buffer a faint blue. The sample was carefully layered directly onto the spacer gel (40% sucrose was sometimes added to make this layering easier). The current was then switched on to give a low current of 1 m/A per tube for the first 5 minutes allowing the sample to penetrate the spacer gel. It was then increased to 5 m/A per tube until the bromophenol blue front was about 1.5cm from the end of the separating gel.

The gel rods were removed by rimming. A syringe fitted with a long needle and filled with water was used for this operation. The needle was pushed into the glass tube containing the gel rod, between the gel and the glass, and water injected during the operation. The injection of water lubricated the gel and allowed it to be removed from the glass tube with ease. The gels were then stained by immersion in either Amido Black 10B or in Coomassie blue, as described earlier. Destaining was carried out either by repeatedly washing the gel in the stain solvent, or by electrophoretic destaining. This latter was carried out in the electrophoresis apparatus with the gels held in wider tubes closed at their base with a plug of polyacrylamide gel. The stained gel rods were placed in a wedge shaped holder (fig 2), then placed

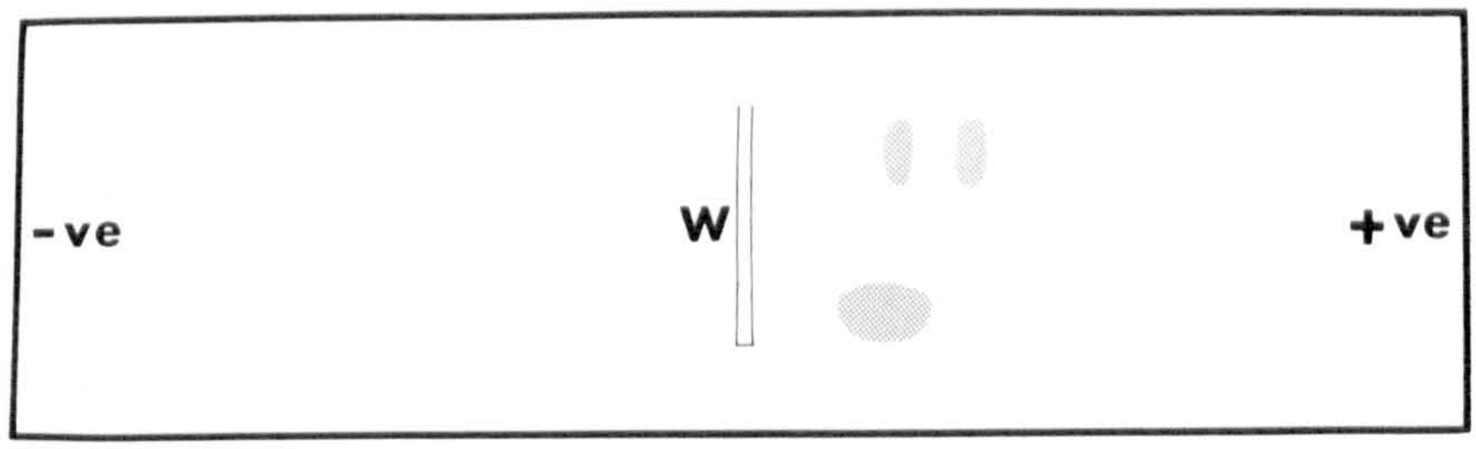
Fig 3 a) Protein pattern obtained by electrophoresis in a fluid film of agar.

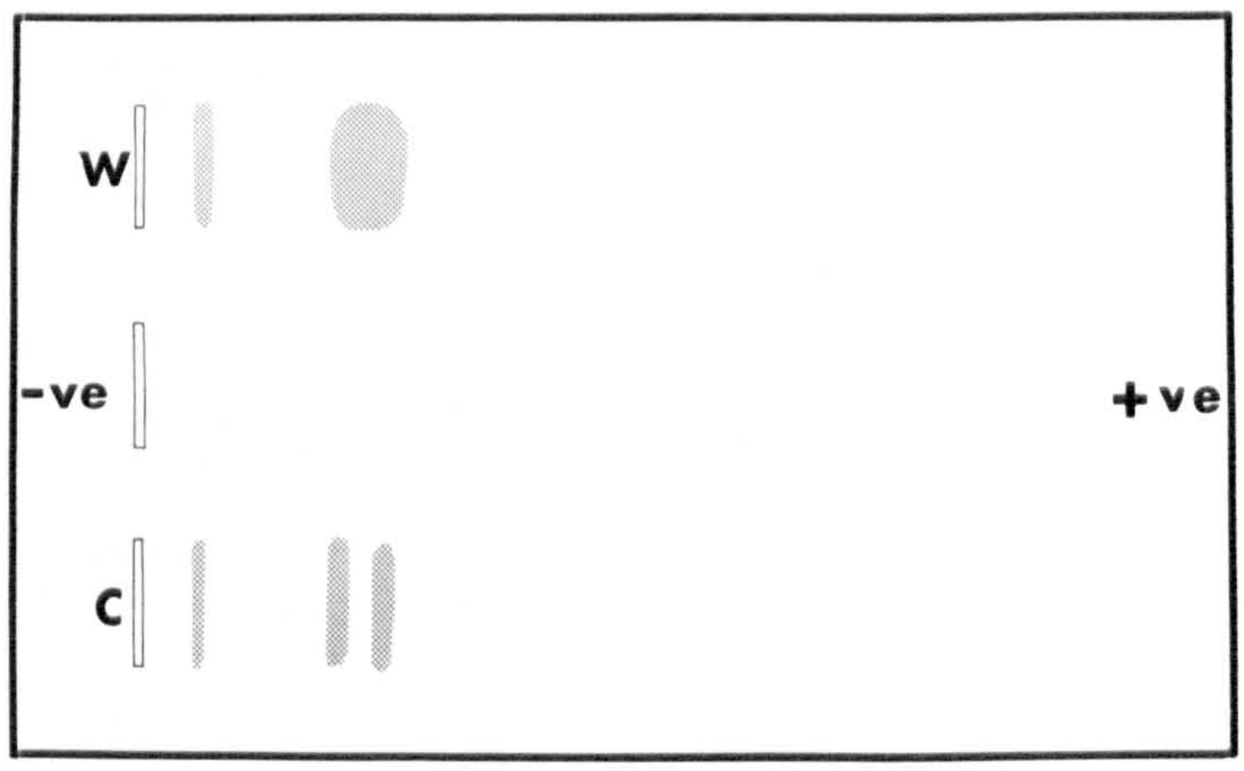
b) Protein pattern obtained by starch gel electrophoresis.

c) Protein pattern obtained by Isoelectrifocussing on 5% polyacrylamide gel. pH values shown at bottom of diagram.

w = whole haemolymph.

c = centrifuged haemolymph.





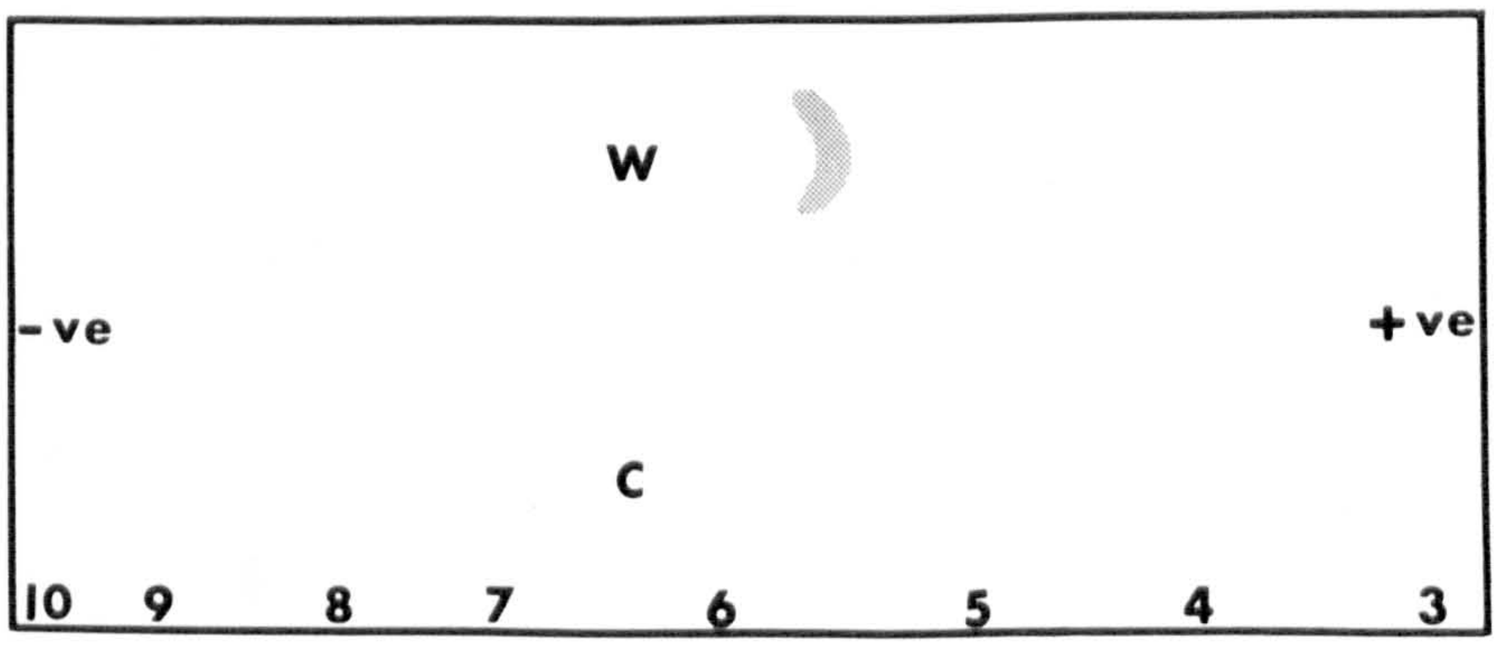


Fig 3

on the travelling stage of a Joyce recording microdensitometer and a tracing of the stained gel obtained.

5. Nitrogen estimations.

Because of the poor resolution of these protein fractionation methods on the small sample available (up to 0.025ml) and the necessity to remove haemocyanin in some cases, it was decided to estimate the protein in the samples. Total nitrogen was determined by digesting samples with hot concentrated sulphuric acid and estimating the resulting ammonia by a Nessler technique (Thompson & Morrison, 1951).

Results.

The results obtained with methods 1,2 and 3 are summarised in fig 3. The modified fluid film agar technique enabled three protein fractions to be observed in whole haemolymph. A two dimensional separation was obtained using this technique. One darkly staining fraction became laterally separated from the other two lightly staining fractions. If centrifuged haemolymph was used then the darkly staining fraction was absent. This fraction is certainly haemocyanin which becomes chromatographically separated from the other more mobile protein fractions when the sample is adsorbed on to the filter paper strip.

Analysis of haemolymph by starch gel electrophoresis

Fig 4 Diagram of the densitometer trace. This was obtained by scanning the protein pattern produced by disc electrophoresis of centrifuged haemolymph in a 5% separating gel.

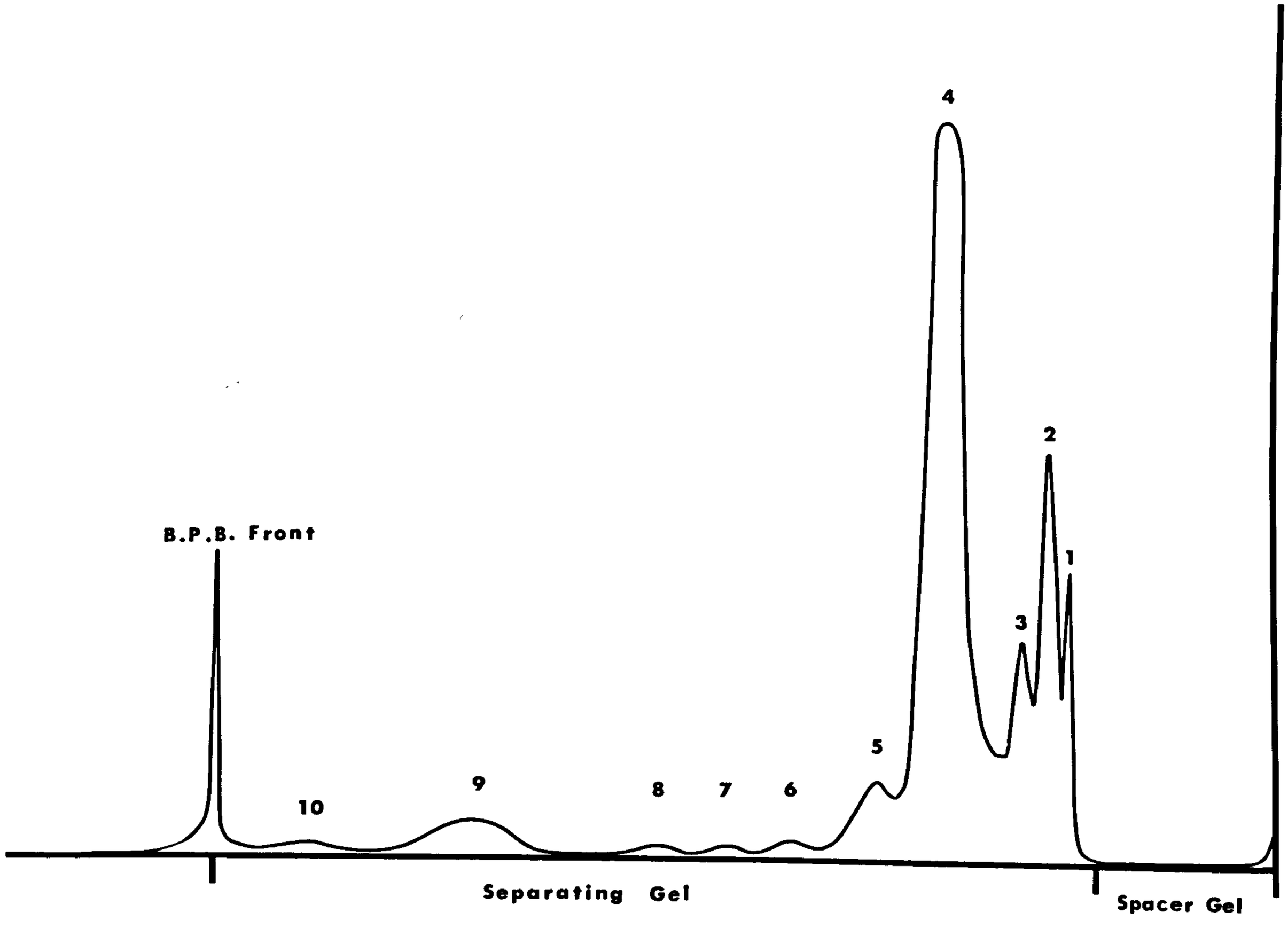


Fig 4

enabled four protein fractions to be observed. Only two fractions were seen in the whole haemolymph sample, while three were seen in the centrifuged sample. The large densely staining haemocyanin fraction obscures two of the proteins when whole haemolymph is used.

Isoelectric focussing gave very poor results, even when using the sensitive Coomassie Blue stain. Only one protein fraction was found and this was at pH 5.6-5.7. It was not present in centrifuged haemolymph and was therefore haemocyanin.

Using disc electrophoresis at least 10 protein fractions could be identified in samples of centrifuged haemolymph, although they could not always be seen in one sample (fig 4). Bands 1 and 2 varied considerably in intensity and band 3 was not always seen if the background staining in this area was too heavy. Band 4 was always the largest and most prominent fraction. Bands 5 and 6 varied considerably in size, 6 usually being the larger. Rarely was one or other of bands 5 and 6 totally absent. Bands 7, 8 and 10 were always very faint and not always seen if the sample size was small. Band 9 was very wide, diffuse and usually faintly staining. The bromophenol blue front was always visible both before and after staining. (It was also seen in controls containing no protein). Rf values (Table 1) of the various fractions have been calculated as a percentage of the distance travelled in the separating gel in relation to the bromophenol blue front.

TABLE I.

Band No.	Rf Value	Band No.	Rf value
1	3%	6	35%
2	5%	7	42%
3	7.5%	8	50%
4	16 - 18%	9	70%
5	26%	10	88%

Rf of bromophenol blue front 100%

Initially a 7.5% polyacrylamide gel was used as the matrix for electrophoresis but this gave poor separation because of the low mobility of the protein fractions. This low mobility was probably due to the relatively small pore size of the gel when compared to the large proteins found in these samples as a much better separation was achieved using a 5% gel. This percentage gel, combined with a 3% spacer gel, was used in all subsequent separations. Using this technique it was found necessary to first remove haemocyanin because the molecules of this protein were too large to enter even the 5% gel lattice. Haemocyanin migrated irregularly down the edge of the gel rod and tended to mask the first five bands. The masked bands could be seen if the gel was sectioned longitudinally. For this reason, centrifuged haemolymph was used in all but the initial runs.

The results of the nitrogen determinations are shown in

table II.

TABLE II.

Sample	g N/ml	mg protein/ml	% total N.
W.H. An 1.	3000	18.75	100
C.H. An 2.	120	0.75	4
	130	0.81	4.3
W.H. An 2.	2400	15.0	100
	2350	14.69	100
	2500	15.63	100
	2500	15.63	100
C.H. An 2.	180	1.13	7.5
W.H. An 3.	3200	10.00	100
	3350	20.94	100
	3350	20.94	100
C.H. An 3.	290	1.81	8
	240	1.50	8
	290	1.81	8
	275	1.72	8
Mean of 10 readings from active Helix aspersa W.H.2050		12.8	100
Mean of 18 readings from aestivating Helix aspersa W.H.4075		25.5	100
C.H. 225		1.4	5.5

C.H. dialysed against			
Hedon-Fleig saline	205	1.3	5.0
Non-proteinaceous Nitrogen	20	-	0.5

W.H. = whole haemolymph C.H. = centrifuged haemolymph.

These experiments were carried out using haemolymph from both Helix aspersa and Agriolimax reticulatus. Any differences in the nitrogen content of the haemolymph of these two animals were masked by the great variation between individuals.

Discussion.

The results of the nitrogen estimations show that the total protein concentration of the haemolymph of Agriolimax reticulatus is about 2%. As much as 95% of this protein is removed by ultracentrifugation and since this procedure removes the haemocyanin, this protein would seem to constitute up to 95% of the proteins in the haemolymph. Estimates of the non-proteinaceous nitrogen were obtained by dialysing centrifuged haemolymph against Hedon-Fleig saline (Lockwood, 1961). The remaining nitrogen in the dialysed centrifuged haemolymph was estimated and the loss of nitrogen calculated. Estimates obtained in this way show that only a small amount (20 g N/ml) of the nitrogen in the haemolymph is non-proteinaceous.

Since the protein concentration of centrifuged haemolymph is 1.3 mg/ml and the maximum sample size available for electrophoresis is 0.025ml, the maximum amount of protein present in any one sample is about 32 g.

Separation of proteins by the first three methods gave poor results in terms of number of fractions isolated. These methods did, however, demonstrate that ultracentrifugation does remove all haemocyanin from the sample without any apparent loss of other protein constituents from the haemolymph. The inability of these techniques to separate more than four fractions, including haemocyanin, would seem to be due to the small size of the sample and its low protein concentration.

The technique of disc gel electrophoresis was found to have a much greater sensitivity than the other techniques tried. A total of 11 fractions could be distinguished, 10 from the centrifuged haemolymph and the haemocyanin which was removed from the sample. Fractions 1 - 4 are constant in all the samples studied. The largest fraction No 4 may consist of more than one protein. In good separations it is possible to see three slight peaks within this fraction (fig 5). These first four fractions account for at least 85% of the area under the graph when this is measured with a planimeter. Hansl (1964) showed that there was a linear relationship between the uptake of stain and protein

concentration when Amido-Schwartz (Amido Black 10B) was used. Therefore, these first four fractions account for at least 85% of the protein in the sample, the remaining six fractions accounting for up to 15%.

Fractions 5 and 6 seem to vary with individuals. In 8 experiments fraction 5 was the largest of the two, fraction 6 being absent in one case. In 15 experiments fraction 6 was the larger, fraction 5 also being absent in one case. Haemolymph was mainly collected from large female stage animals. Haemolymph from an animal with its hermaphrodite gland at the post-reproductive stage H (Runham & Laryea, 1968) showed fraction 6 larger than fraction 5. Conversely, haemolymph from an animal with its hermaphrodite gland at the late spermatozoa stage E showed fraction 5 larger than fraction 6. It is not yet known how many hormones there are in the haemolymph of this animal, or even if they are proteins. However, fractions 5 and 6 would certainly seem to warrant further investigation as possible proteinaceous sex hormones in this slug.

The remaining fractions 7 - 10 stain very weakly and are probably present in very small amounts. However they do not appear to be very variable.

Of all the proteins which have been isolated by the technique of disc electrophoresis, fractions 5 and 6 are the only

ones which appear to vary. Davis & Lindsay (1967) were able to separate more fractions from the haemolymph of immature Helix pomatia than from the adults. Thus it may be possible to find more fractions in the haemolymph of immature A.reticulatus and some of these may be correlated with reproductive development. However, before this can be carried out either the sensitivity of the technique, or the volume of haemolymph which can be collected from small animals must be increased.

Bibliography.

AWDEH, Z.L., WILLIAMSON, A.R. & ASKONAS, B.A. (1968) Isoelectric focussing in polyacrylamide gel and its application to immunoglobins. Nature, Lond. 219: 66-67.

BAILEY, T.G. (1969) A new anaesthetic technique for slugs. Experientia, 25: 1225.

CHOQUET, M. (1965) Rechcrches en culture organotypique sur la spermatogenèse chez Patella vulgata L. (Mollusque Gastéropode). Rôle des cérébröides et des tentacles. C.r.hebd.Séanc.Acad.Sci.Paris, 261: 4521-4524.

CHRAMBACH, A., REISFELD, R.A., WYCKOFF, M. & ZACCARI, J. (1967) A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. Analyt. Biochem. 20: 150-154.

DAVIS, B.J. (1964) Disc electrophoresis-II, Method and application to human serum proteins, Ann. N.Y. Acad. Sci., 121: 404-427.

DAVIS, G.M. & LINDSAY, G.K. (1967) Disc electrophoretic analysis of molluscan individuals and populations. Malacologia, 5: 311-334.

GOMOT, L. & GUYARD, A. (1964) Evolution en culture in vitro de la glande hermaphrodite de jeunes escargots de l'espèce Helix aspersa Mull. C.r.hebd.Séanc.Acad.Sci. Paris, 258: 2902-2905.

GUYARD, A. (1967) Féminisation de la glande hermaphrodite juvénile d'Helix aspersa Mull associée in vitro au ganglion cérébroïde d'escargot adulte ou de Paludine femelle. C.r.hebd.Séanc.Acad.Sci.Paris. 265: 147-149.

GUYARD, A. (1969) Autodifférenciation femelle de l'ébauche gonadique de l'escargot Helix aspersa (Mull) cultivée sur milieu anhormonal. C.r.hebd.Séanc.Acad.Sci.Paris. 268: 966-969.

HANSL, R. (1964) Review of some new applications for disc electrophoresis and for the criteria for quantitative densitometry. Ann. Acad. Sci. 121: 391-403.

LAVIOLETTE, P. (1954a) Rôle de la gonade dans le déterminisme hormonal de la maturité glandulaire du tractus génital chez quelques gastéropodes Arionidae et Limacidae. Bull.Biol.Fr.Belg. 88: 311-332.

LAVIOLETTE, P. (1954b) Étude cytologique et expérimentale de la régénération germinale après castration chez Arion rufus. Ann.Biol. 30: 481-492.

LOCKWOOD, A.P.M. (1961) Ringer Solutions and some notes on the physiological basis of their ionic composition. Comp. Biochem.Physiol. 2: 241-

ORNSTEIN, L. (1962) Theory of disc electrophoresis. Reprint from Canalco, Bethesda, Maryland. pp.1-44.

PAUL, J. (1959) Cell and tissue culture. Livingstone, Edinburgh & London.

PELLUET, D. & LANE, N.J. (1961) The relation between neurosecretion and cell differentiation in the ovotestis of slugs. Can.J.Zool. 39: 789-805.

PELLUET, D. (1964) On the hormonal control of cell differentiation in the ovotestis of slugs. Can.J.Zool.42: 195-199.

RESSLER, N. & ZAK, B. (1956) Electrophoresis in a fluid film. Clin.Chim.Acta. 1: 392-

RUNHAM, N.W. & LARYEA, A.A. (1968) Studies on the maturation of

the reproductive system of Agriolimax reticulatus (Pulmonata: Limacidae). Malacologia 7: 93-108.

RUNHAM, N.W., BAILEY, T.G. & LARYEA, A.A. (Unpublished).

SMITHIES, O. (1959) An improved procedure for starch gel electrophoresis. Further variations in the serum proteins of normal individuals. Biochem.J. 71: 585-587.

STREIFF, W. (1966) Étude endocrinologique du déterminisme du cycle sexuel chez un mollusque hermaphrodite protandre Calyptraea sinensis L. I. Mise en évidence par culture in vitro de facteurs hormonaux conditionnant l'évolution du tractus génital mâle. Ann.Endocrinol. 27: 385-400.

STREIFF, W. (1967a) II. Mise en évidence par culture in vitro de facteurs hormonaux conditionnant l'évolution du tractus femelle. Ann.Endocrinol. 28: 461-472.

STREIFF, W. (1967b) III. Mise en évidence par culture in vitro de facteurs hormonaux conditionnant l'évolution de la gonade. Ann. Endocrinol. 28: 641-656.

THOMPSON, J.F. & MORRISON, G.R. (1951) Determination of organic nitrogen. Control of variables in the use of Nessler's Reagent. Anal. Chem. 23: 1153-1157.

WILD, A.E. (1963) Proteins of rabbit foetal fluids. PhD Thesis, University of Wales.

The establishment of an in vitro organ culture technique for organs of the slug Agriolimax reticulatus

Introduction.

Techniques for the culture of vertebrate cells and organs have been available for many years and well developed synthetic media have been developed. In the last decade, Workers have been increasingly interested in these techniques as tools for research into cell biology, virology, parasitology and endocrinology. The media used for the culture of invertebrate cells and organs have often been adapted from those developed for vertebrate culture. Much of the early work was with marine organisms where media based on filtered sea water, with or without added nutrients were used.

Early tissue culture techniques applied to molluscs, especially terrestrial forms, met with little success. Despite the powers of regeneration which these animals possess, it proved very difficult to obtain proliferation of mollusc cells in vitro. However, migration of cells from the explant did occur, (Gatenby 1932). It is only in the last decade that cell proliferation in invertebrate tissue culture has been overcome (review by Jones 1962).

The study of invertebrate organ culture techniques is even more recent, little work having been carried out before the 1950's.

The majority of this invertebrate organ culture work has been with insects, a reflection of their economic importance.

The development of reliable techniques for molluscan organ culture are by no means as well advanced as with methods for insects. Work by Gatenby et al (1931, 1932, 1934a and 1934b) demonstrated the ease with which cells will migrate from pieces of mantle tissue of Helix aspersa, even in non-sterile conditions. He used either Helix haemolymph or Hedon-Fleig saline as a medium for these experiments. However, because of the lack of cell division (Gatenby 1934b) this work may be described as cell maintainance, rather than cell culture (which implies cell proliferation in vitro). Bohuslav, (1932 - reviewed by Gatenby 1934b), used a saline with the addition of either an extract of the explanted tissue or a 3% solution of peptones prepared by peptic digest of ox fibrin. The pH of this medium was adjusted to the pH of the haemolymph, which according to Bohuslav, was between 6.8 - 7.4. However, this pH is known to be too low for some of the species he used, e.g. Helix pomatia haemolymph has a pH of 7.6 - 7.9 (Roach 1963), and this fact might explain the less extensive outgrowths obtained by Bohuslav. No mention was made of cell division, and this again is probably an example of cell maintainance rather than cell culture.

Workers on molluscan culture prior to 1950 concentrated on the maintainance of cells in physiological media. In the last

decade, with the improvement of techniques in other fields of culture, interest has been renewed in molluscan culture. Vago & Chastang (1958) succeeded in producing cell lines of Helix aspersa and H. pomatia using a fairly simple medium consisting of a physiological saline with the addition of Casein hydrolysate, glutamine, yeast extract, choline and antibiotics. Small numbers of mitoses were observed. They (Vago & Chastang 1960) later succeeded in culturing oyster cells again obtaining mitoses, although the frequency was much less than can be obtained in vertebrate cell cultures.

Karnik & Kamat (1961), working on Lamellidens corrianus, made observations on the growth of epithelial cells from the digestive diverticulae in tissue culture. They used Hedon-Fleig saline (pH 7.0) as their medium with a hanging drop technique. It would seem that they had trouble in effecting complete sterility, as they added 0.001% propionic acid and 0.025% mercuric chloride to the medium. However they do report having seen large numbers of cell divisions in their cultures using aceto-orcein preparations.

Flandre & Vago (1963) obtained outgrowths of cells from Helix aspersa tissues. They succeeded in subculturing the cells from these outgrowths and obtained active cell division. They used chicken plasma coagulum as a support for the primary cultures and a complex medium incorporating snail extract and chick embryo

extract.

Necco & Martin (1963) succeeded in obtaining mitoses in Octopus white body cells using a modified Parkers TC 199 medium (in which the osmotic concentration of the medium was adjusted to correspond with that of octopus body fluids with sodium chloride).

Chardonnet & Peres (1964) cultured Mytilus galloprovincialis heart and muscle cells after dissociating them with tripsin. Using a simple medium containing up to 10% of young Mytilus tissue extract, they succeeded in obtaining cell maintainance but never observed any cell multiplication.

Burch and Cuadros (1965) using a complex medium, succeeded in culturing gonadal cells of Helix pomatia, Planorbina glabrata and Pomatiopsis lapidaria and promoting their growth for 60 days. During this time mitotic and meiotic stages of cell division were seen.

Perkins & Menzel (1964) succeeded in maintaining oyster cells for up to 42 days in a medium containing various oyster tissue extracts. They reported that there was no evidence of mitoses in these cultures.

Tripp, Bisignani & Kenny (1966), using a variety of media containing chicken serum, succeeded in maintaining oyster amoebocytes. No cell divisions were seen ~~and~~ in the cultured amoebocytes, although a few mitotic figures were seen in

"fibroblast-like" cells. However Li & Stewart (1966) using more complex media containing vertebrate sera succeeded in obtaining multiplication of oyster cells.

Rannou (1967) made long term cultures of cells from three marine molluscs in media containing 20% homologous serum. Numerous subcultures of epitholial and conjunctive cells were made but no mention was made of cell divisions, although cells were often binucleate.

Molluscan organ culture has only been attempted in the last few years, much of this work involving marine animals. One can distinguish two types of method for the culture of mollusc organs.

a) Organ culture on a solid medium.

The methods for culturing mollusc organs on a solid medium have been adapted from the method of Wolff & Haffen (1952), first developed for the culture of embryonic organs of vertebrates. This method was first used by Sengel (1961) for the culture of various organs of the marine invertebrates. He succeeded in maintaining brachial lamellae and intestinal diverticulae of Barnea candida for up to 7 days.

Stréeff & Peyro (1963) and Streiff (1963, 1964, 1966a, 1966b, 1967a and 1967b) have developed this technique for organs of the marine prosobranch mollusc, Calyptraea sinensis. With

this technique Streiff has shed much light on the hormonal relationships between the reproductive tract and various organs controlling the sexual cycle in this protandric hermaphrodite mollusc.

Guyard & Gomot (1963), Gomot & Guyard (1964) and Guyard (1967, 1969a, 1969b) adapted this technique for the culture of gonads of juvenile Helix aspersa. They studied the survival and differentiation of this organ when cultured alone, with the cerebral ganglion of an adult Helix aspersa or with the cerebral ganglion of a female Viviparus viviparus.

Choquet (1964, 1965), culturing the gonads of Patella vulgata succeeded in obtaining gametogenesis and then studied the role of the cerebral ganglia and tentacles in controlling its development. The culture of a variety of marine annelids and mollusc tissues was carried out by Durchon & Schaller (1963) and Durchon (1964) again using this same technique. Durchon & Richard (1967) studied the endocrine role of the optic gland in the ovarian maturation of Sepia officinalis.

b) Organ culture on a liquid medium.

Bevelander & Martin (1949) working on the mechanisms of calcification in Pintata radiata were able to maintain muscular and ciliary activity for up to 6 days in mantle tissue strips using mollusc blood as the medium.

Long term culture of molluscan organs in a liquid medium was first attempted by Benex (1961, 1964, 1965, 1966, 1967a and 1967b). This worker studied the survival of Australorbis glabratus and Planorbis corneus explants, using a technique developed from that used by J.A.Thomas (1941).

Hollande (1968) has used a liquid medium to study the development of secretory granules in the cells of the mucus gland of Helix pomatia. Vianey-Liaud & Lancastre (1968) have also developed a culture technique for the organs of Australorbis glabratus using a diphasic medium.

The present study was undertaken to develop a culture medium for organs of the slug Agriolimax reticulatus in order to investigate the control of the development of the reproductive tract. As there are many observations in the literature that active hormonal substances are transmitted in the haemolymph of molluscs (Laviolette, Pelluet & Lane, Pelluet, Streiff, Guyard, Choquet) it was considered preferable for the basic medium to contain no molluscan protein. It was decided to develop a liquid culture medium for the following reasons:-

1. The complete medium is easily sterilised by filtration.

2. It is easy to change the medium in long term experiments without disturbing the explants.

3. The medium is more homologous and stable.

Results. Development of Saline.

The pH of the haemolymph was found to be very high pH 8.86, but similar to that found in Arion ater by Roach (1963). This was assumed to be the correct pH value and the initial development of the saline and medium for Agriolimax reticulatus was based on this figure. (During the course of experiments it became obvious that this value was incorrect. Organs survived in a medium whose pH was 7.7 - 7.8 and subsequent in vivo pH measurements demonstrated that the true pH of the haemolymph does in fact lie within these limits). In consequence, the physiological saline upon which the medium is to be based must have a low concentration of bicarbonate and phosphate ions because these ions will readily precipitate calcium in such alkaline solutions.

The salines considered for this purpose were:-

1) The saline used by Flandre & Vago (1963) for studies on Helix tissues.

2) Hedon-Fleig saline (Lockwood 1961, Goddard 1960, Gatenby 1932).

3) The saline devised by Roach (1963) for Arion ater.

The Flandre & Vago saline was discarded because of its high calcium and bicarbonate content (Table I). The abilities of H edon-Fleig saline, as recommended by Goddard (1961) for Helix,

and the saline devised by Roach for Arion ater to maintain the heart beat in the excised heart of A.reticulatus and the contractile properties of the leading edge of the mantle were compared. Both the tissues were immersed in saline in a non-sterile culture chamber. The pH of both solutions was adjusted to the same value.

TABLE I.

Salt	Hedon-Fleig	Roach's	Flandre & Vago
NaCl	7.0 g/l	2.52 g/l	7.2 g/l
KCl	0.3 g/l	0.26 g/l	0.4 g/l
$CaCl_2$	0.1 g/l	0.336 g/l	0.3 g/l
Na_2HPO_4	0.5 g/l	0.03 g/l	-
NaH_2PO_4	-	-	0.2 g/l
$NaHCO_3$	1.5 g/l	0.76 g/l	5.5 g/l
$MgSO_4$	0.3 g/l	0.51 g/l	0.2 g/l
Glucose	1.0 g/l	11.5 g/l	-
$NaSO_4$	-	0.53 g/l	-
NaOH	-	0.064 g/l	-

Calculated g/l anhydrous salts.

Bacterial contamination was not noticed until about 60 hours after the start of each experiment. Roach's saline was found to

maintain the heart beat for up to 2 hours with a slowing down of the rate of beating during the second hour. Contractions of the leading edge of the mantle continued rhythmically for about 24 hours and then on stimulation for about another 12 hours.

In Hedon-Fleig saline the heart beat was maintained for 24 hours but the rate of beating gradually slowed after the first hour, eventually becoming intermittent by 24 hours. Thereafter, beating would start if the heart was stimulated, it was maintained for a few minutes at a high rate, then stopped. No beating could be induced after 48 hours. The contractions of the mantle edge followed a similar course, continuing rhythmically for about 48 hours, thereafter needing some stimulation. No contraction occurred after 72 hours. Slight variation in the rates of contraction was noticed during the course of this experiment and the figures stated, and those in table II are maxima.

As a result of these experiments, it was decided to adopt the Hedon-Fleig saline as the basis of the physiological saline in the medium.

TABLE II.

Saline													
	H/B.p.min.	40	36	28	24	21	18	10	48	-	-	-	-
Hedon-Fleig	Mantle	++	++	++	++	++	++	++	++	++	+	+	-
	H/B.p.min.	38	29	-	-	-	-	-	-	-	-	-	-
Roach's	Mantle	++	++	++	++	++	++	++	++	+	-	-	-
	Time hrs	0	1	2	3	6	12	18	24	36	48	60	72

++ = spontaneous contraction of mantle

+ = contraction of mantle when stimulated

H/B.p.min. = Heart beats per minute.

The requirement for a liquid medium free from molluscan protein limited the choice of a medium which could serve as a basis for the development of an organ culture medium for A.reticulatus. The media of Flandre & Vago (1963) and Burch & Cuadros (1965) satisfied these requirements most closely. A comparison of these media (Table III) shows the greater complexity of the Burch & Cuadros medium with the level of animal extracts at about 9%. On the other hand, the Flandre & Vago medium conteins a very high (at least 40%) level of animal extracts. Although not necessarily a desirable factor in a culture medium, the greater complexity of the Burch & Cuadros medium, especially the inclusion of the synthetic vertebrate medium 199 (increasing the number of

chemicals known to have a beneficial effect on cell growth) and the lower concentration of animal extracts (reducing the number of unknown chemicals present), would seem a desirable factor in this case.

TABLE III.

A comparison of some inorganic salts in pulmonate culture media. B & C = Burch & Cuadros medium, F & V = Flandre & Vago medium, H/F = Hedon-Fleig saline, T.1. = Medium T.1. All values stated are mg %.

	B & C	T.1.	F & V	H/F
NaCl	3.4	7.0	7.2	7.0
KCl	0.18	0.28	0.4	0.3
$CaCl_2$	0.09	0.09	0.3	0.1
NaH_2PO_4	0.11	0.11	0.2	-
Na_2HPO_4	0.54	0.54	-	0.5
KH_2PO_4	0.27	0.27	-	-
K_2HPO_4	0.11	0.11	-	-
$MgSO_4$	0.12	0.21	0.2	0.30
$NaHCO_3$	0.98	1.38	5.5	1.5
$MnSO_4$	0.002	0.002	-	-
Animal extracts	9%	9%	40%	-

See Table IV for additional ingredients common to B & C and T.1 media only.

Fig 1 Diagram of Glove Box in which the organ culture technique was carried out.

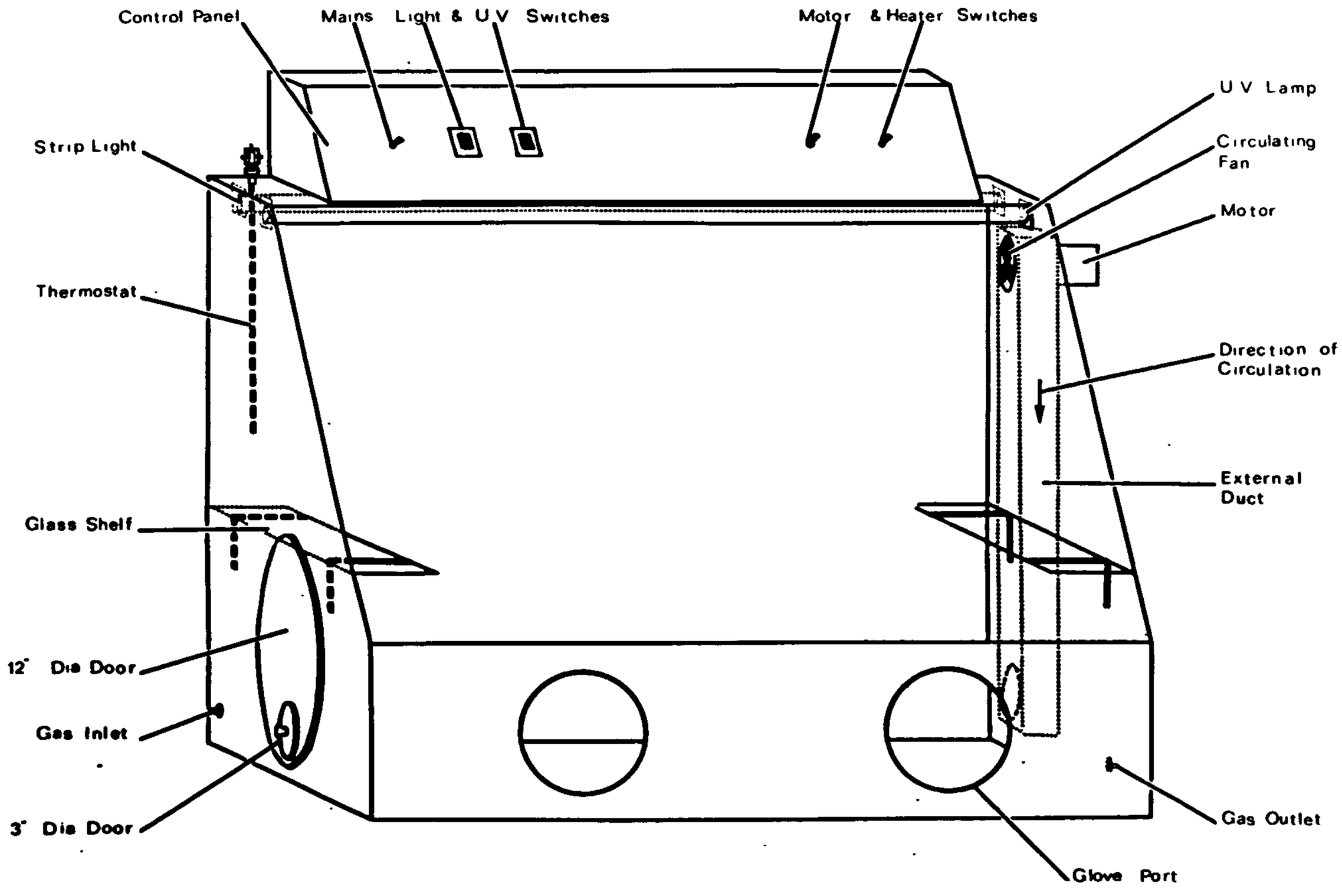

Control Panel
Mains Light & U V Switches
Motor & Heater Switches
U V Lamp
Strip Light
Circulating Fan
Motor
Thermostat
Direction of Circulation
External Duct
Glass Shelf
12" Dia Door
Gas Inlet
3" Dia Door
Gas Outlet
Glove Port

Fig 1

Fig 2 Diagram of organ culture chamber.

Fig 3 Histogram showing the survival of the various cell types found in the isolated hermaphrodite gland. Explants were cultured in media B & C or T.L.

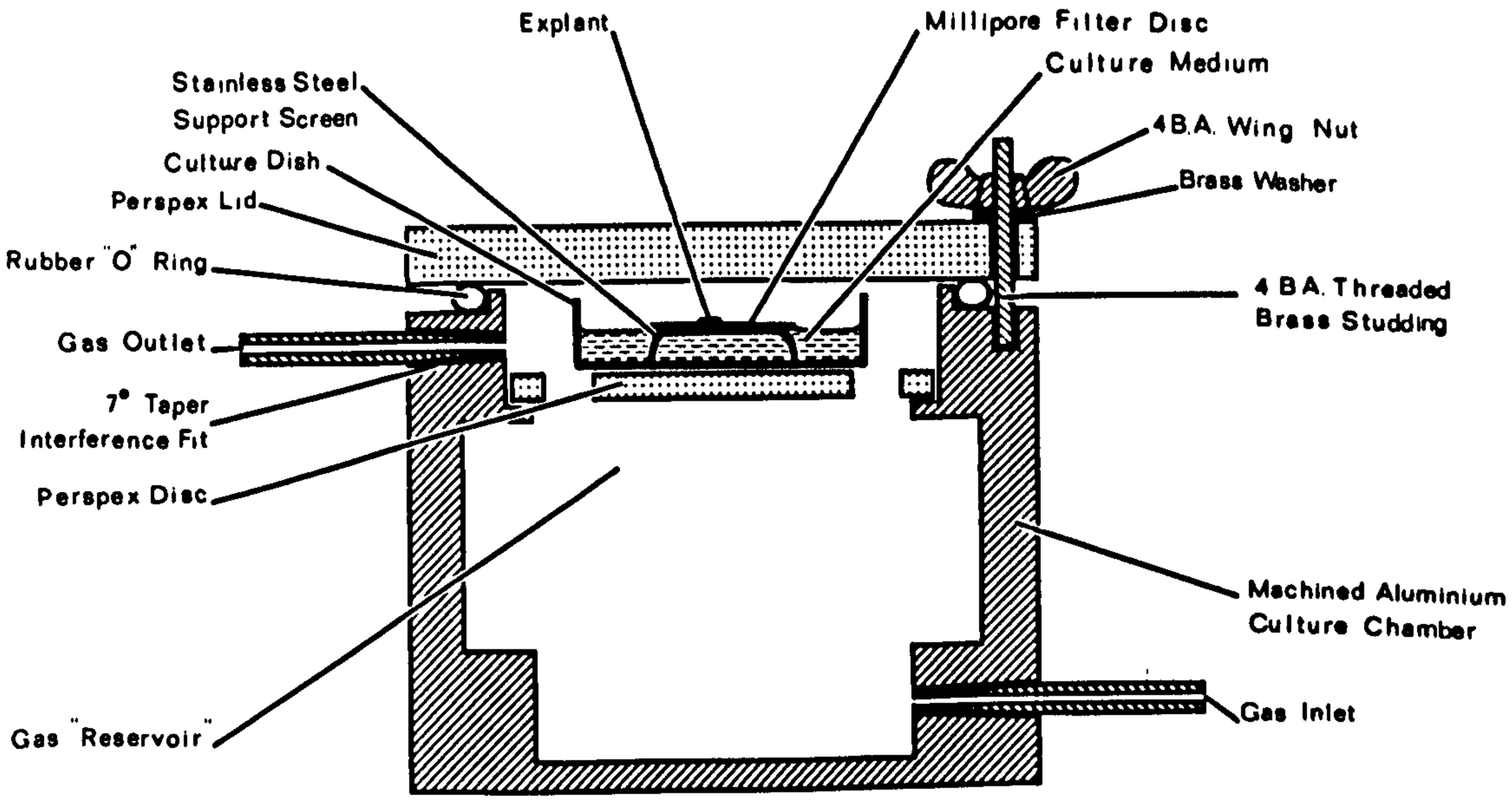

Explant
Millipore Filter Disc
Culture Medium
Stainless Steel
Support Screen
Culture Dish
Perspex Lid
Rubber "O" Ring
Gas Outlet
7° Taper
Interference Fit
Perspex Disc
Gas "Reservoir"
4B.A. Wing Nut
Brass Washer
4 B.A. Threaded
Brass Studding
Machined Aluminium
Culture Chamber
Gas Inlet

Fig 2

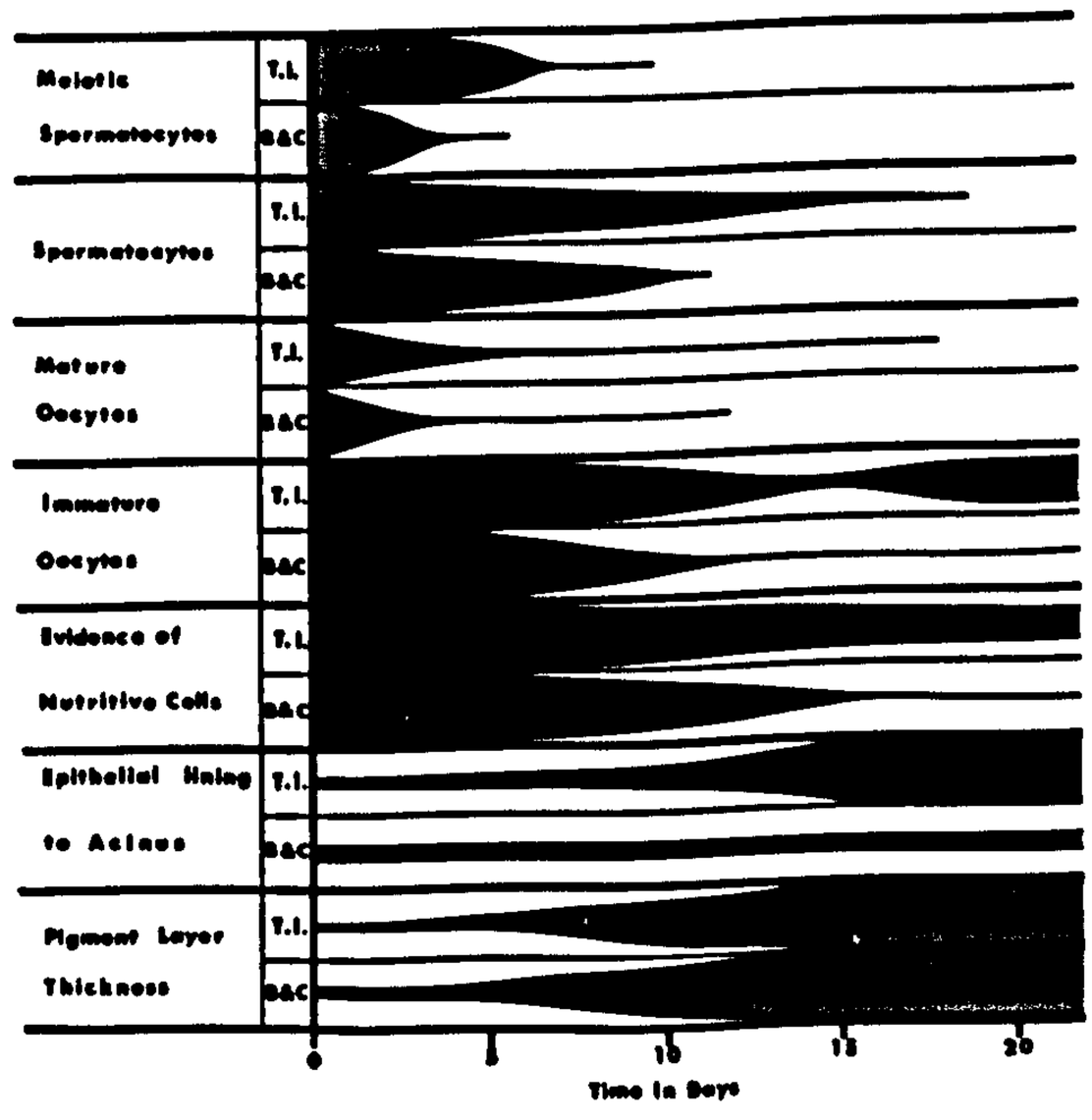

Meiotic
Spermatocytes
T.I.
B&C
Spermatocytes
T.I.
B&C
Mature
Oocytes
T.I.
B&C
Immature
Oocytes
T.I.
B&C
Evidence of
Nutritive Cells
T.I.
B&C
Epithelial lining
to Acinus
T.I.
B&C
Pigment Layer
Thickness
T.I.
B&C
0
5
10
15
20
Time in Days

Fig 3

Development of Culture technique.

It was decided to adapt the culture chambers (fig 2) of Trovell (1959). These were machined from 3" diameter solid aluminium bar. Three equidistant holes were drilled around the upper rim of the chamber, and tapped to accept 4 B.A. threaded brass rod. A $\frac{1}{4}$" perspex disc 3" in diameter was cut and holes drilled to correspond with those in the upper rim of the chamber. A lip of 3/32" was made on the upper rim of the chamber to fit a 1/8" O ring of 2 3/16" diameter (VIT 1159 Edwards high Vacuum Ltd.). A perspex dividing disc inside the chamber supported the culture dish, a Monax 5cm glass petri dish cut to a depth of $\frac{1}{4}$". The explants were grown in contact with a 1" diameter sterile millipore filter which was supported on a piece of expanded stainless steel mesh.

All culture operations, other than dissection, were carried out inside a glove box (fig 1) which was made from $\frac{3}{4}$" thick Formica faced chipboard. This box was illuminated by a 60 watt fluorescent light. Sterility was ensured and maintained by means of an ultraviolet germicidal strip light. Ducting was fitted on one side of the glove box with openings at the top and the bottom. Inside this duct were two small 50 watt heating elements controlled by a thermostat (mercury contact type). Circulation was effected by means of a small motor-driven fan

situated in the top opening of the duct which gave a down draught in the duct. This system enabled a constant temperature, above ambient, to be maintained in the glove box. All gas entered the box through the gas inlet at a rate of 1.5 - 2 litres/min. It was then directed either into the atmosphere of the glove box in the case of nitrogen, or into the manifold system, in the case of the gas mixture used in the culture chambers, by means of a three way glass tap. A plastic filter holder containing a 1" millipore filter membrane type GA-10 (0.05μ) was placed on each arm of this tap. A similar three way tap was fitted to the gas outlet but no millipore filters were used on this outlet as it was only opened when there was a positive pressure in either of the gas systems. The gas mixture was distributed to the culture chambers through a 12 branch glass manifold. At first the exhaust gas from the culture chambers was allowed to escape into the atmosphere of the box. It was noticed, however, that a high level of ozone in this atmosphere resulted from the action of the ultra-violet radiation on the high concentration of oxygen in the glove box. Another 12 branch manifold was then connected to the exhaust outlets of the culture chambers and this carried waste gases directly to the exterior of the glove box via the three way tap connected to the gas outlet. The atmosphere of the glove box was regularly flushed out with pure nitrogen and always flushed

out prior to the opening of any of the culture chambers. Thus a low concentration of oxygen was always maintained in the glove box. All gas entering the system passed through a sintered glass dreschel bottle containing distilled, sterile water, before passing through the millipore filters. Thus the gases were saturated with water and completely sterile.

All experiments were carried out with organs of the slug Agriolimax reticulatus. All the animals were freshly captured in the vicinity of the laboratories in Bangor, North Wales. Explants were usually made from animals which were anaesthetised (Bailey 1969) although some were made from non-anaesthetised animals. Dissection was carried out in sterile Hedon-Fleig saline or latterly in old medium. All instruments were sterilised by washing them in 70% alcohol and then flaming them. Once the explants had been obtained they were transferred to a sterile millipore filter, using a platinum "spud" (as recommended by Paul 1959) mounted in a glass handle. The millipore filter was contained in a 5cm petri dish and kept moist with a little medium. The petri dish was quickly transferred to the glove box, in which the ultra violet lamp had been switched on. Irradiation of the explants (after Gatenby 1935) took place about 50cm from the source. After two minutes the explant was turned over with a fresh sterile "spud" and irradiated for a further two minutes.

Using a further sterile "spud" each explant was carefully transferred from the filter paper to a series of sterile washes:- 10,000/10,000/200; 5,000/5,000/100; 2,500/2,500/50; 1,000/1,000/20; 500/500/10; 250/250/5; IU penicillin/mg streptomycin/ gm Fungizone; made from stock solutions then diluted with medium. The explants were washed in each of these solutions for 5 minutes, finally being transferred to the millipore filters in the culture chambers. The medium and the gas phase were changed at intervals as stated later. Culture was carried out at room temperature, 20-23°C, heating being used when the temperature fell below this value.

All apparatus was cleaned by boiling in Calgon/sodium metasilicate solution (Paul 1959). Apparatus was sterilised by autoclaving at 20 lbs per sq in for 30 minutes unless otherwise stated. Perspex and other non-autoclavable substances were sterilised by washing them in 70% alcohol. Ultra violet irradiation was always carried out when any article was placed in the glove box, and at the same time the atmosphere was gased with nitrogen.

The ingredients for all media were mixed in an Erlenmeyer flask, in the order stated in the formulae. The medium was filtered through a Gelman 2" pressure filter using 47mm dia Millipore filters (pore size GA-10) and a pressure of 60 p.s.i.

of nitrogen. It was found advantageous to incorporate a prefilter into the system to enable up to 250 ml of medium to be filtered at any one time. The prefilter was made from a 6 cm dia Ford's Sterimat 7 GS/PH cut to the appropriate size. The pressure filter assembly (including the 300 ml bottle to collect the filtrate) was autoclaved at 10 p.s.i. for 30 minutes. After filtration the bottle containing the filtrate was washed on its outside with 70% alcohol. The bottle, together with the filter assembly, was passed into the glove box and the filter assembly was then withdrawn from the box. This procedure ensured that the contents of the bottle remained sterile. The medium was then dispensed into 28 ml McCartney bottles for storage in a deep freeze at -20°C, one bottle being kept at room temperature as a sterility control.

Histological techniques.

The criterion for the survival of the organs was their histological appearance when compared with that of the controls. Tissues for examination with the light microscope were treated as follows unless otherwise stated. Tissues were fixed by immersion for 24 hours in Heidenhein's Susa, then dehydrated by washing in two changes, 24 hours each, of cellosolve (2-ethoxy-

ethanol). They were embedded in ester wax (1960), after immersion in stirred baths of cellosolve/ester wax (50/50) and two changes of ester wax for 30 minutes each. 4μ sections were stained in Heidenhein's Azan. Tissues for examination in the electron microscope were fixed in 1% osmium tetroxide in veronal buffer (Palade 1951) at 0°C for 1 hour. Tissues were then washed for 1 hour in distilled water before dehydration in ethanol (25%, 50%, 75% and 100%) and embedding in araldite. Thick araldite sections for light microscopy were cut at 1μ and stained in toludine blue. For examination in the electron microscope, sections were cut at 500Å-700Å on a L.K.B. ultratome and stained in uranyl acetate and lead citrate. The material was examined in an E.M.6.M. electron microscope.

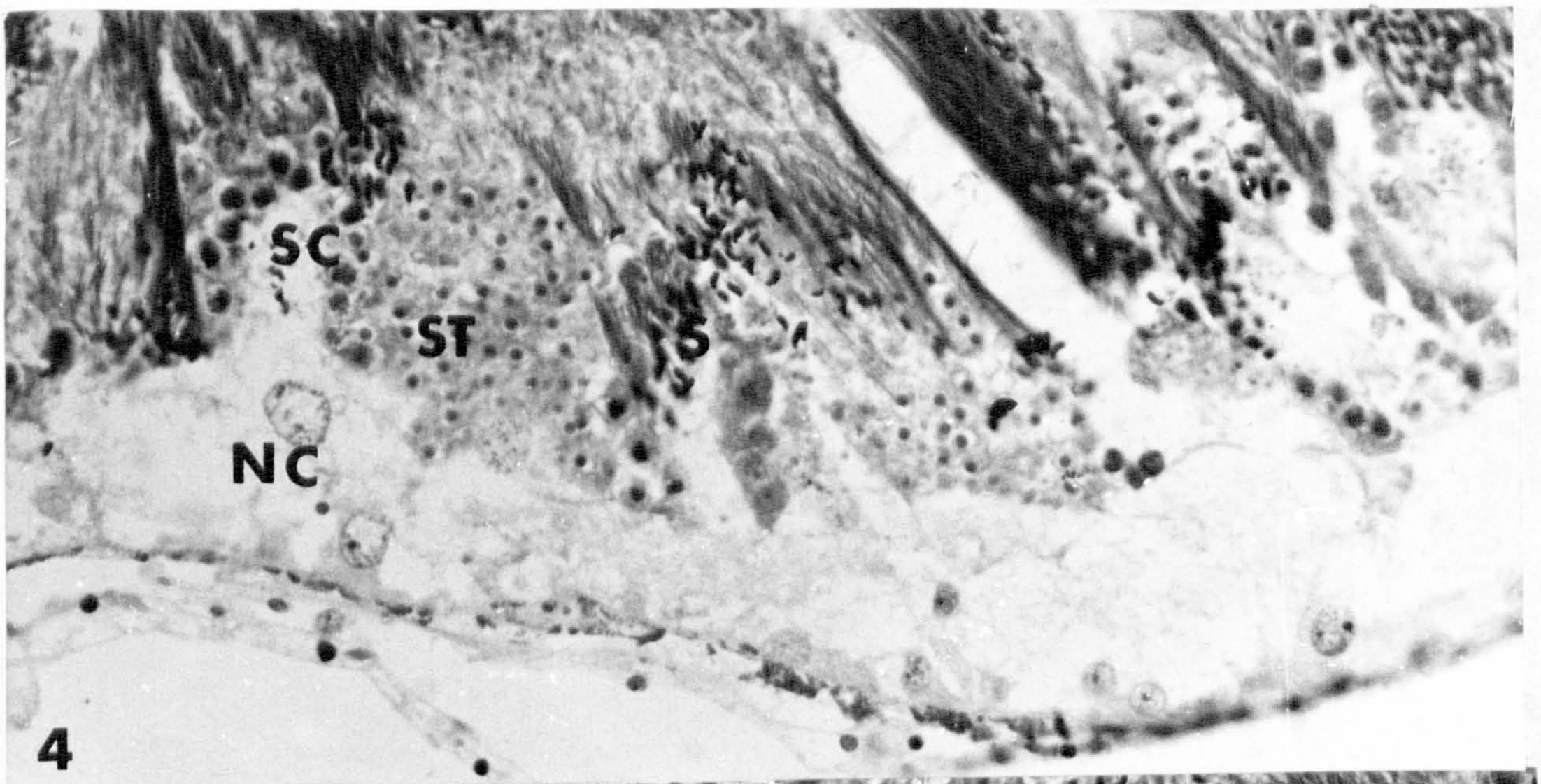
Control tissues were fixed immediately prior to the remainder of the organ being placed in culture.

In order to find the optimum conditions for the survival of organs of Agriolimax reticulatus, a series of experiments was undertaken to test the experimental procedure and the media used. From the results obtained from these and other experiments on the haemolymph of this slug, modifications were made to this procedure until a satisfactory organ culture method was developed.

Fig 4 Hermaphrodite gland cultured for 11 hours in Hedon Fleig saline at pH 8.6. X 200.

Fig 5 Control hermaphrodite gland. X 200.

Figs 6, 7 and 8 Hermaphrodite cultured in Hedon Fleig saline at pH 7.7 for 3, 4 and 7 days respectively. X 200.



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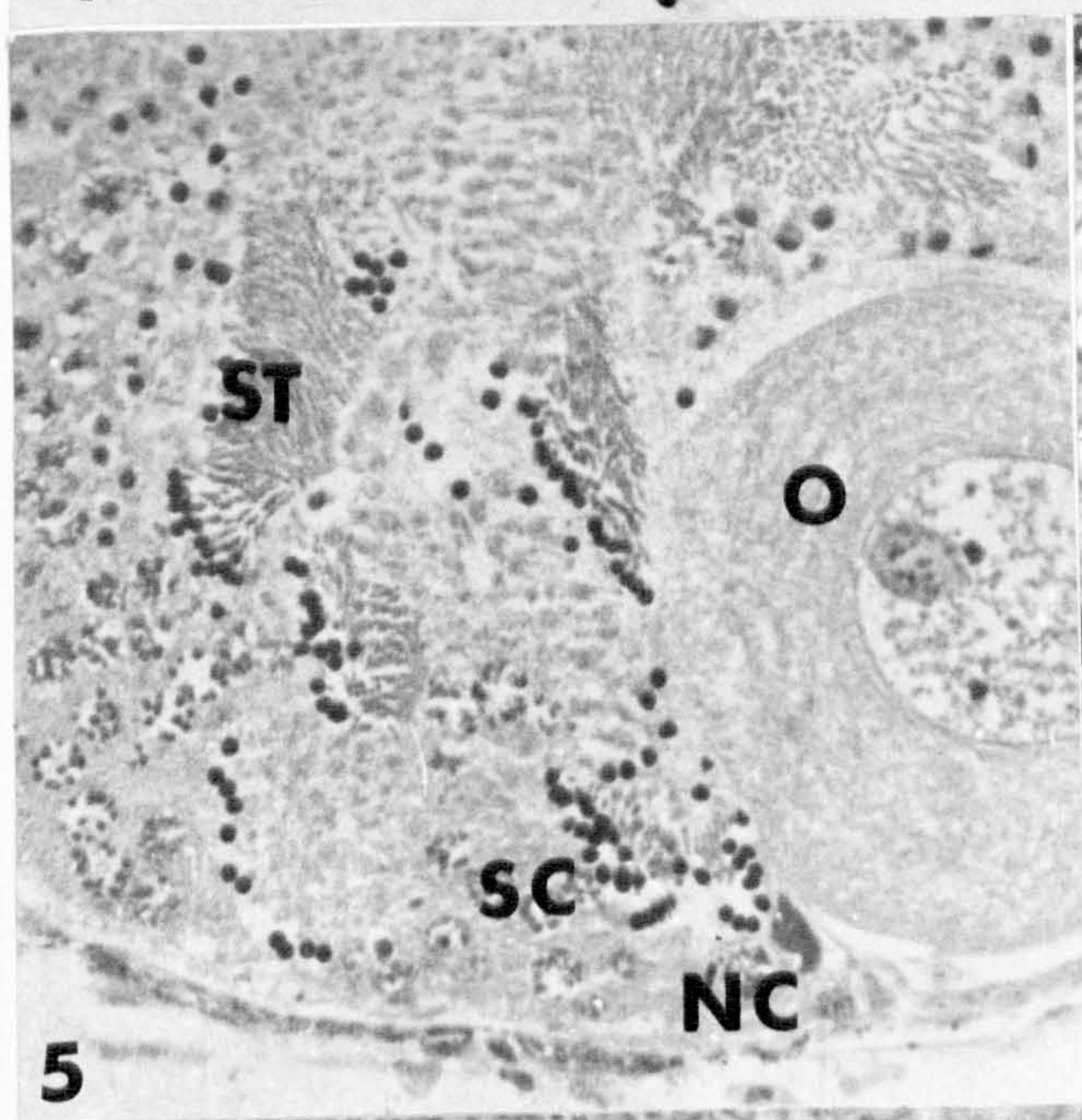

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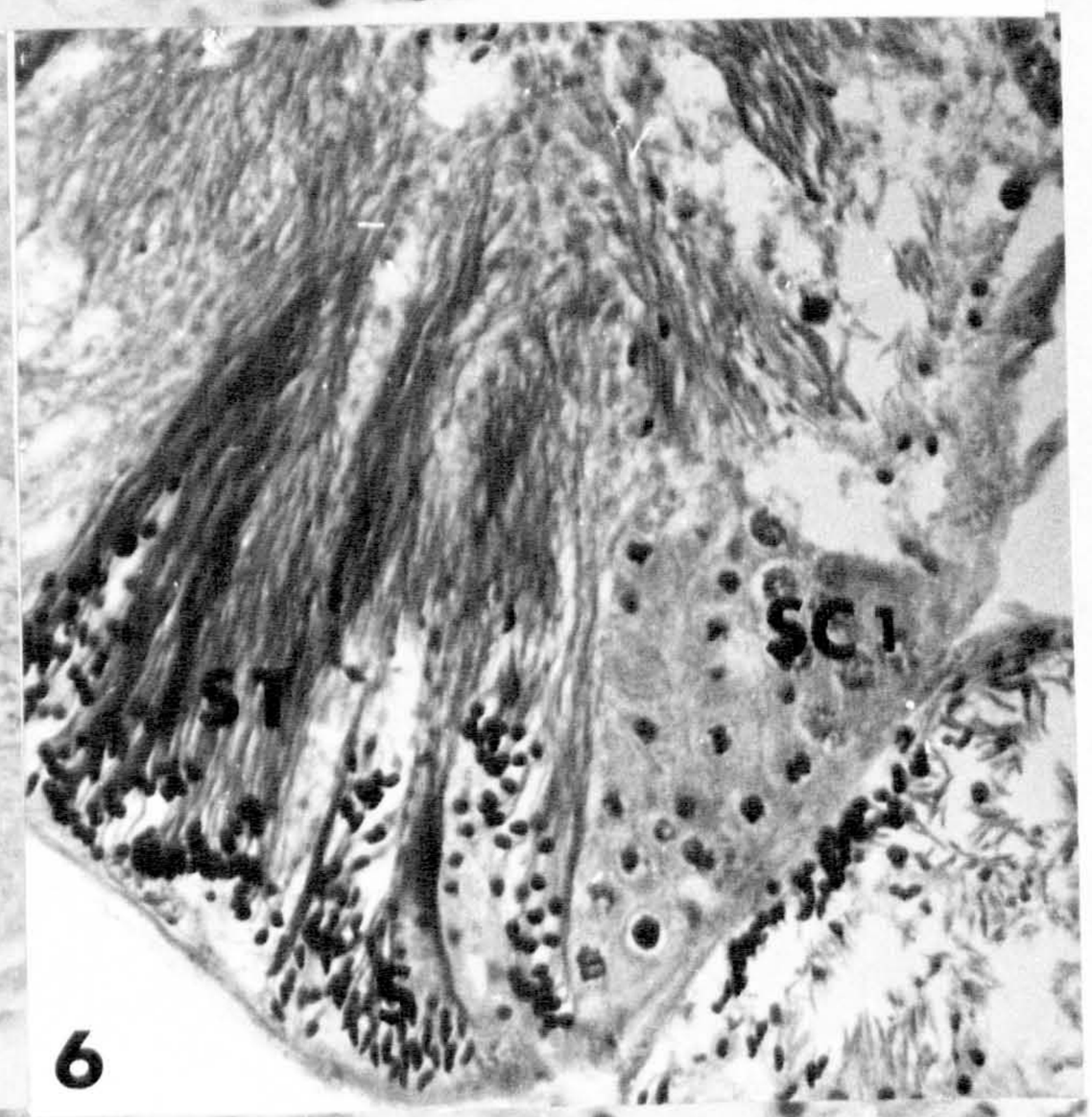

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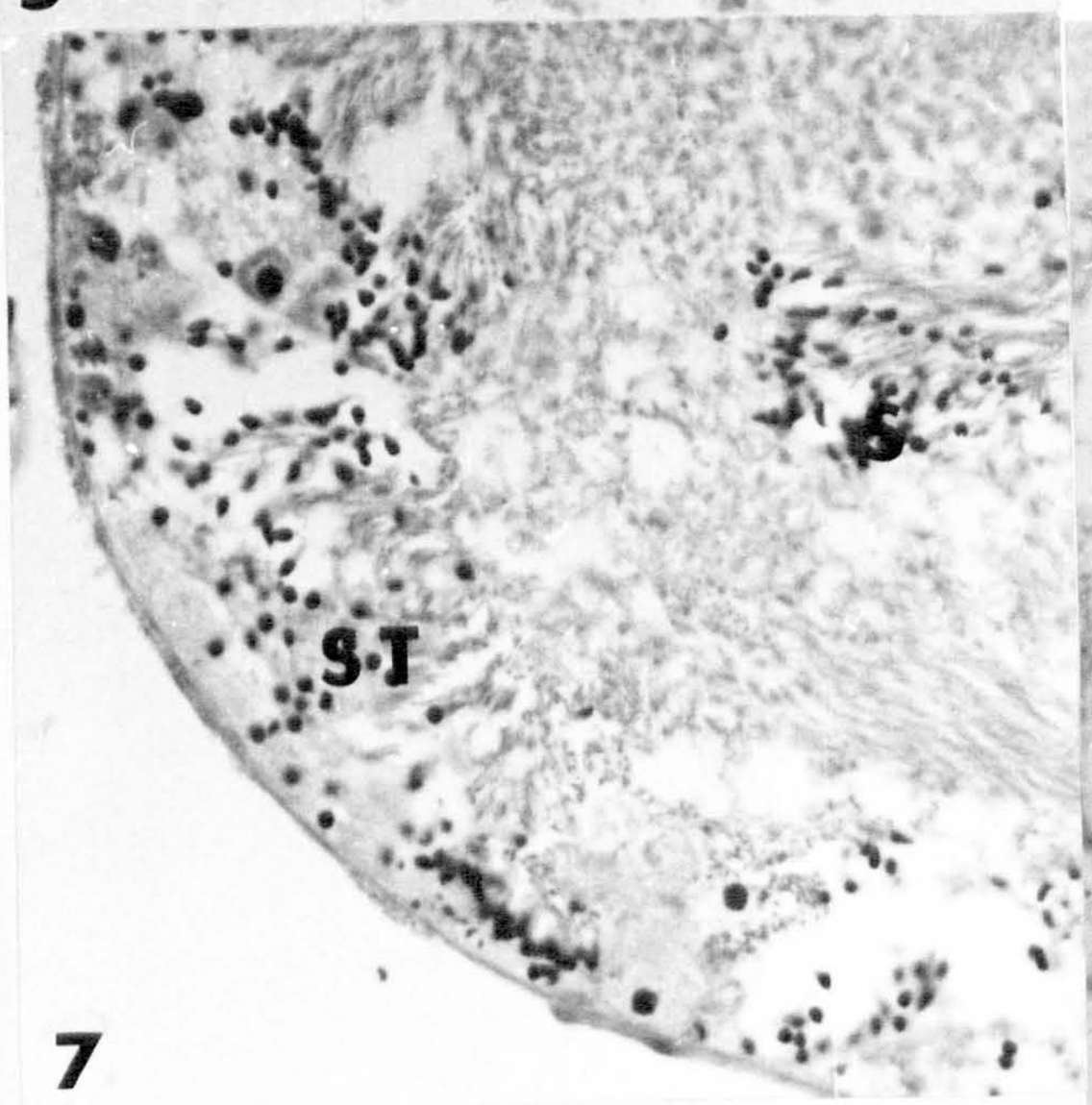

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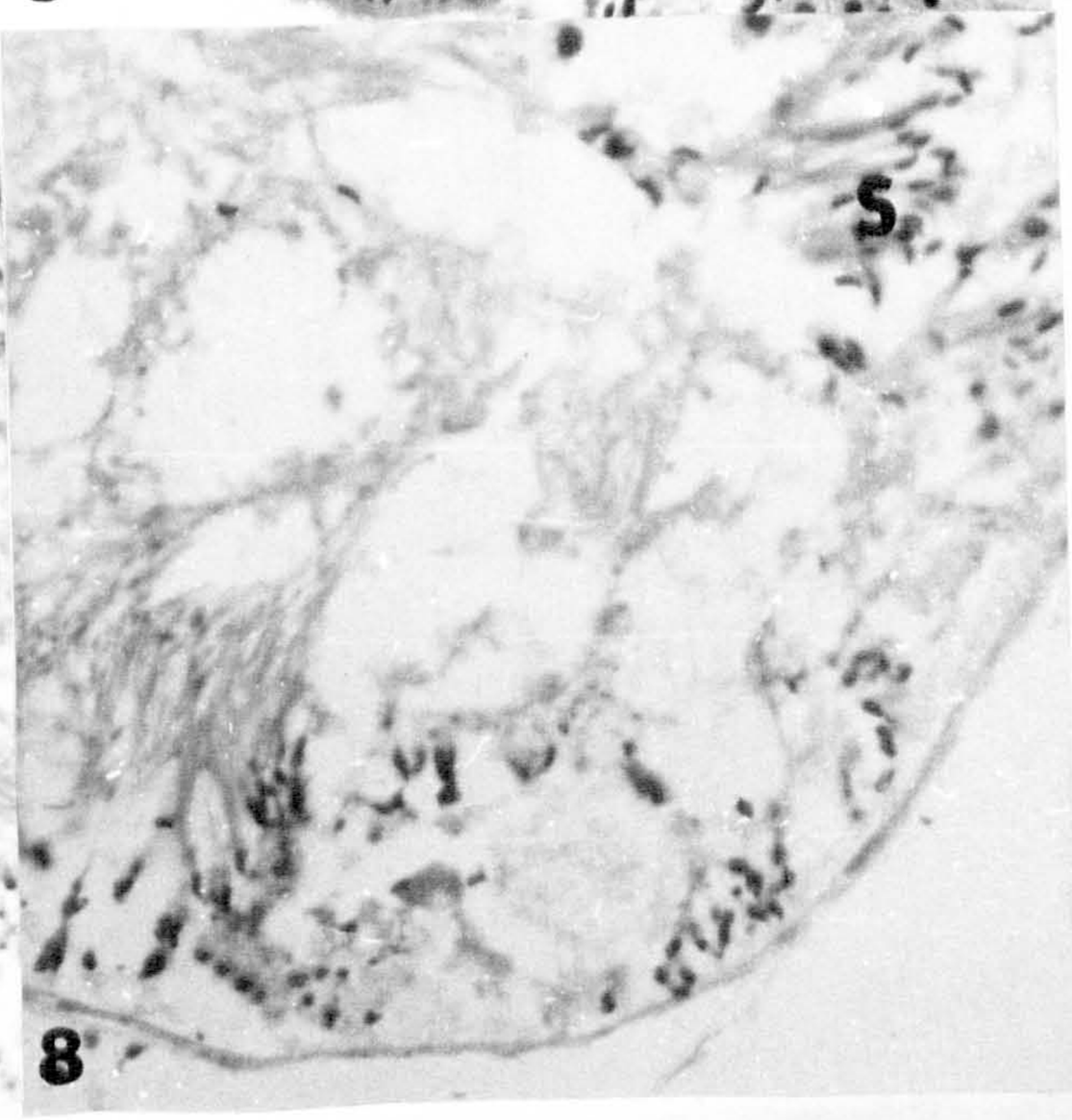


8

SERIES 1. (Figs 4 - 8)

The first series of experiments was undertaken in order to provide a control series of organ explants. Explants of the hermaphrodite gland of A.reticulatus in the spermatid and spermatozoa stages, were cultured in Hedon-Fleig physiological saline, to which had been added the antibiotic mixture used in the medium of Burch & Cuadros (table 4). The saline was adjusted to either pH 7.7 or 8.6 and a gas mixture of 90% air/10% oxygen was used. Seven explants were made from each hermaphrodite gland, one of each being fixed after 0,1,2,3,4,7 and 10 days respectively. The medium and gas mixture was changed at 3 day intervals.

Table 4

A comparison of the medium of Burch & Cuadros and medium T.1.

	B & C	T.1.
Double distilled water	480 ml	480 ml
199 + 0.5% peptone	500 ml	500 ml
B.H.I. bacteriological broth	20 ml	20 ml
S.L. broth (Table a)	20 ml	20 ml
M-9 stock A (Table b)	4 ml	4 ml
M-9 stock B (Table b)	8 ml	8 ml
Salt solution C (Table c)	0	100 ml
Sodium bicarbonate 4 g/l	0	100 ml
Calf fetal serum	40 ml	40 ml
Snail extract	40 ml	0
Chick embryo extract	0	40 ml
Antibiotic mixture (table d)	8 ml	8 ml

Table 4 (cont'd).

Table a - S.L. broth made up to 1 litre with double distilled water.

Tripticase	10 g
Yeast extract	5 g
K_2HPO_4	6 g
di-Ammonium hydrogen citrate	2 g
Glucose	20 g
$NaC_2H_3O_2$ $3H_2O$	25 g
Glacial acetic acid	1.32 ml
Salt solution (Table e)	5 ml
Tween 80	1 g

Table b - M-9 stock solutions.

Solution A -	Na_2H PO_4 (Anhydrous)	150 g
	KH_2PO_4 (Anhydrous)	75 g

made up to 1000 ml with water.

Solution B -	$MgSO_4 7HO$	20g
	NaCl	50 g
	NH_4Cl	100 g

made up to 1000 ml with water.

Table 4 (cont'd).

Table c - Salt solution c.

NaCl	36 g
KCl	1 g
$MgSO_4$	2 g

made up to 1000 ml with water.

Table d - Antibiotic mixture.

Fungizone	50 mg
Penicillin	1,000,000 I.U.
Streptomycin	1 g

made up to 100 ml with water.

Table e - Salt solution for S.L. broth.

$MgSO_4 7H_2O$	11.5 g
$MnSO_4\ H_2O$	2.86 g

made up to 100 ml with water.

At pH 8.6, the explants became disorganised after 1 day in culture. The sertoli cells, which normally play a considerable part in the characteristic arrangement of the germ cells, seemed to break down and only small groups of germ cells could be seen in the lumen of the acinus. Many of the spermatocytes had degeneratod, their nuclei having rounded up to form dense staining bodies. After 2 days the only normal appearing cells

were late spermatids and mature spermatozoa. All other types of cell, including the connective tissue cells, were degenerating.

At pH 7.7, the acini appeared similar to the controls until the second day of culture, with only very slight vacuolation appearing in the lumen of the acinus between the sperm tails. At 3 days, degeneration began, being most evident in the spermatocytes. The oocytes were shrunken and the characteristic appearance of the sperm masses was becoming disorientated as degeneration of the sertoli cells started. The cells in the connective tissue sheath in the factors of the hermaphrodite ductules and in the walls of the blood vessels all appeared normal at this time. Degeneration continued until at day 7 the connective tissue cells had degenerated and their nuclei were pycnotic. At this time only mature sperm and advanced, tailed spermatids appeared normal. Such mature sperm never degenerated, even after 70 days in culture at an abnormal pH.

Similar results were obtained when this experiment was repeated with Hedon-Fleig saline at 4:1 dilution with double distilled water. Degeneration thus takes place between 3 and 7 days after culture. This delay in the onset of degeneration is probably due to the presence of nutritive substances present in the tissues themselves at the time of explantation. It is

especially evident that the germ cells inside the acinus degenerate more or less randomly.

SERIES 2. (Figs 9 - 12)

This series of experiments was designed to compare the survival of various explants in the media of Burch & Cuadros and T.1. (table 4) at pH's varying from 7.4 - 8.8 using a gas mixture of 90% air/10% oxygen. Explants were made of hermaphrodite gland, common duct, albumen gland, spermatheca and penial sac complex at various stages in reproductive development, and also of the brain and optic tentacle complex, radular gland and ventricle.

Explants of hermaphrodite gland were cultured in groups of up to 20 on a millipore filter disc. Each gland was divided into a maximum of 9 pieces. One was fixed as a control and the others were placed one in each culture chamber. The optimum pH for culture was found to be 7.7 - 7.8. A pH change of 0.2 units was enough to cause a rapid degeneration, signs of this being visible in the tissues after 90 minutes. To ensure that these media remained within such limits the media were changed at 1 - 3 day intervals.

The results obtained with hermaphrodite gland explants are summarised in Fig 3. During the first few days of culture some

Fig 9 Mature prostate gland cultured for 10 days in medium T.1. at pH 7.7. X 300.

Fig 10 Muscle outgrowth obtained in medium T.1. at pH 7.7 after 15 days. X 2500.

L = Lumen of Prostate diverticulum.

M.F. = Millipore filter.

Mus = Muscle.

N = Nucleus.

S = Secretion of prostate.

(↖) = area of attachment of muscle to surface of millipore filter.

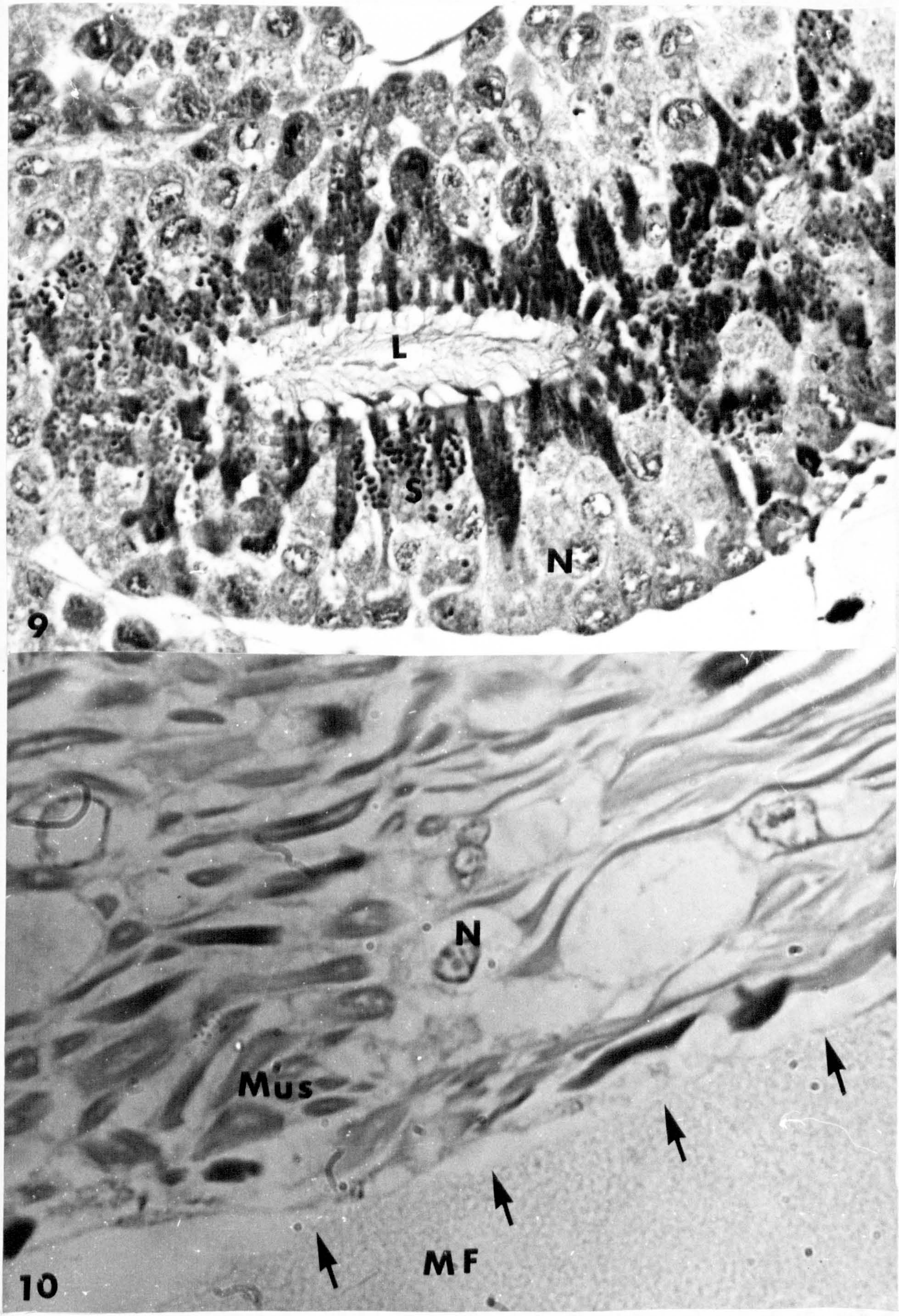

L
S
N
9
N
Mus
MF
10

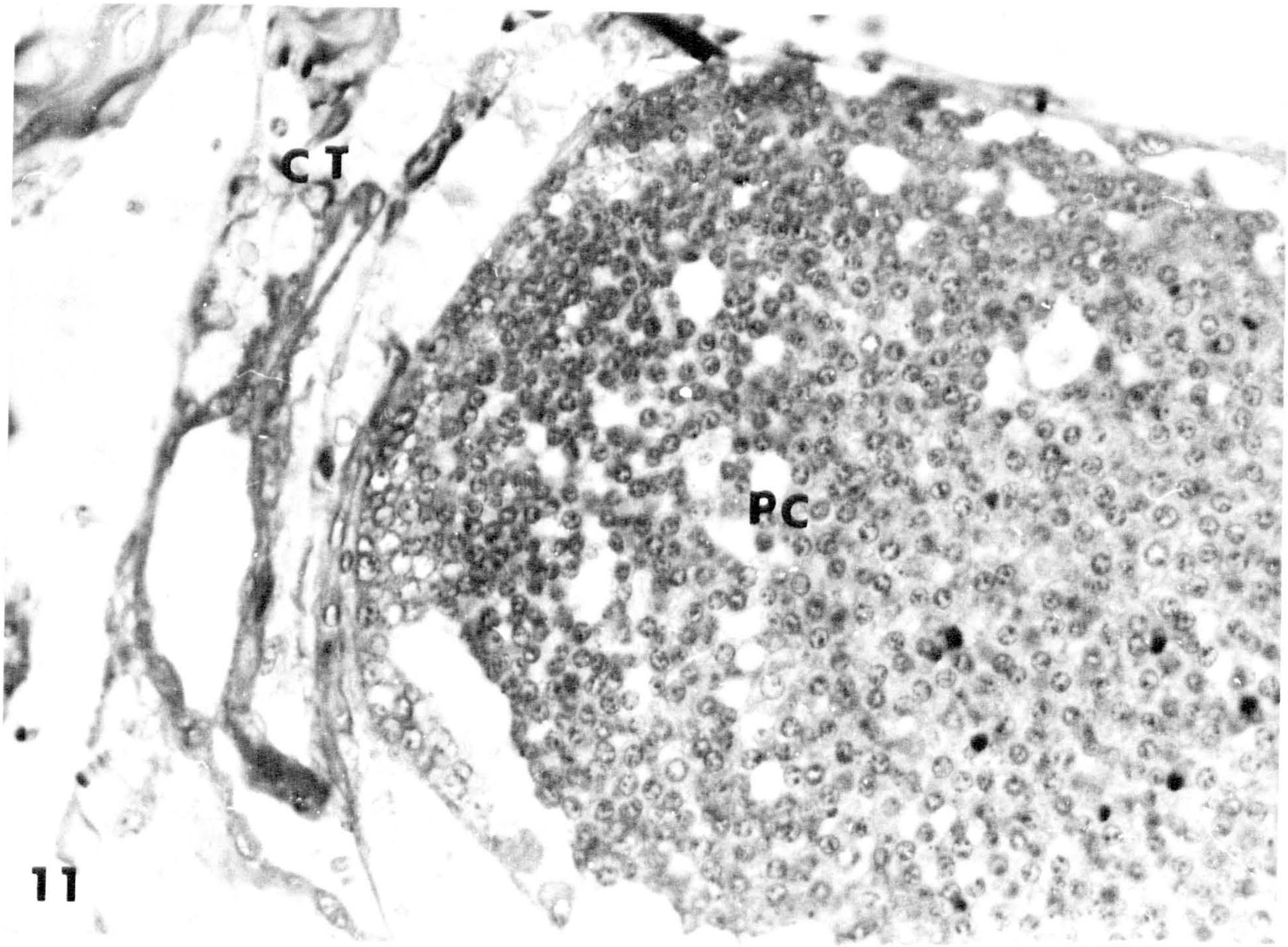
Fig 11 Procerebrum after 21 days culture in medium T.1. at pH 7.7. X 300.

Fig 12 Cells in part of pedal ganglion after 15 days culture in medium T.1. at pH 7.7. X 300.

C.T. = connective tissue.

Np = Neuropile.

P.C. = Procerebral cells.



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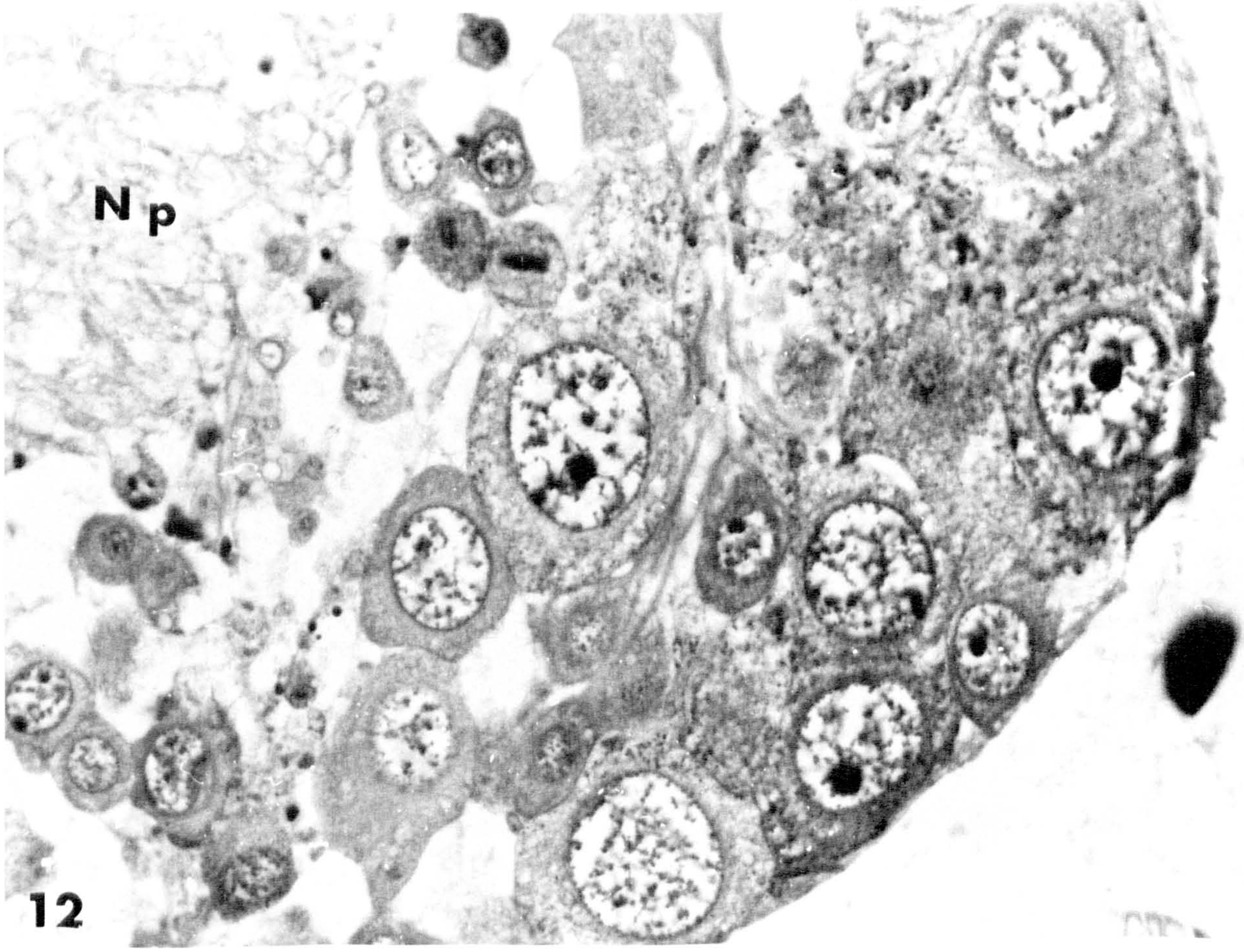


12

small areas of the acini showed a tendency to degenerate. After about 3 days, however, this degeneration ceased and only certain cell types then degenerated. The others remained fairly constant in their numbers while a few appeared to increase to a limited extent. Connective tissue cells survived very well during this time. By the 22nd day of culture the lumen of the hermaphrodite gland tended to have certain areas where cells were healthy and other areas devoid of "normal" cells, except for the very persistent sperm. From Fig 3 it can be seen that the medium T.1. maintains the explants of the hermaphrodite gland better than does the medium of Burch & Cuadros. It was also found that grouped explants survived better than single explants, presumably because of the increase in nutrients contained in the larger volume of tissue.

Explants of other parts of the reproductive tract showed excellent survival and cells were seen to be normal in appearance in both the light and the electron microscopes. However in the larger explants, e.g. albumen gland, cells from the central area of the tissues showed signs of necrosis presumably due to oxygen starvation. Since the mature common duct also showed these necrotic areas, the culture of this mature organ was not attempted in future experiments. With these explants there was little difference between the two media used.

Explants of the brain and optic tentacle complex, radular gland and ventricle also showed an optimum pH for survival at pH 7.7. Muscle and connective tissue survived well and in some cases muscle cells grew actively and adhered to the surface of the millipore filter. The cells of the procerebrum usually survived well but the cell bodies in other parts of the brain showed a very variable survival. Apparently healthy cells were randomly distributed between degenerating cells and large vacuoles appeared in the neuropyle. This degeneration occurred in the first few days of culture and then little change took place in the remaining time. The brain cells degenerated to a lesser extent in medium T.1. The optic tentacles survived quite well in both media.

It was concluded that both media sustain tissues of Agriolimax reticulatus, but that medium T.1. allowed active growth and cell division.

Series 3. (fig 13)

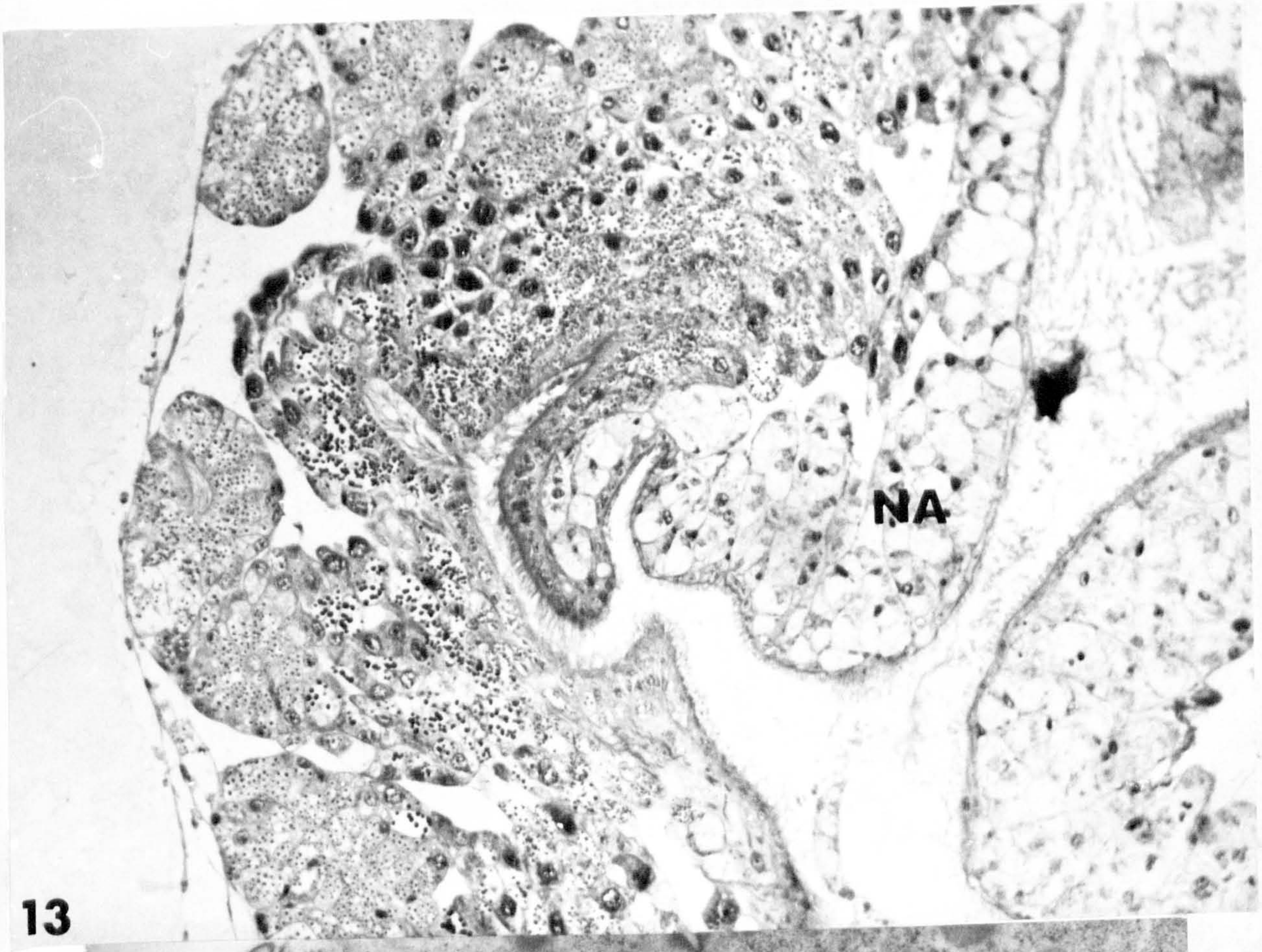
In this series of experiments cultures were undertaken with explants either at the gas/liquid interphase, or submerged in the medium, supported on millipore filters. Gas mixtures of 95% oxygen/5% carbon dioxide, 95% oxygen/5% nitrogen and 90% air/ 10% oxygen were used. Both the T.1. and the Burch & Cuadros media were used.

Fig 13 Common duct explant after 10 days showing necrotic area. (NA). X 300.

Fig 14 Cell division in epithelium of immature oviducal gland. Cultured for 14 days in medium T1 + 10% chick embryo extract at pH 7.7. X 15,000.

cen = centrosome.

M.P. = Metaphase plate.



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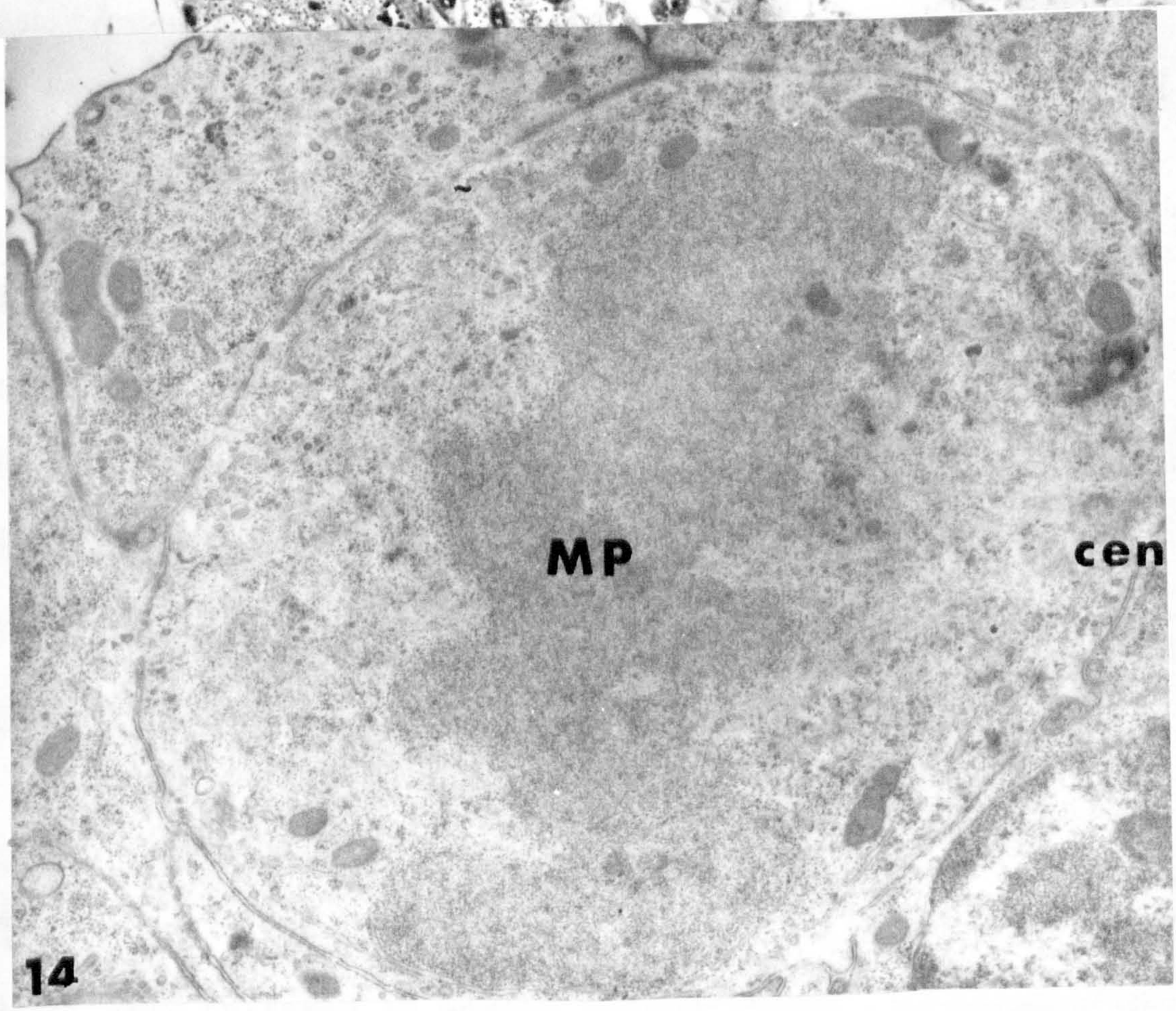

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The 95% oxygen/5% carbon dioxide gas mixture was soon rejected because it rapidly caused the media to become too acidic, especially the medium T.1. Submerged explants survived best with a gas mixture of 95% oxygen/5% nitrogen. However, even with this high oxygen content all but the smallest explants exhibited necrosis at their centres, presumably due to oxygen starvation. In the same gas mixture, but at the gas/liquid interphase, explants showed a zone of pycnotic cells around their periphery while bulky explants still showed a small zone of necrosis in their centre. This peripheral zone of pycnotic cells may possibly be due to oxygen poisoning. Surface explants cultured in 90% air/10% oxygen showed a slightly enlarged area of necrosis in the centre of the bulky explants, when compared with those cultured in the high oxygen gas phase, but there was no zone of pycnotic cells at their periphery. It was decided that the 90% air/10% oxygen gas mixture produced the best results and that the explants should be supported at the gas/liquid interphase.

SERIES 4.

This series of experiments was designed to test the effect, if any, of varying the concentration of the medium T.1. Explants were made of the hermaphrodite gland, common duct and albumen gland. These were cultured in medium T.1. at 100%, 90%, 80% and 70%

dilution.

The differences between cultures at these various dilutions, with both hermaphrodite gland and reproductive tract were not very marked. However, there was a slight improvement in the number and the length of survival of the spermatocytes, and less of a tendency to produce vacuoles at a dilution of 80%. Since the Δt of this diluted medium is in the range of that found for the haemolymph, it was decided to adopt this dilution of 80% in subsequent media.

SERIES 5.

This series of experiments was undertaken in order to eliminate infection problems which, hitherto, had caused experiments to be terminated after a maximum of 28 days.

a) Karnik & Kamat (1961) recommended the addition of mercuric chloride 0.025% and propionic acid to the culture medium stating that it prevents bacterial and fungal infection. This addition was made to the dissection medium and the washes only, none being added to the culture medium. Explants of the hermaphrodite gland and common duct were made and cultured in T.1. at 80% dilution and in the gas mixture decided upon in series 3. On fixing these explants, even after 5 days, no "normal" cells were seen. This addition did however prevent any infection.

b) Attempts were made to prevent fungal infection by means of Fungizone (Squibb l.t.d.).Fresh solutions of Fungizone were added to culture dishes to give a concentration of 10 g/ml of medium. This addition delayed the appearance of fungal growth for 4 days but did not reduce the incidence of infection. The fungicidal properties of Fungizone were tested by injecting the solution into bottles containing medium to give concentrations of 50-1.25 g/ml, and these bottles were then innoculated with spores of the fungus Penicillium brevi-compactum, one of the most common species causing infection. None of these concentrations killed the fungus and eventually the fungus developed, even if the Fungizone was repeatedly added every 4 days.

c) Finally Fungizone was injected into Agriolimax reticulatus in concentrations of 320, 160, 80, 50, 40, 30, 25, 10, 5, 2.5 and 1.25 g/gm of slug (live wt) using an Agla syringe. The animals which survived, those injected with less than 50 g/gm slug, were used as a source of explants of hermaphrodite gland and common duct. These were cultured as before, using the normal washes. The infection rate was still not altered.

It was thus decided that the only way to limit infection of the culture chambers was to maintain absolute sterility in the culture chambers and if possible in the glove box, since the antifungicidal agent Fungizone will not prevent fungal infection

and bacterial infections are only slightly delayed by using Penicillin and Streptomycin at acceptable concentrations for *in vivo* culture. One source of fungal infection was found to be the stock solution of 100,000 I.U. penicillin and 100,000 mg streptomycin per ml of water, used as a stock solution in making up sterile washes.

d) If explants were cultured in medium from which the antibiotic mixture had been omitted, and the culture was for longer than about 10 days, no change was noticed in the infection rate. Thus, in subsequent culture media the antibiotic mixture was omitted.

At the present time, the main source of infection of explants is mainly fungal and from the lumena of such organs as the common duct. There seems no effective means of preventing this.

SERIES 6.

It was decided to test the treatments to which the explants were subjected before being placed into culture. Hermaphrodite glands were divided into 6 explants; 1 control and 1 explant for each of the following treatments;

Dissection in medium T.1.	U.V. for 2 min per side	sterile washes	culture in T.1. at pH 8.6.
1 +++	-	-	+++
2 +++	-	+++	+++
3 +++	+++	-	+++
4 +++	+++	+++	+++
5 +++	placed for 10 mins in macerated digestive gland		+++
6 +++	fixed as a control.		

Explant 5, which was allowed to remain in macerated digestive gland tissue, acted as a control for any damage which might be caused to the hermaphrodite gland explants by damage to the digestive gland during dissection.

The effects of these treatments were similar although the infection rate increased when parts of the sterile procedure were omitted. No difference was observed after 2 hours, although at the "wrong" pH, signs of pycnosis are clearly visible after 1½ hours. Even after 1 and 2 days, no difference was noticed between explants. It was concluded that the preliminary sterile procedure for the explants had no significant detrimental effect on the tissues.

SERIES 7. (Fig 14)

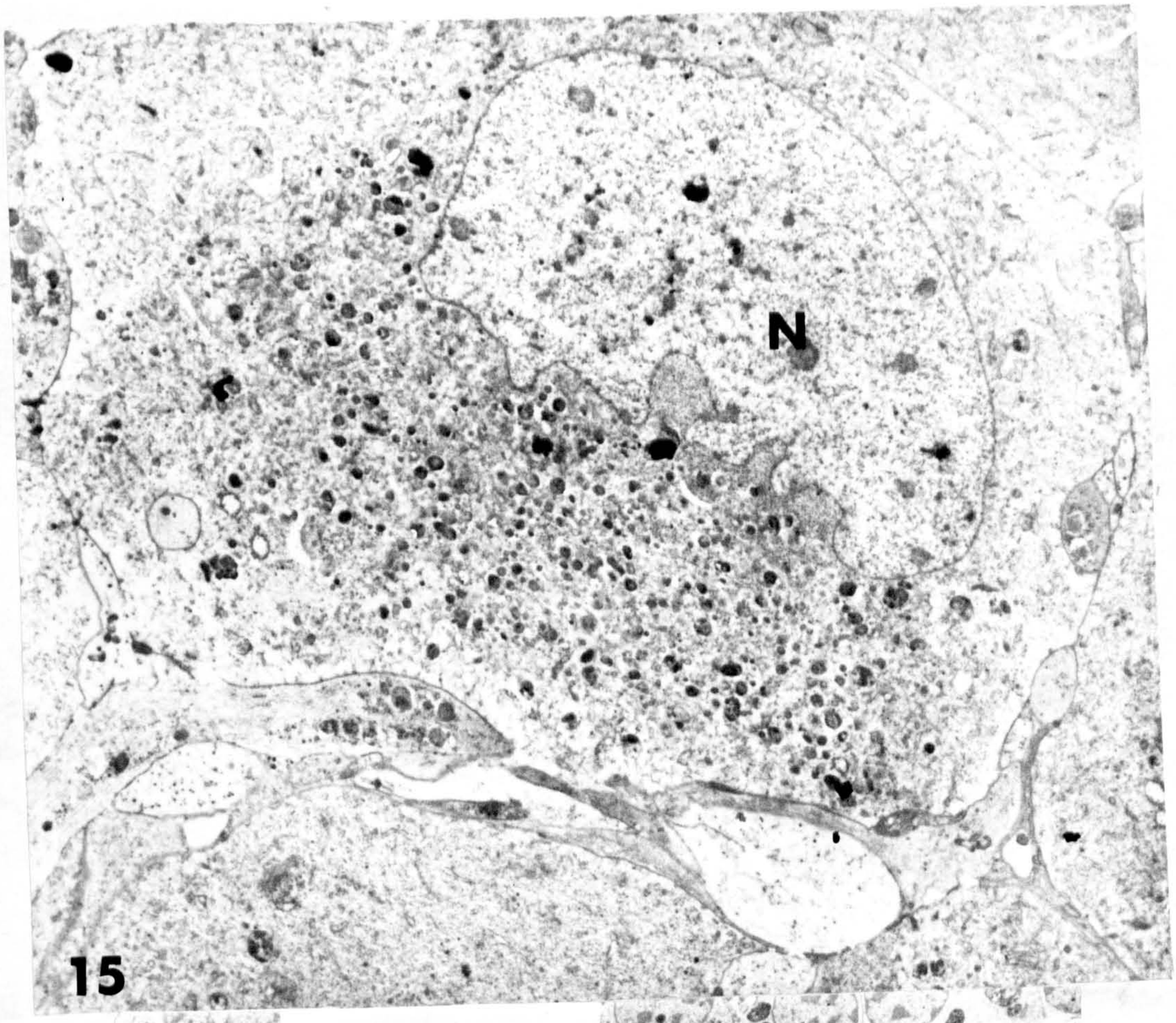
In the results obtained from the previous series of

experiments it was found that no cell divisions were seen after an interval of about 10 days although cells, other than spermatocytes, were apparently healthy. In an attempt to induce cell divisions, especially in the spermatocytes, chick embryo extract (Difco) was used to replace the human serum in the medium T.1.

Explants were made of hermaphrodite gland, common duct, albumen gland and brain and optic tentacle complex. These were cultured in this modified medium for up to 28 days, tissues being fixed at 0,7,14,21 and 28 days. In no cases were cell divisions observed when explants were separated from one another and results were similar to those obtained for medium T.1. in series 2. This medium is, however, capable of supporting meiotic divisions of the spermatocytes as was shown when explants of hermaphrodite gland were cultured in contact with brain and optic tentacle complexes. These complexes did however exhibit an improved survival and chick embryo extract was substituted for human serum in the medium T.1.

SERIES 8. (Figs 15 - 23)

The modified medium T.1. is thus capable of maintaining cells, supporting active growth and cell divisions. However, the pH of this medium, in conjunction with the 90% air/10% oxygen



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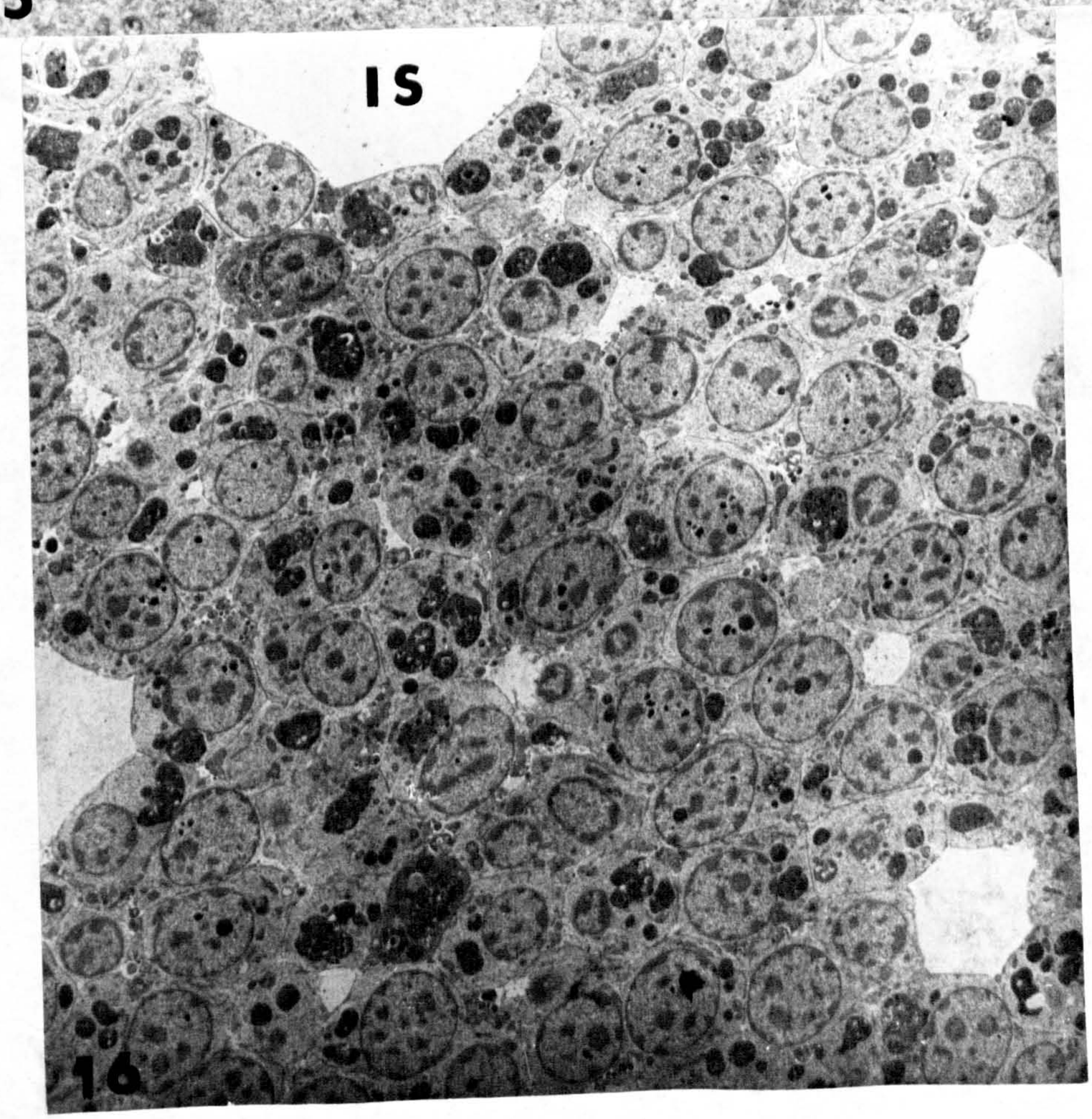

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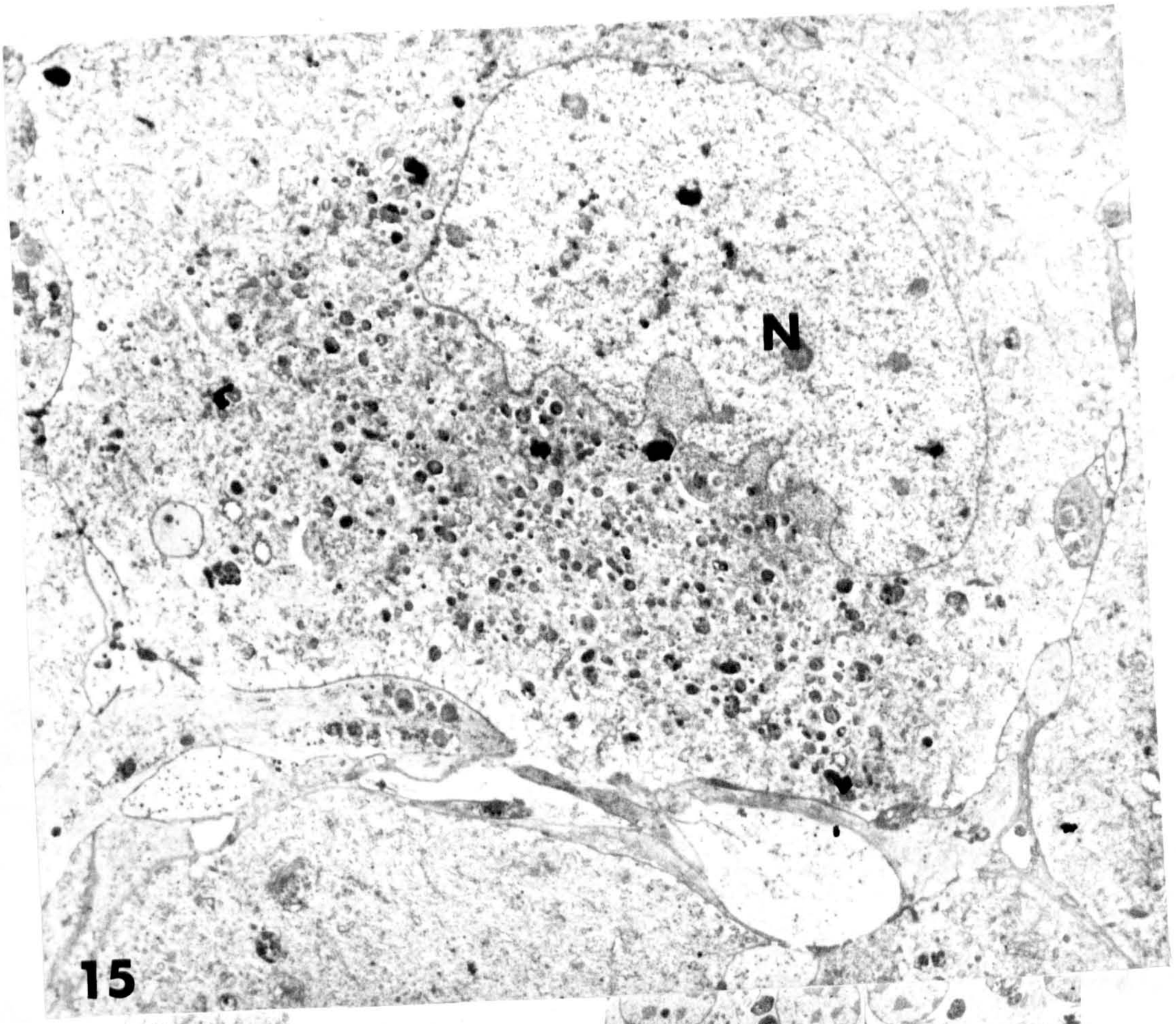
Fig 15 Cell in parietal ganglion after 17 days culture in fully developed medium at pH 7.7. X 3000.

Fig 16 Procerebral cells after 17 days culture in fully developed medium at pH 7.7. X 3000.

N = Nucleus.

IS = Intercellular Space.


N
15

IS
16

Fig 17 Epithelial lining of acinus.
Isolated explant cultured for
28 days in fully developed
medium at pH 7.7. X 3000.

Fig 18 Apical detail from fig 17. X 35,000.

Ep = Epithelial Cells. F = Fat.

LA = Lumen of Acinus. M = Mitochondrion.

W = Wall of acinus.

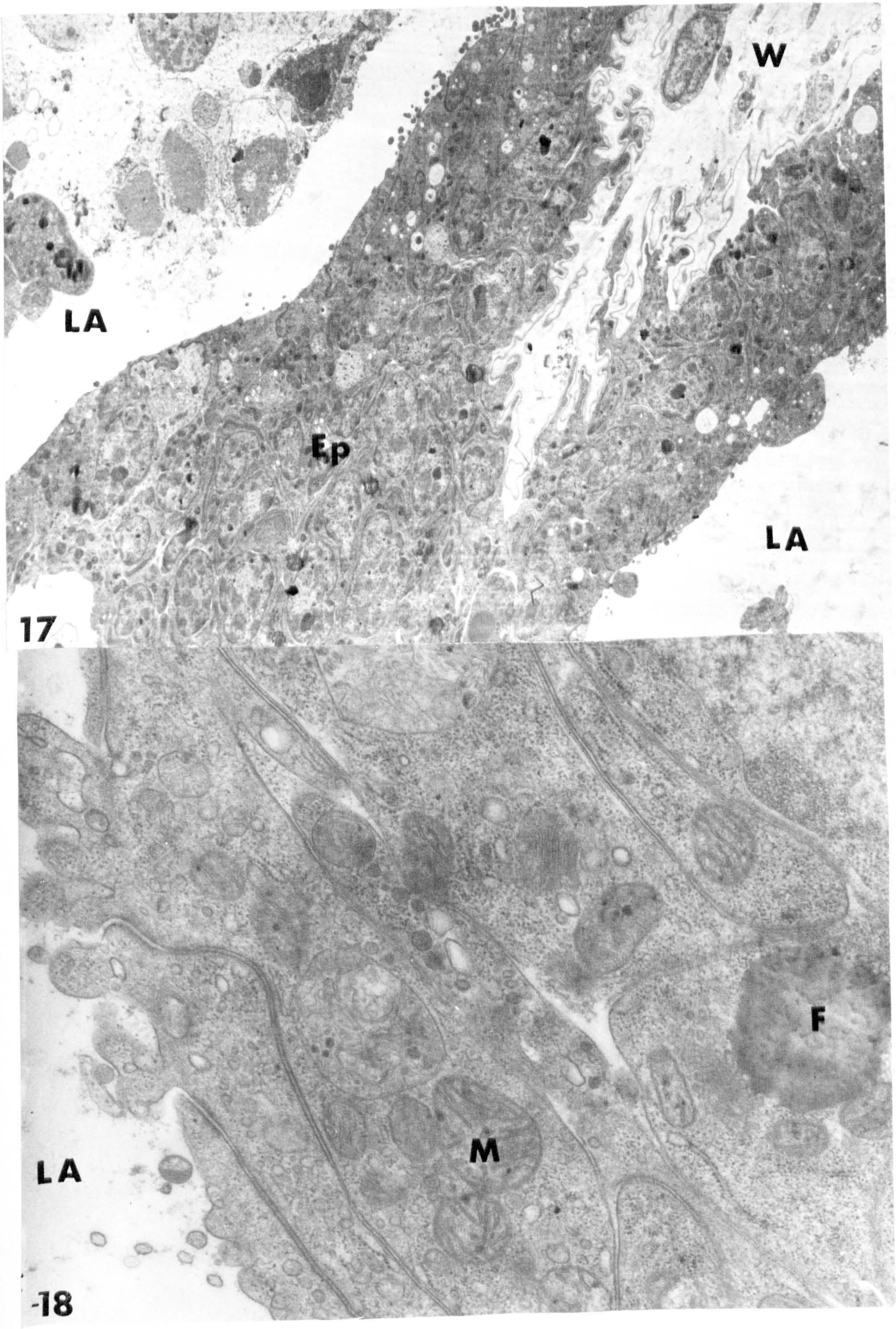

W
LA
Ep
LA
17
LA
M
F
18

Fig 19 Epithelium of male groove of differentiating common duct cultured in association with the gonad and brain of the same animal for 28 days in fully developed medium at pH 7.7. X 8,000.

Fig 20 Explant of Stage E hermaphrodite gland cultured with the brain/tentacle complex of an animal whose hermaphrodite gland was at Stage C. Explant cultured for 21 days in fully developed medium at pH 7.7. X 300.

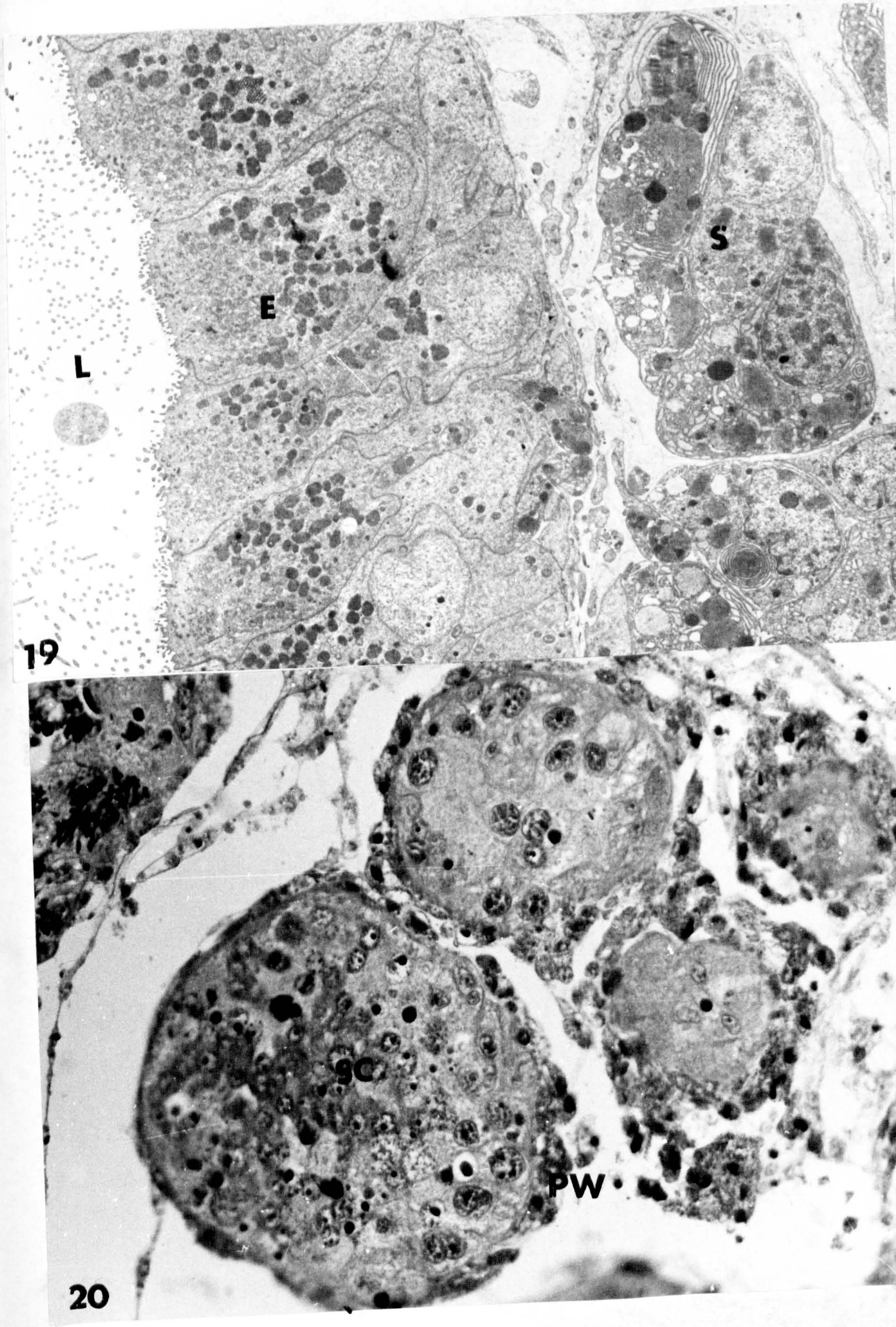
E = Epithelial Cells of male groove.

L = Lumen.

PW = Pigment Wall of Acinus.

S = Secretory cell of male groove.

SC = Spermatocytes.



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20

Fig 21 Explant of Stage D hermaphrodite gland cultured in isolation for 21 days in fully developed medium with the addition of 10% common duct extract. X 300.

Fig 22 Detail from Fig 20 showing division of primary spermatocyte. X 2500.

MP = Metaphase plate.

O = Oocyte.

Scl = Primary Spermatocyte.

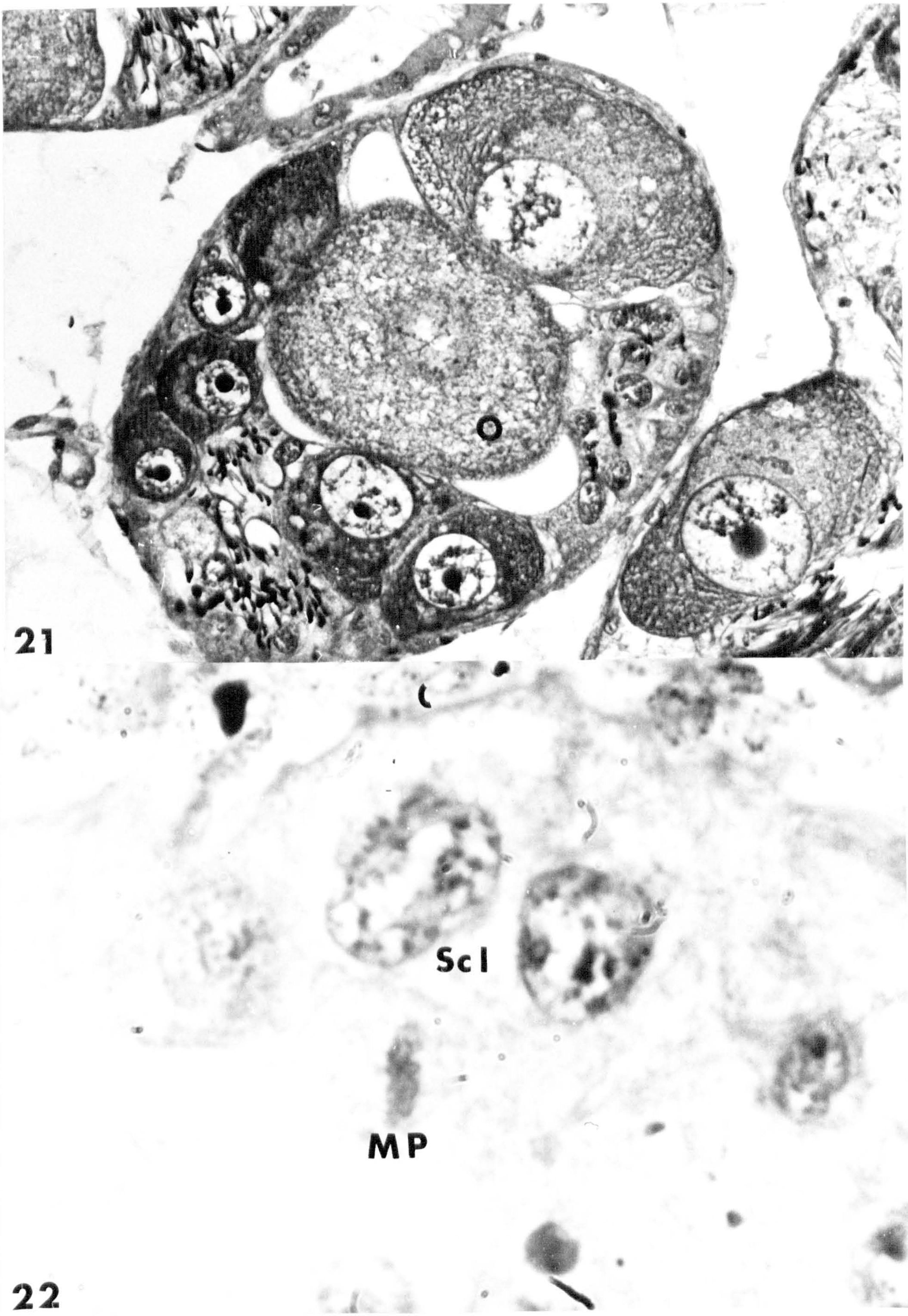

21
Scl
MP
22

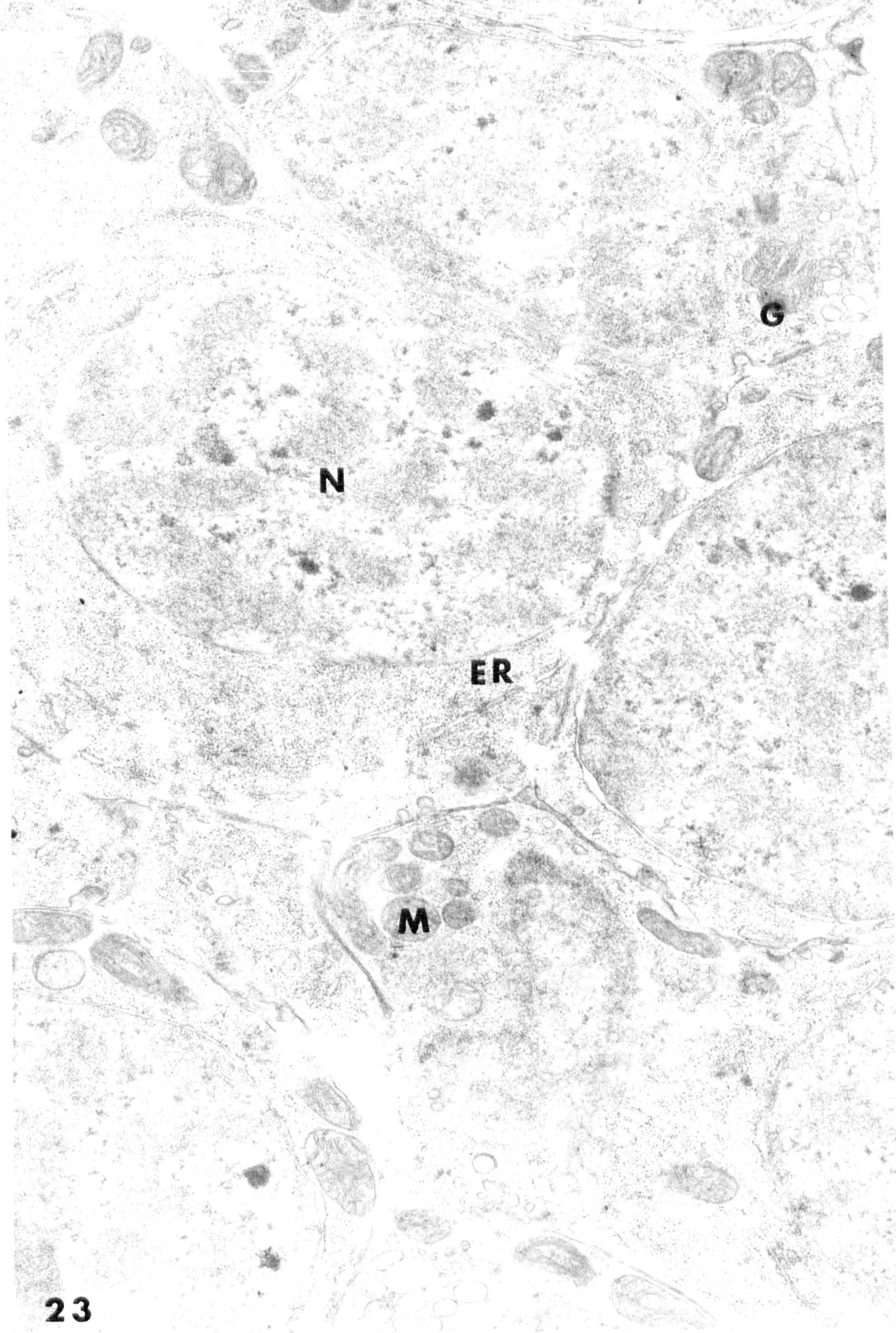
Fig 23 Primary Spermatocytes from acinus cultured in association with the brain/tentacle complex from the same animal. Explants cultured for 18 days in fully developed medium. X 20,000.

E.R. = Endoplasmic Reticulum.

G = Golgi body.

M = Mitochondrion.

N = Nucleus.


G
N
ER
M

gas mixture, is not as stable as might be desired. The medium was developed for use with, among other things, extracts of tissues of Agriolimax roticulatus, and since the pH is only maintained within the desired limits by frequent changing of the medium (every 1 - 3 days), this procedure would be very wasteful of tissue extracts. Attempts were therefore made to stabilise the pH so that the medium would only need changing at longer intervals.

The change in the pH seems due to the decomposition of bicarbonate, since it only occurs if the medium is open to a large atmosphere. When the concentration of bicarbonate was reduced, then the pH of the medium was found to be more stable. However, the bicarbonate ion constitutes a major portion of the inorganic ion content of the haemolymph of slugs (Roach 1963) and its presence in high concentrations would therefore seem a necessary constituent of a satisfactory physiological saline or culture medium. It was thus considered undesirable to reduce the sodium bicarbonate content of the medium T.1. since its concentration was already quite low when compared with that of the haemolymph.

Attempts were therefore made to reduce the decomposition of bicarbonate by the introduction of carbon dioxide into the air/oxygen gas mixture. Carbon dioxide was mixed with the

air/oxygen gas mixture using flowmeters (Rotameter, 1100 series. MFG Co Ltd) calibrated for 0 - 2.5 litres/min Air and 0 - 100cc/min carbon dioxide. The gas from the flow meters was mixed and passed into the chambers. A slow continuous flow was passed into the culture chambers at a rate of 50cc/min/chamber. Within 24 hours the pH of all media subjected to a carbon dioxide concentration of less than 20% had risen from pH 7.7 to 7.95 while above 20% the pH had fallen to below 7.0. This was therefore an unsatisfactory method of reducing the decomposition of the bicarbonate.

Further attempts to stabilise the pH were undertaken. The NaH_2PO_4/K_2HPO_4 ratio was varied but this had little effect on the pH of the medium.

If the medium in the McCartney bottles is gased with nitrogen for about 24 hours the pH change will take place and then the pH remains constant. If the pH is then adjusted to the required value it will remain constant when exposed to the gas mixture in culture. This is not a very satisfactory method for the stabilisation of the pH since the stabilisation is probably due to the decomposition of the bicarbonate ion and is in effect a reduction in the concentration of bicarbonate.

Recently Gardner (1969) described the use of Tricine as a buffer in animal tissue cultures. The buffering

capacity of Tricine (N-tris((hydroxymethyl)) methyl glycine) was therefore tested. A 1.25 molar solution of Tricine (BDH) was made and sterilised by autoclaving at 20 p.s.i. for 20 minutes. This solution was then added to the culture medium to give concentrations of 50, 25 and 12.5mmoles/1 (50m moles/1 being the concentration recommended by Gardner). This resulting medium was then placed in culture chambers and subjected to a slow but continuous flow of nitrogen. Even after 3 days of continuous gasing, the buffering capacity of 25 and 50m moles/1 was very good, and any pH change was less than 0.1 units. Organs were therefore cultured in medium containing 25m moles of tricine/1. This medium was left unchanged for 15 days after which the tissues were fixed and examined in both the light and the electron microscopes. There was no apparent detrimental effect on the tissues when compared to the results of culture in medium lacking Tricine. The pH of the used culture medium was measured and it was found to have risen by 0.05 units. It was therefore decided to incorporate Tricine into the culture medium at a concentration of 25m moles/1.

CONCLUSIONS.

1) The culture medium adopted for culturing organs of the slug Agriolimax reticulatus is shown in table 6.

2) The procedures of the culture technique were tested and have to have no ill effects on the explanted organs.

3) 90% air/10% oxygen was found the most satisfactory gas mixture tested.

4) Antibiotics were ineffective in affecting sterility. No other satisfactory method was found which prevented infection once introduced into the culture chambers.

5) It was found necessary to obtain absolute sterility in the culture procedure.

6) The only source of infection was from the explants themselves. However, a combination of ultra violet radiation and sterile antibiotic washes proved effective in reducing infection rates to acceptable levels.

7) Tricine was found to be the most effective buffer tested.

TABLE 6.

Medium adopted for the culture of organs of A. reticulatus

Medium 199 + 0.5% peptone	500 ml
Brain Heart Infusion (Difco)	20 ml
S.L. broth table a	20 ml
M.9 stock solution A table b	8 ml
M.9 stock solution B table b	8 ml
Salt solution C table c	100 ml
Sodium bicarbonate 4 g/l	100 ml
Calf fetal serum (Difco)	40 ml
Chick embryo extract (Difco)	40 ml
Double distilled water	403 ml
Tricine (1.25 molar aqueous solution)	25 ml

Table a - S.L. Broth

Casein hydrolysate	10 g
Yeast extract	5 g
K_2HPO_4	6 g
di-Ammonium hydrogen citrate	2 g
Glucose	20 g
Sodium acetate (Hydrated)	25 g
Glacial acetic acid	1.32 ml
Salt solution Table e	5 ml
Tween 80	1 g
Double distilled water	to 1000 ml

TABLE 6 (cont'd.)

Table b - M-9 solution A		Na_2HPO_4 (anhydrous)	75 g
		KH_2PO_4 (anhydrous)	37.5 g
	made up to 1000 ml with double distilled water.		
	solution B	NaCl	50 g
		$MgSO_4$	20 g
		NH_4Cl	100 g
	made up to 1000 ml with double distilled water.		
Table c - Salt solution C.		NaCl	36 g
		KCl	1 g
		$MgSO_4$	2 g
	made up to 1000 ml with double distilled water.		

Table e - Salt solution for S.L. broth.

$MgSO_4$ $7H_2O$	11.5 g
$MnSO_4$ H_2O	2.86 g

made up to 100 ml with double distilled water.

References.

BAILEY, T.G. 1969. A new anaesthetic technique for slugs, Experientia, 25: 1225.

BENEX, J. 1961, La survie d'explants de Planorbes (Australorbis glabratus) en milieu synthétique antiseptique et nutritif, C.r. hebd. Séanc. Sci. Paris, 253: 734-6.

- 1964, Sur la dedifférenciation des tentacles de Planorbis en survie: Rôle de la présence d'éléments nerveux sur le retard de cette dedifférenciation. C.r. hebd. Séanc. Sci. Paris, 258: 2193-2196.

- 1965, Action comparée de certains sulfamides à noyau pyrimidique, sur des explants de Mollusques 261: 5233-5236.

- 1966, Les possibilitiés de la culture organotypique au milieu liquide dans l'étude des problèmes parasitaires. Annls. Parasit. hum. comp., 41: 351-378.

- 1967a, Importance de la structure chimique et de la configuration stérique dans l'augmentation de la survie d'explants maintenus en culture organotypique. C.r. hebd. Séanc. Sci. Paris, 265: 631-634.

- 1967b, Substances susceptibles d'augmenter la survie d'explants maintenus en culture organotypique. C.r. hebd. Séanc. Sci. Paris, 265: 571-574.

BEVELANDER, G. & MARTIN, J. 1949, Culture of mantle tissue of marine molluscs, Anat. Rec., 105: 614.

BOHUSLAV, P. 1933, Die Gewebezuchtung des postembryonalen Verdauungstraktus, der Glandula salivalis und des Receptaculum seminis bei Mollusken aus der Familie Helicidae. Arch. exp. Zellforsch, 13: 673- .

BURCH, J.B. & CUADROS, C. 1965, A culture medium for snail cells and tissues, Nature, Lond, 206: 637-638.

CHARDONNET, Y. & PERES, G. 1964, Essai de culture de cellules provenant d'un Mollusque "Mytilus". C.r. Séanc Soc Biol. 157: 1593-1595.

CHOQUET, M. 1964, Culture organotypique de gonades de Patella vulgata. C.r. hebd. Séanc Sci. Paris, 258: 1089-1091.

- 1965, Recherches en culture organotypique, sur la spermatogenèse chez Patella vulgata L. (Mollusque. gastéropode). Rôle des ganglions cérébroïdes et des tentacles. C.r. hebd. Séanc.Sci. Paris, 261: 4521-4524.

DURCHON, M. 1964, Résultats de cultures organotypiques chez les annélides et les mollusques gastéropodes, Bull.Soc. Zool.Fr., 89: 45-48.

DURCHON, M. & RICHARD, A. 1967, Étude, en culture organotypique, du rôle endocrine de la glande optique dans la maturation ovarienne chez Sepia officinalis. C.r. Hebd. Séanc.Sci. Paris, 264: 1497-1500.

DURCHON, M. & SCHALLER, F. 1963, Application de la méthode de culture organotypique aux recherches endocrinologiques chez les annélides polychètes. C.r.hebd.Séanc.Sci. Paris, 256: 5615-5617.

FLANDRE, O. & VAGO, C. 1963, Culture de tissues de gastéropodes en coagulum plasmatique. Ann.Epiphyties., 14: 23-25.

GARDENER, R.S. 1969, The use of Tricine buffer in animal tissue cultures. J.Cell.Biol., 42: 320-321.

GATENBY, J.B. 1931, Outgrowths from pieces of Helix aspersa, the common snail. Nature. Lond., 128: 1002-1003.

- 1932, A technique for studying growth and movement in explants from Helix aspersa. Arch. exp.Zellforsch., 13: 666-672.

GATENBY, J.B. & HILL, J.C. 1934, Improved technique for non-aseptic tissue culture of Helix aspersa, with notes on molluscan cytology. Q.Jl.Microsc.Sci.,76: 331-352.

GATENBY, J.B., HILL, J.C. & MACDOUGALD, T.J. 1935, On the behaviour and structure of cells of Helix aspersa in aseptic and non-aseptic tissue culture. Q.Jl.microsc. Sci. 77: 129-166.

GODDARD, C.K. 1960, Physiological media for tissues of Helix, Nature. Lond., 187: 68-69.

GUYOT, L. & GUYARD, A. 1964, Évolution en culture in vitro de la glande hermaphrodite de jeunes escargots de

l'espòce Helix aspersa Mull. C.r.hebd.Séanc.Sci.Paris., 258: 2902-5.

GUYARD, A. 1967, Féminisation de la glande hermaphrodite juvénile d'Helix aspersa Mull associée in vitro au ganglion cérébroïde d'escargot adulte ou de Paludine femelle. C.r. hebd. Séanc.Sci. Paris, 265: 147-149.

- 1969a, Élaboration d'un milieu synthétique enrichi destiné à la culture d'organes de Mollusques. C.r.hebd. Séanc.Sci.Paris, 268: 162-164.

- 1969b, Autodifférenciation femelle de l'ébauche gonadique de l'escargot Helix aspersa Mull. cultivée sur milieu anhormonal. C.r. hebd. Séanc.Sci.Paris, 268: 966-969.

GUYARD, A. & GOMOT, L. 1963, Survie et différenciation de la gonade juvénile d'Helix aspersa Mull en culture organotypique. Bull.Soc.Zool.Fr., 89: 48-56.

HOLLANDE, Et. 1968, Évolution des grains de sécrétion dans les cellules des glandes multifides d'Helix pomatia maintenues en survie expérimentale. C.r.hebd.Séanc.Sci. Paris., 267: 1054-1057.

JONES, B.M. 1966, Invertebrate tissue and organ culture in cell research, Chap 7, Cells and tissues in culture, Methods in Biology and Physiology III. Acad. Press.

KARNIK, A.G. & KANAT, D.N. 1961, Tissue culture studies in the lamellibranchs. I. Preliminary observations on the growth of epithelial cells from Lamellidens corrianus Lea, Univ. Bombay., N.S. 29B: 172-178.

LAVIOLETTE, P. 1950, Rôle de la gonade dans la morphogenèse du tractus génital, chez quelques Mollusques Limacidae et Arionidae. C.r. hebd. Séanc.Sci.Paris, 231: 1567-1569.

- 1950, Sur un retard de la maturité génitale observé chez Arion rufus, Bull.Soc.Limn.Lyon,19: 52-56.

- 1951, Régénérats germinaux multiples chez Arion rufus. C.r. hebd. Séanc.Sci.Paris. 233: 1139-1141.

- 1953, Régénération germinale après castration chez un mollusque, gastéropode, pulmoné Arion rufus, XIV Internat. Cong. Zool., 233-235.

- 1954, Rôle de la gonade dans le déterminisme hormonal de la maturité glandulaire du tractus génital chez quelques gastéropodes Arionidae et Limacidae. Bull. biol.Fr.Belg., 88: 311-332.

- 1954, Étude cytologique et expérimentale de la régénération germinale après castration chez Arion rufus Annls.Biol., 30: 481-492.

1956, Rôle de la gonade dans la maturation glandulaire du tractus génital chez quelques gastéropodes. Annls. Sci. nat., 18: 171-173.

LI, M.F. & STEWART, J.E. 1966, In vitro cultivation of cells of the oyster Crassostrea virginica, J.Fish.Res.Bd.Canada., 23: 595-599.

LOCKWOOD, A.P.M. 1961, Ringer solutions and some notes on the physiological basis of their ionic composition. Comp. Biochem. Physiol., 2:241-

MECCO, A. & MARTIN, R. 1963, The mitotic activity of the white body cells of Octopus vulgaris in vitro. Ann Epiphyties 14: 23-25.

PALADE, G.E. 1952, A study of fixation for electron microscopy. J.exp.Med., 95: 285-297.

PAUL, J. 1959, Cell and tissue culture, 3rd Ed. Livingstone, Edinburgh and London.

PELLUET, D. 1964, On the hormonal control of cell differentiation in the ovotestis of slugs. Can.J.Zool.,42: 195-199.

PELLUET, D. & LANE, N.J. 1961, The relation between neurosecretion and cell differentiation in the ovotestis of slugs. Can.J.Zool., 39: 789-805.

PERKINS, F.D. & MENZEL, R.W. 1964, Maintainance of Oyster cells *in vitro*. Nature, Lond., 204: 1106-1107.

RANNOU, M. 1967, Culture de cellules de Mollusques Marines. Vie Milieu, 18: 525-530.

ROACH, D.K. 1963, Analysis of the haemolymph of *Arion ater*. J. exp. Biol., 40: 613-623.

SENGEL, P. 1961, Survie en culture *in vitro* de diverses organes d'invertébrés marins adultes. C.r. hebd. Séanc.Acad. Sci.Paris, 252: 3666-3669.

STREIFF, W. 1963, Survie d'organes de Mollusques marins en culture *in vitro*. Présentation des milieux A6, A7, A7c and B2. Gen. Comp. Endocrinol., 3: 733-734.

- 1964, Survie d'organes de Mollusques marins en culture *in vitro*. Résultats obtenus sur les melieux A6, A7, A7c and A8c. Bull.Soc.Zool.Fr.,89: 56-59.

- 1966, Autodifférenciation ovarienne chez un mollusque prosobranche hermaphrodite protandre *Calyptraea sinensis* (L). C.r.hebd.Séance.Acad.Sci.Paris., 263: 539-542.

- 1966, Étude endocrinologique du déterminisme du cycle sexuel chez un mollusque hermaphrodite protandre *Calypyraea sinensis* (L). I. Mise en évidence par culture *in vitro* de facteurs hormonaux conditionnant

l'évolution du tractus femelle. Annls.Endocr.,28: 461-472.

- 1967, Étude endocrinologique du déterminisme du cycle sexuel chez un mollusque hermaphrodite protandre Calyptraea sinensis L. III. Mise en évidence par culture in vitro de facteurs hormonaux conditionnant l'évolution de la gonade. Annls. Endocr.,28: 641-656.

STREIFF, W. & PEYRE, A. 1963, Survie en culture in vitro d'organes de Calyptraea sinensis L. C.r.hebd.Séanc.Acad. Sci. Paris., 256: 292-294.

THOMAS, J.A. 1941, La survie d'organites de Stélérides in vitro. C.r.hebd.Séanc.Acad.Sci.Paris.,213: 85-88.

TRIPP, M.R., BISIGNANI, L.A. & KENNY, M.T. 1963, Oyster Amoebocytes in vitro. J.Invert.Pathol.,8: 137-140.

TROWELL, O.A. 1959, Culture of mature organs in synthetic medium. Exp. Cell. Res. 16: 118-147.

VAGO, C. & CHASTANG, S. 1958, Obtention de lignees cellulaires en culture de tissues d'invertébrés. Experientia., 14: 110.

VAGO, C. & CHASTANG, S. 1960, Culture de tissues d'huîtres. C.r.hebd.Séanc.Acad.Sci.Paris., 150: 2751-

VIANEY-LIAUD, M. & LANCASTRE, F. 1968, Utilisation d'un milieu diphasique pour la culture organotypique d'explants Australorbis glabratus. C.r.hebd.Séanc.Acad.Sci.Paris., 266: 1317-1319.

WOLFF, Et. & HAFFEN, K. 1952, Sur une méthode de culture d'organes embryonaires *in vitro*. J.exp.Zool., 119: 381-404.

The structure and development of the Hermaphrodite gland, hermaphrodite duct and common duct of Agriolimax reticulatus.

Introduction.

This study was undertaken so that a detailed knowledge of the structure and development of these organs might be acquired. This information was necessary in order to help interpret any changes that might be observed during the in vitro culture of these organs.

Histologically the structure and development of slug reproductive organs is well documented (Gatenby 1918, Rosenwald 1927, Richter, 1935, Filhol 1938, Abeloos 1944, Laviolette 1954, Pelluet & Lane 1961, Lusis 1961, Martoja 1964, Pelluet 1964, Galangau 1964, Kugler 1965, Smith 1966, Quattrini & Lanza 1965, Quattrini 1967 and Runham & Laryea 1968). However the only work describing the ultrastructure of these organs is that of Quattrini & Lanza (1965) on the hermaphrodite gland of Vaginulus borellianus and Laevicaulis alte and Quattrini (1967) on the prostate of Milax gagates.

Materials and Methods.

A.reticulatus were collected from grassy areas and from under stones in the vicinity of the laboratory. Organs were quickly dissected from the animals, some of which were first

anaesthetised in carbon dioxide (Bailey 1969). Fixation for light microscopy was carried out in Susa (12 - 24 hrs) followed by two washes in cellosolve of 24 hrs each. The tissues were embedded in ester wax (1960), serial sections were cut at 4μ and stained in Heidenheines Azan.

For examination in the electron microscope, tissue was fixed in buffered 1% osmium tetroxide (Palades fixative) for one hour at 0°C. Occasionally fixation was carried out for 3 hours in phosphate buffered Gluteraldehyde at 0°C followed by overnight washing in phosphate buffer. This treatment was followed by post-osmification in 1% buffered osmium tetroxide. The results obtained were generally inferior to those obtained with Palades fixative, and some shrinkage of the tissues was noticed. After both methods of fixation tissues were washed in distilled water, dehydrated in ethanol and embedded in Araldite. Sections were cut at 1μ using an L.K.B. Pyramatome or L.K.B. Ultratome and stained in toluidine blue for study with the light microscope. For examination in an A.E.I. E.M. 6. electron microscope, tissues were sectioned at 600 - 700Å on an L.K.B. Ultratome and stained in uranyl acetate and lead citrate (Peace 1964).

Results.

A. Hermaphrodite Gland.

The hermaphrodite gland of A.reticulatus consists of numerous sac-like acini, joined together by small factors of the hermaphrodite duct. These ductules join to form the hermaphrodite duct, so that the overall structure has the appearance of a bunch of grapes. Each acinus consists of a connective tissue wall enclosing a number of different cell types (Fig 1).

During the course of its development, the hermaphrodite gland passes through a definite sequence of stages which are recognised by the relative numbers of the different cell types that are present. Richter 1935 and Runham & Laryea 1968 have made detailed studies of the structure and development of the hermaphrodite gland of A. agrestis (probably A. reticulatus) and A. reticulatus respectively. These stages, as described by Runham & Laryea, are briefly as follows:-

Undifferentiated stage (A) (Fig 2)

The gland consists of a solid elongated cluster of undifferentiated cells enclosed ih a connective tissue sheath. This sheath contains only a few pigment cells.

Spermatocyte stage (B) (Figs 3 & 4)

The connective tissue sheath has now acquired some pigment and the gland appears much darker. Outgrowths develop from the

Fig 1 Stage F. hermaphrodite gland. X 500.

HD = Hermaphrodite ductule.

N = Nucleus of Nutritive cell.

O = Oocyte.

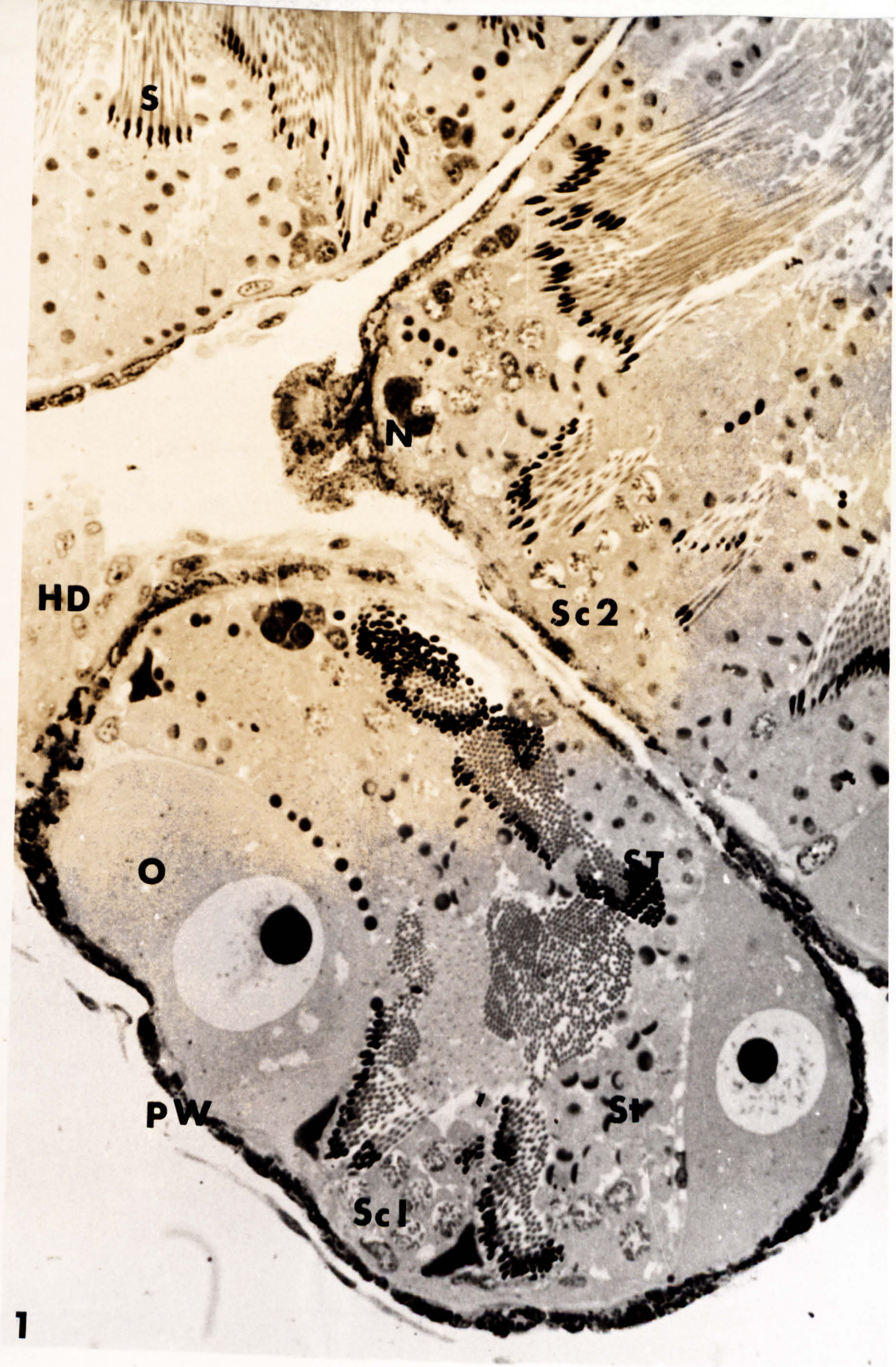
S = Mature Spermatozoa.

Sc1 = Primary Spermatocytes.

Sc2 = Secondary Spermatocytes.

St = Spermatids (early).

S.T. = Spermatids (late).



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Hermaphrodite Gland Stages. X 200.

Fig 2 Stage A

Fig 3 Stage B

Fig 4 Stage C (early)

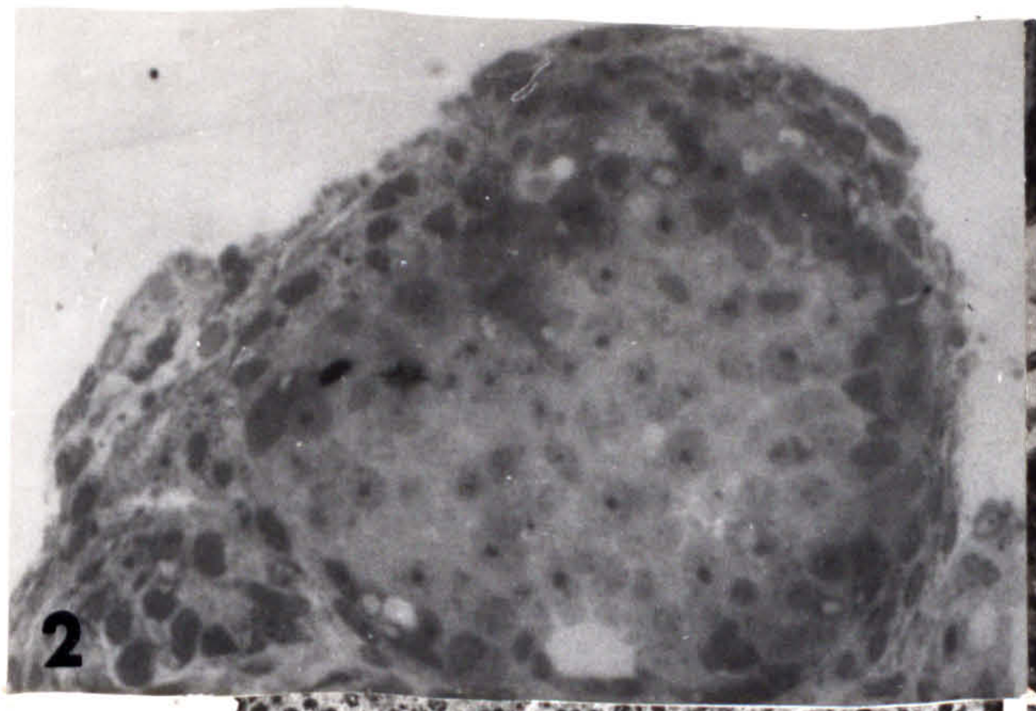
Fig 5 Stage C (late)

Fig 6 Stage D

Fig 7 Stage E

Fig 8 Stage G

Fig 9 Stage H.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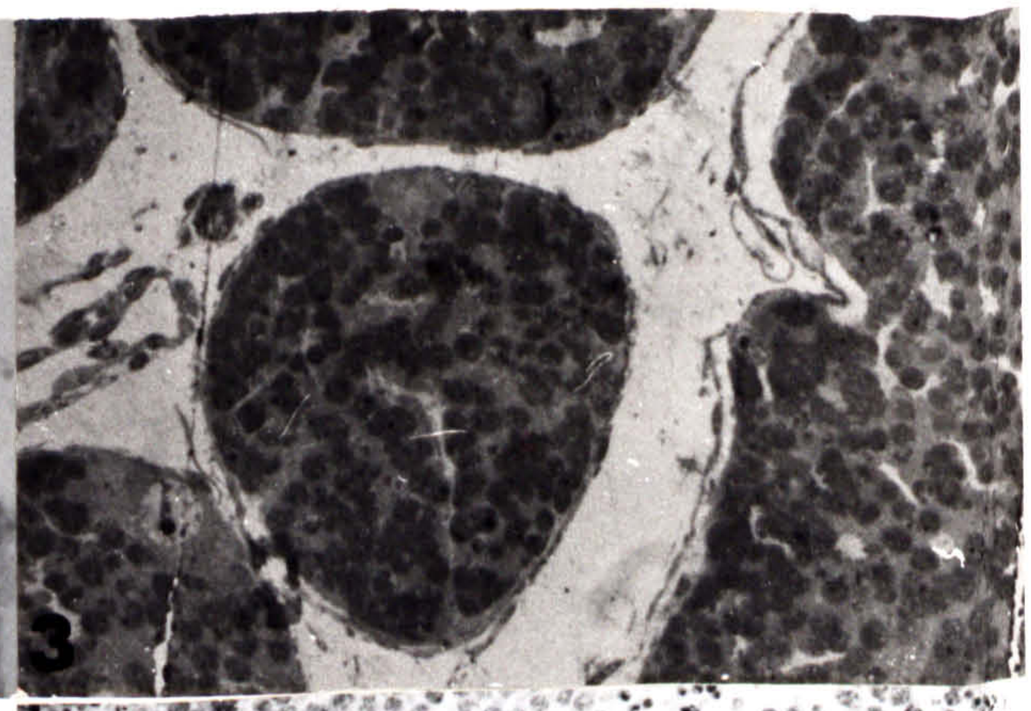
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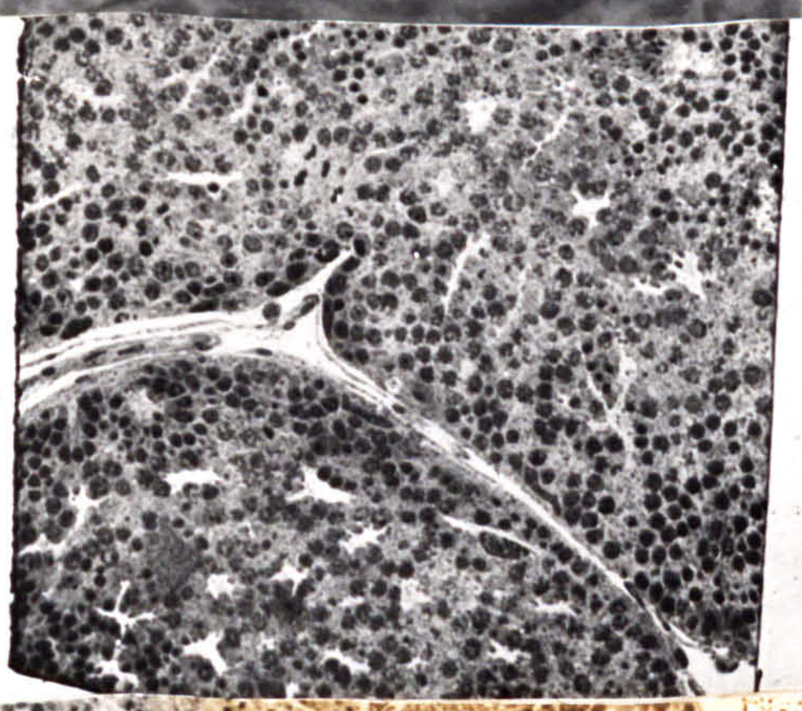
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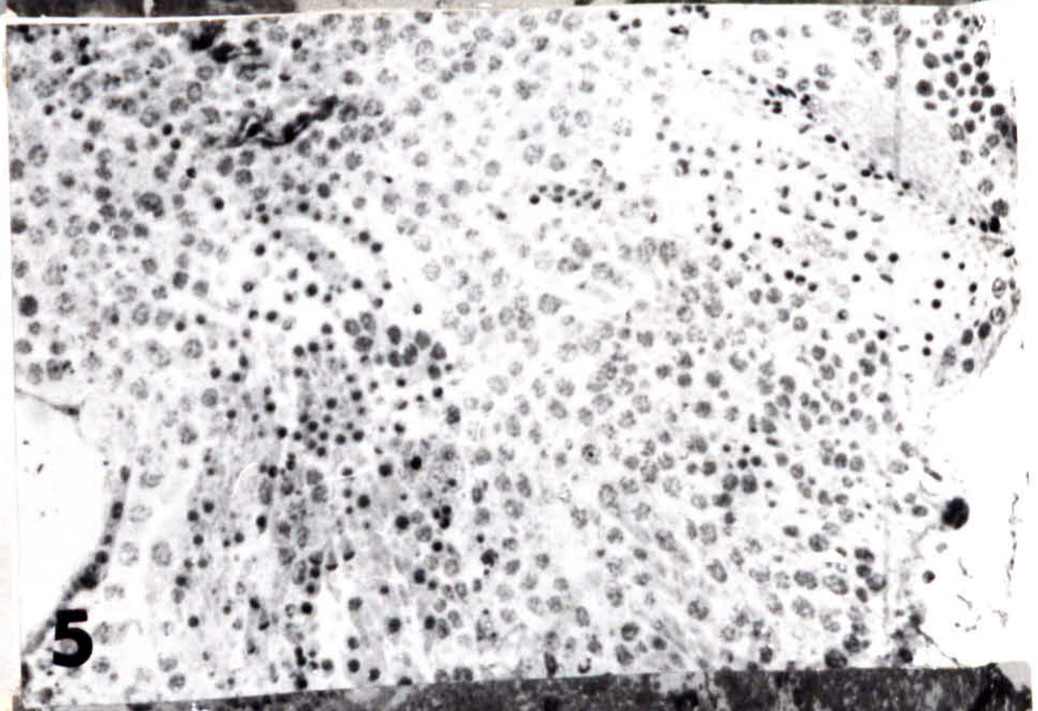
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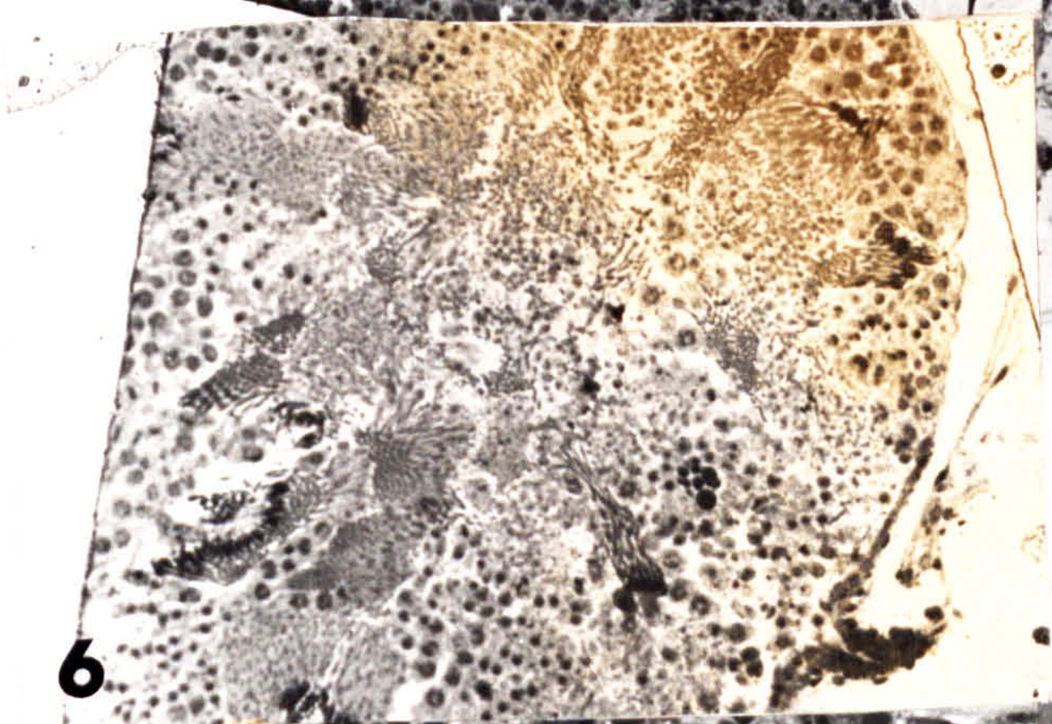
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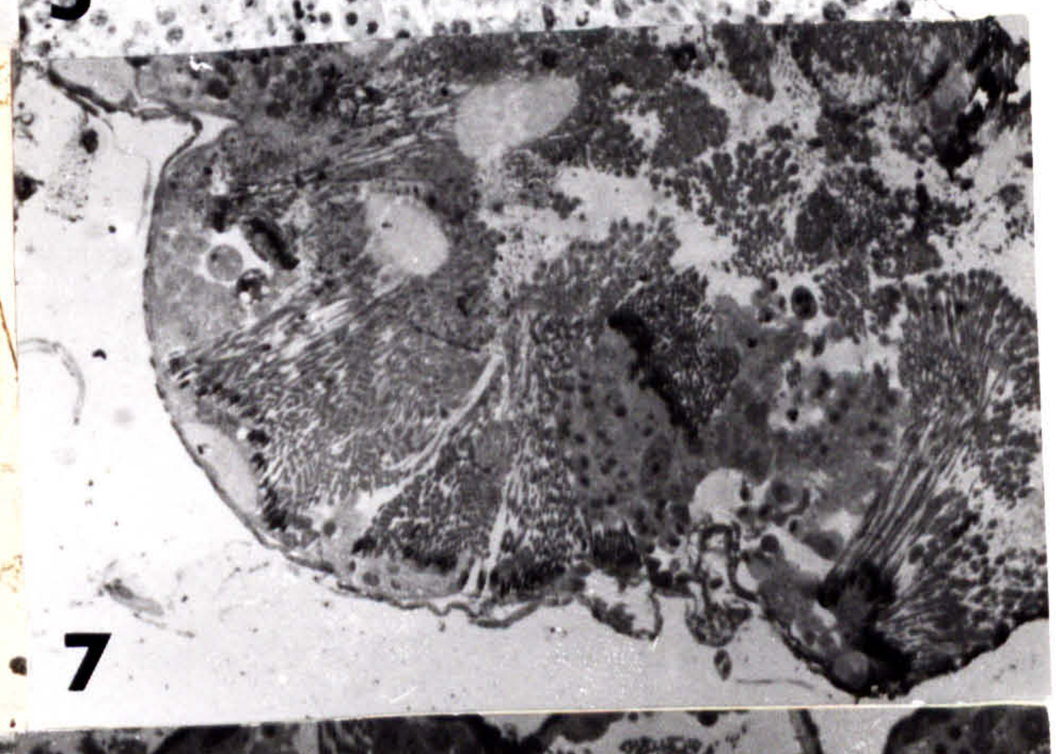
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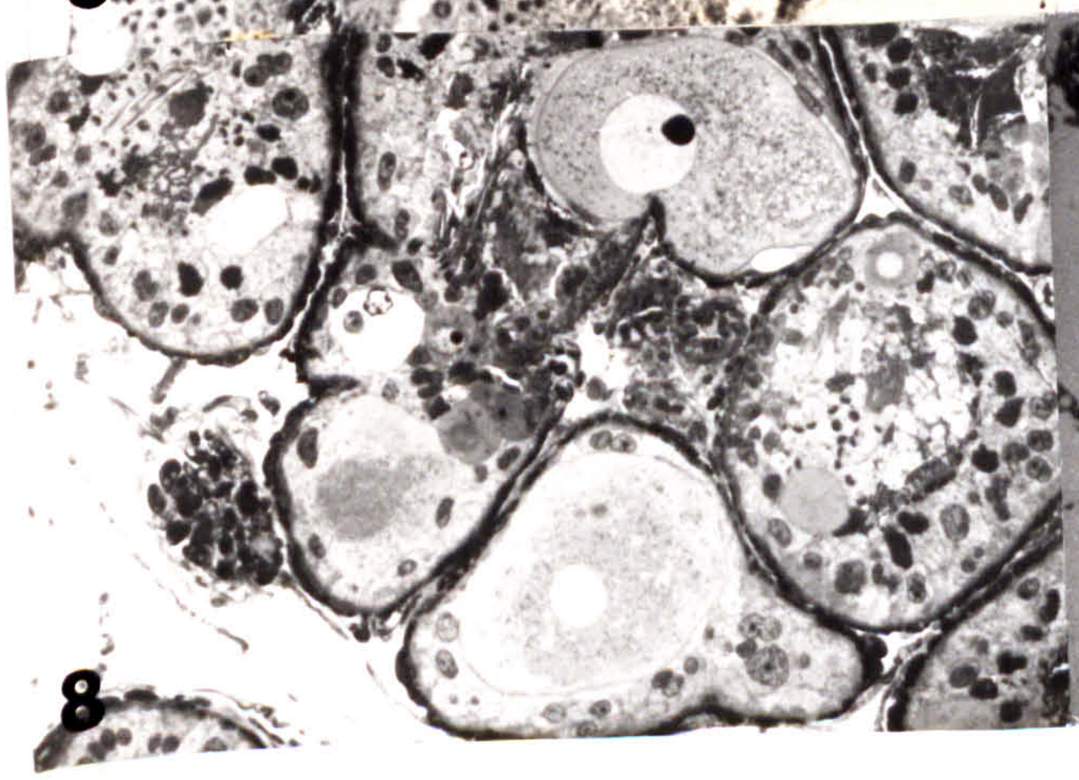
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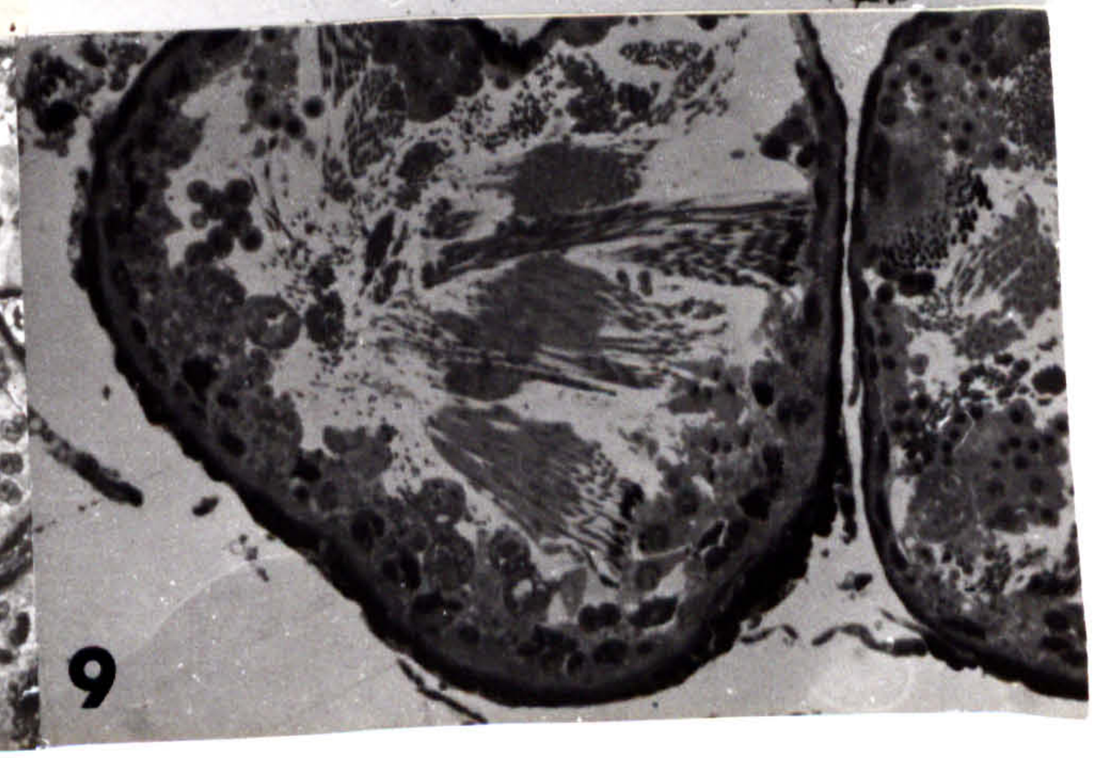
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solid mass of cells and form solid acini. As each acinus enlarges, the undifferentiated cells remain at the base of the acinus, and an epithelial lining forms around the wall of the neck region. This neck region may function as a germinal epithelium. Further enlargement of the acinus does not result in any increase in the diameter of the neck region. As the acinus enlarges, the undifferentiated germ cells multiply and begin to differentiate. Several cell types can be recognised at this time; undifferentiated germ cells; spermatogonia; and primary spermatocytes; are found towards the centre of the acinus; oocytes, nurse cells; and the epithelium of undifferentiated cells restricted to the neck region are found at the periphery of the acinus.

Spermatid stage (C).(Fig 5)

In addition to the cell types mentioned above, spermatids are found in groups, usually towards the centre of the acinus where a lumen is forming. These groups are found in association with processes from the nurse cells.

Early spermatozoon stage (D). (Fig 6)

The acinus is now largely filled with spermatids. Some mature spermatozoa are to be found towards the centre of the acinus and the sperm tails fill the lumen.

Late spermatozoon stage (E). (Fig 7)

A central lumen, containing free mature spermatozoa, is now clearly visible. The layers of spermatogenic stages are now much thinner. Many small oocytes are visible around the periphery of the acinus.

Early oocyte stage (F). (Fig 1)

Oocytes are now enlarging and a follicle can be seen surrounding them. Small oocytes are present particularly in the neck region of the acinus. Sperm stages are present, often in quite high numbers.

Late oocyte stage (G). (Fig 8)

The number of spermatogenic stages ~~are~~ is considerably reduced and oocytes are found free in the lumen.

Post reproductive stage (H). (Fig 9)

This is characterised by the presence of a cuboidal epithelium lining the acinus. It is found in this stage and to a limited extent in the late stage G. There is great variation in the number of sperm and oocytes present in the acinus at this stage. There often appears to be considerable overlap between these last four stages E, F, G and H.

The only published work dealing with the ultrastructure of the pulmonate hermaphrodite gland is that of Quattrini & Lanza (1965)

on Vaginulus boreallianus and Laevicaulis alte. However, ultrastructural studies have been made on limited aspects of the development of the hermaphrodite gland, e.g. Oogenesis (Taylor & Anderson 1969, Bedford 1966), and oocyte/follicle cell differentiation (Anderson 1969, Nieland & Goudsmit 1969).

Acinus wall.

Using the light microscope the wall of the acinus appears a simple structure consisting of an outer pigment layer and an inner layer staining blue with azan. With the higher magnifications of the electron microscope, the wall of the acinus can be resolved into a thin layer of collagen fibres bounded on its inner surface by a basement membrane 500Å thick (Fig 19 etc.). In the matrix of collagen fibers ~~are~~ is found a number of apparently randomly arranged smooth muscle cells and some nerve axons. Embedded in the surface of this collagenous layer is a discontinuous layer of pigment cells. The thickness of this pigment layer varies greatly (0 - 15μ), tending to increase in thickness as the acinus matures.

The cytoplasm of the pigment cells (Fig 19) is very extensive and contains a varying number of pigment granules. These granules are flat discoid structures with a regular size of 0.6μ in diameter and 0.25μ in thickness. In addition, the cytoplasm contains masses of irregularly shaped electron dense material,

comprising aggregates of smaller particles of around 200Å, similar to glycogen granules. Small rings of endoplasmic reticulum are to be found, together with scattered mitochondria. The nucleus is flattened (3μ in thickness) with peripherially arranged chromatin and a rather pale nucleolus.

In the undifferentiated hermaphrodite gland and to a lesser extent at the spermatocyte stage the acinus wall contains much less collagen and a far greater proportion of cellular elements. These connective tissue cells contain a considerable number of glycogen-like granules and the pigment cells contain few pigment granules. It would appear that the wall is rapidly growing at this time, keeping pace with the increase in the size and number of the acini.

As the acinus reaches maturity, its wall is seen to contain an increased number of collagen fibers and pigment granules. However the wall remains thin and it rarely exceeds 5μ in thickness.

As the mature germ cells are shed, the acinus begins to shrink and the wall appears to thicken. This thickening reaches a maximum in the post reproductive stage acinus (Fig 31). In such acini the pigment layer is much thicker and the pigment cells appear more rounded. The basement membrane and collagen are very convoluted. Thus the thickening in the latter stages of acinus

differentiation appears due to a shrinkage of the acinus rather than to an increase in the amount of pigment present.

Undifferentiated Germ Cells. (Fig 10)

Many types of cell are found enclosed within this acinus wall. Undifferentiated germ cells are rather irregular in shape and are 8 - 10μ across their widest part. The cytoplasm contains mitochondria, vesicles and occasionally free ribosomes, endoplasmic reticulum, deposits of glycogen-like granules and is restricted to a thin layer around the nucleus. The surface of these cells is usually covered with microvilli. The nucleus is large and irregular and chromatin is only visible when the cell is dividing. One, or occasionally two, large dense nucleoli are present towards the centre of the nucleus. These cells divide to form the spermatogonia, oogonia and possibly nutritive cells.

Male germ cells.

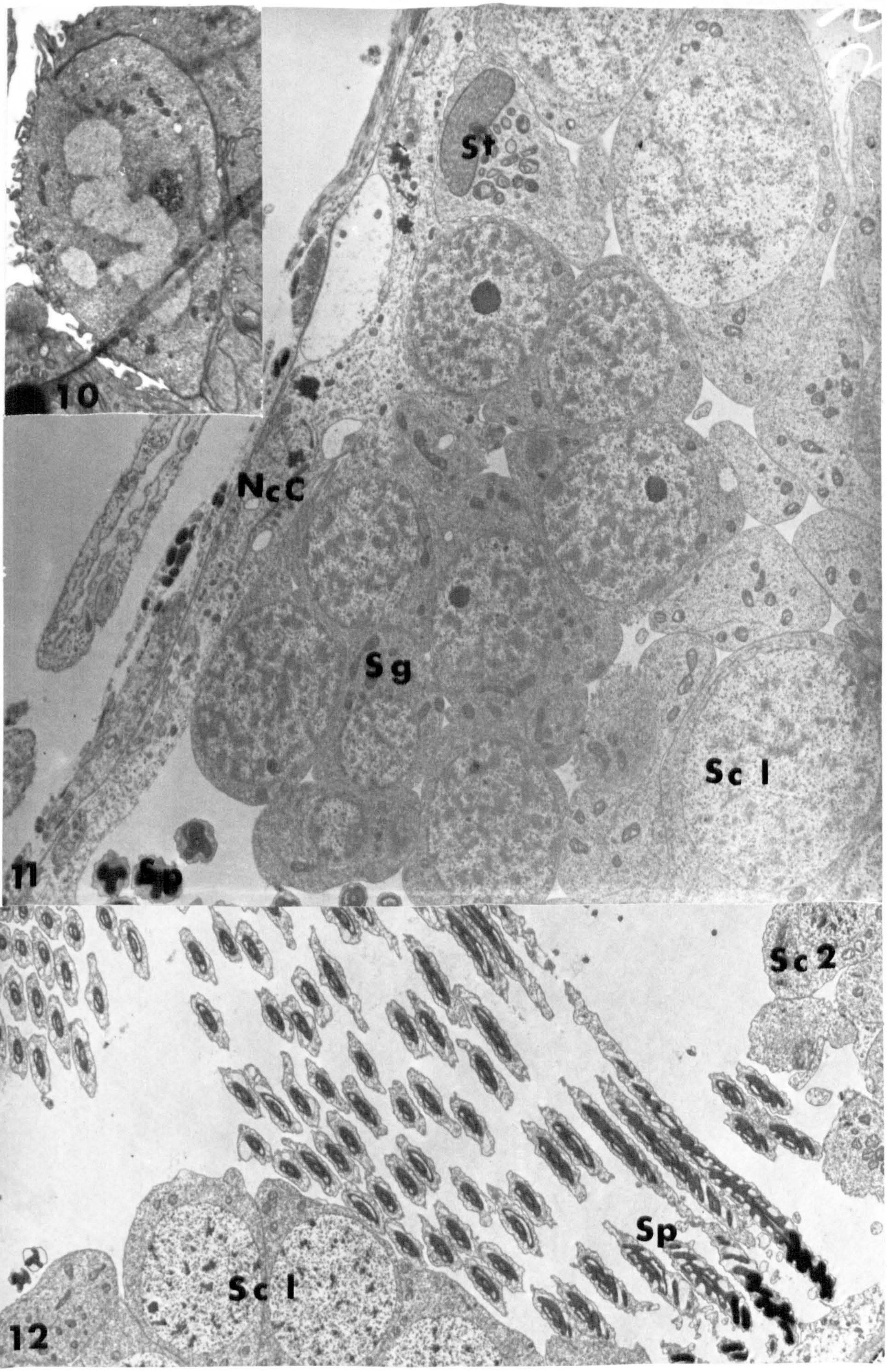
Spermatogonia (Fig 11 etc.) are most abundant in the early stages of acinus differentiation, but are found at later stages in small numbers. These cells are of similar dimensions to the undifferentiated germ cells but are more regular in shape and tend to be associated with cytoplasmic processes from the nutritive cells. The cytoplasm of these spermatogonia contains mitochondria, some smooth and some rough endoplasmic reticulum, free ribosomes,

Fig 10 Undifferentiated Germ Cell. X 10,000.

Fig 11 Part of acinus of stage C hermaphrodite gland. X 8,000.

Fig 12 Part of acinus of Stage E hermaphrodite gland. X 8,000.

NcC Nutritive cell cytoplasm.
Sg Spermatogonium.
Sc1 Primary Spermatocytes.
Sc2 Secondary spermatocytes.
Sp Mature Spermatozoa.
St Early spermatid.


St
10
NcC
Sg
Sc 1
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Sp
Sc 2
Sc 1
Sp
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and a few vesicles. The large nucleus is more or less spherical and contains a number of discontinuous patches of more densely staining chromatin in addition to the one or two nucleoli which are often to be found at the periphery of the nucleus. There are no microvilli on the surface of these cells.

The primary spermatocytes (Fig 11 etc.) are slightly larger than the spermatogonia and have a more extensive cytoplasm. At this stage the developing male gametes are at their maximum size and they become smaller with further development. They are round or slightly oval in shape and are found in close contact with the cytoplasmic processes of the nutritive cells (as are all subsequent stages of spermatogenesis). The nucleus is spherical and large but contains no nucleolus. The darker staining chromatin within these nuclei forms a reticulum or network. The cytoplasm is usually aggregated into a lobe on one side giving the cell an asymmetrical appearance. The nuclear membrane is surrounded by several concentric rings of smooth reticulum which branch into the cytoplasm. The mitochondria are numerous and tend to be aggregated in the cytoplasmic lobe.

The primary spermatocytes undergo the first of the meiotic divisions to produce the secondary spermatocytes (Fig 12 etc.). These cells are smaller than any of the previous stages of spermatogenesis (5 - 7μ in diameter). The nucleus of these

cells is 2 - 3μ in diameter and contains a chromatin network which represents the chromosomes seen with the light microscope. The two daughter cells remain joined by their cytoplasm after the cell division is completed. This cytoplasm is again aggregated towards one side of the nucleus. This cytoplasmic lobe, with its enclosed mitochondria, is situated adjacent to the cytoplasmic process of the nutritive cell. These cells are not very numerous at any stage in the development of the acinus and it would appear that this stage in the maturation of the spermatozoon is short lived.

The secondary spermatocytes undergo a further meiotic division to produce two spermatids. Since the secondary spermatocytes are still joined, and the result of their division is two joined spermatids, these spermatids are usually found in groups of four joined by their cytoplasm (Fig 16). Apparently, immediately after division the nuclear material of the spermatid rapidly condenses to form a small and denser spherical nucleus to the side of the cell in contact with the nutritive cell cytoplasm. This is the start of the development of the spermatid head. The mitochondria and other cytoplasmic inclusions aggregate in the larger area of cytoplasm away from the nucleus. At opposite sides of the nucleus, there is an apparent fusion of the double nuclear membrane resulting in a thickened membrane at the

Fig 13 Part of acinus of stage G hermaphrodite gland. X 10,000.

Fig 14 Junction between spermatid head and nutritive cell. X 25,000.

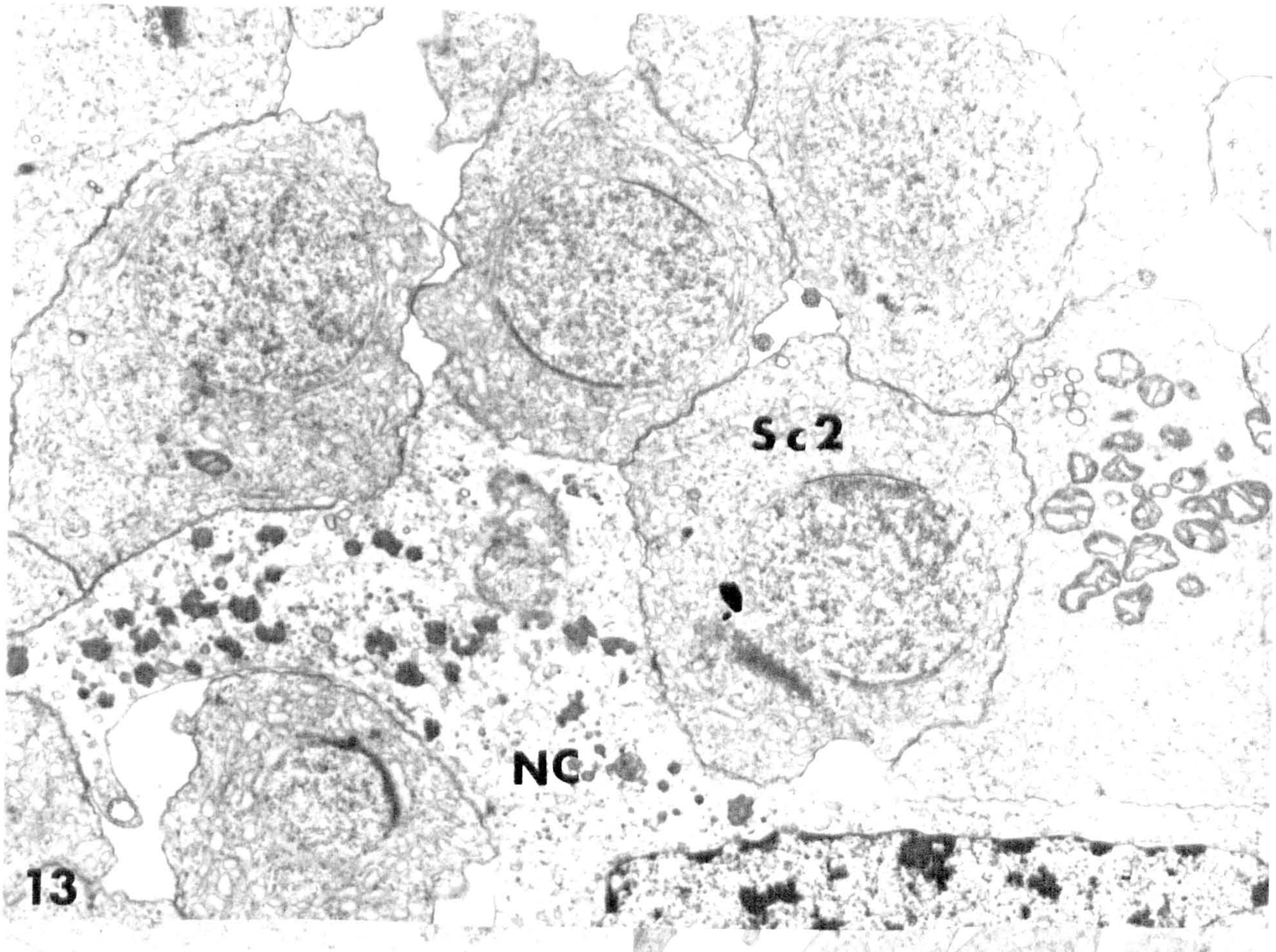
Fig 15 Cytoplasm on spermatid tails. X 15,000.

N.C. = Nutritive cell cytoplasm.

Sc2 = Secondary spermatocyte.

St = Spermatid.

S.T. = Spermatid Tail.



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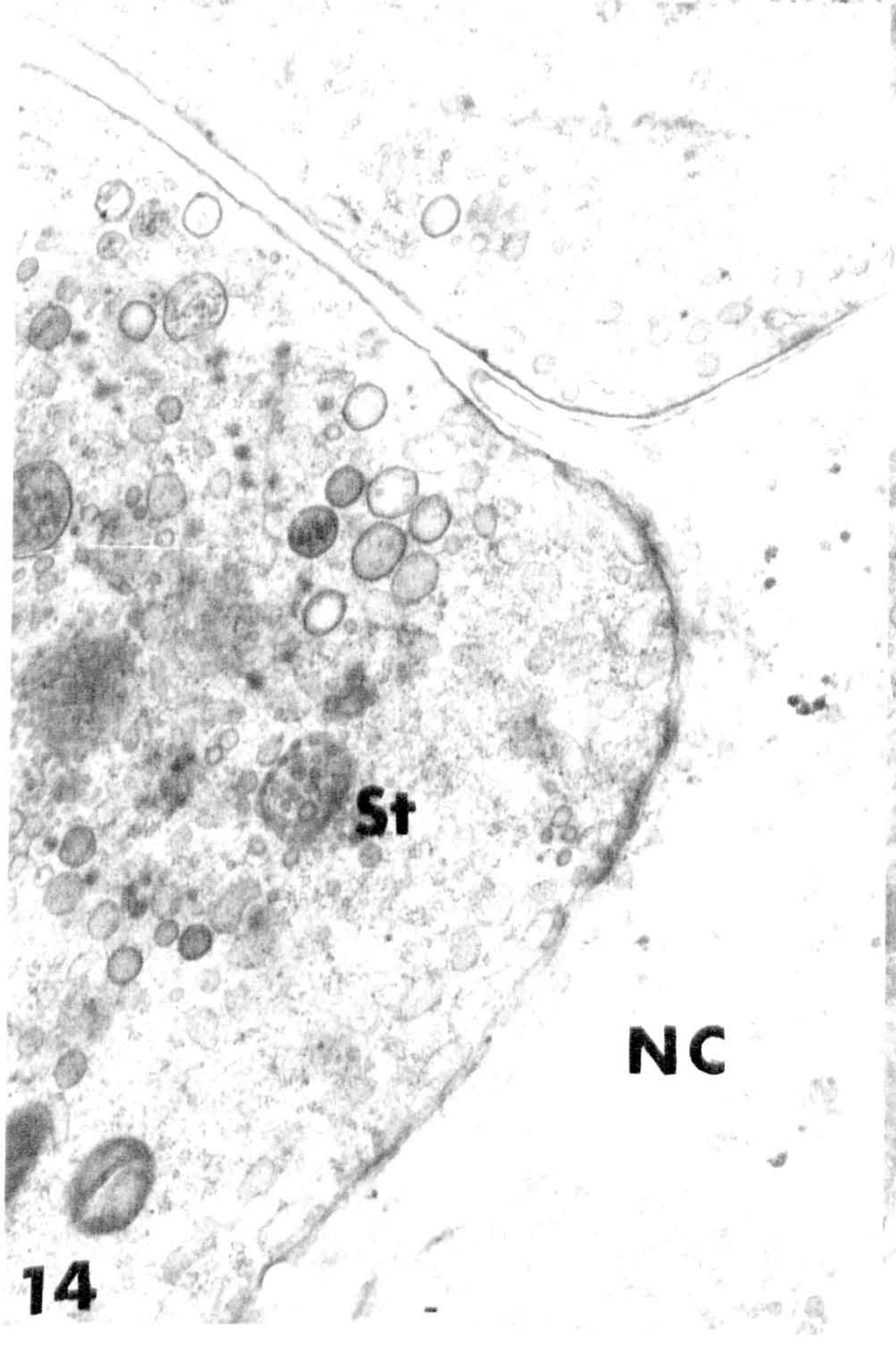

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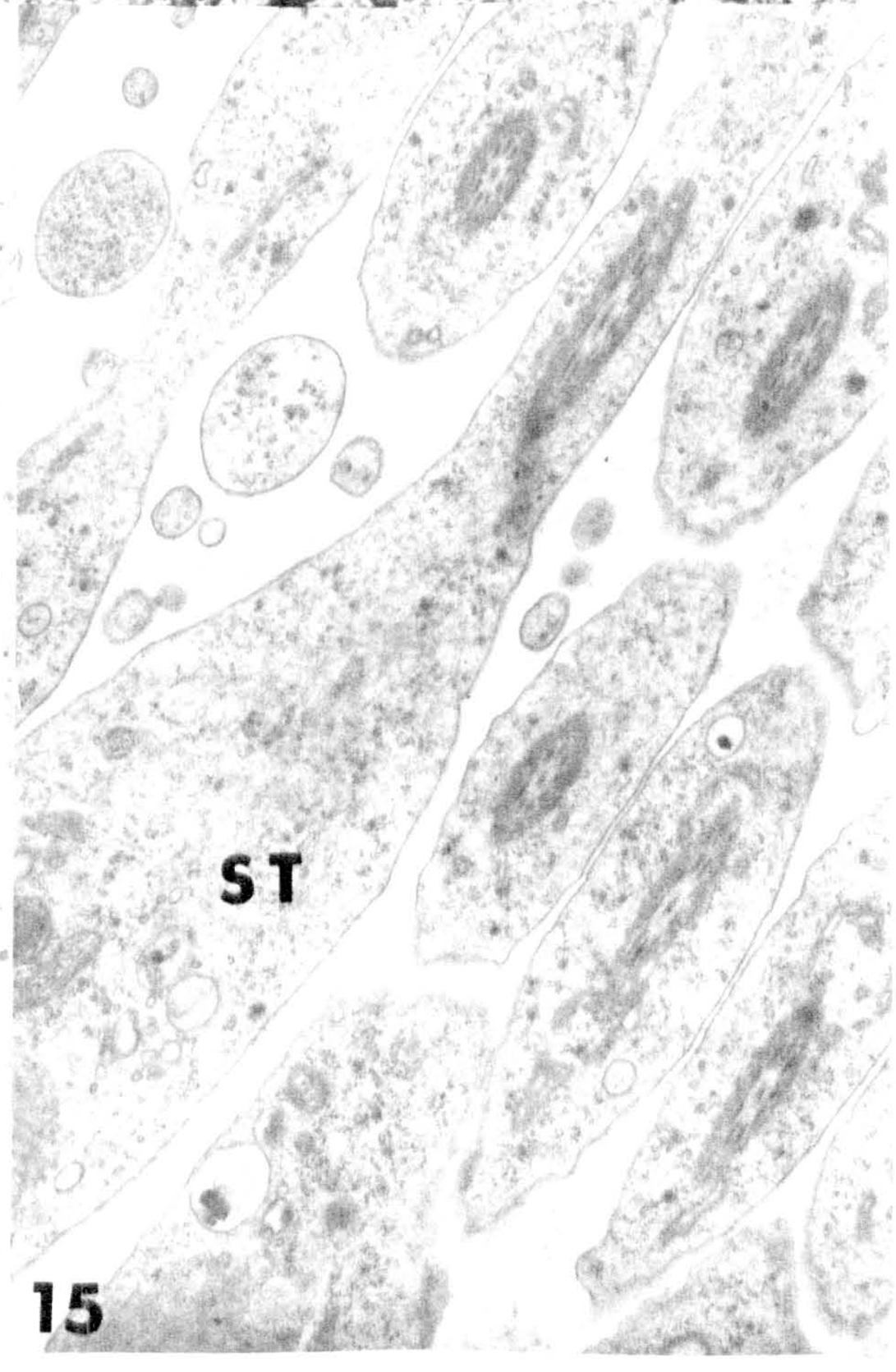

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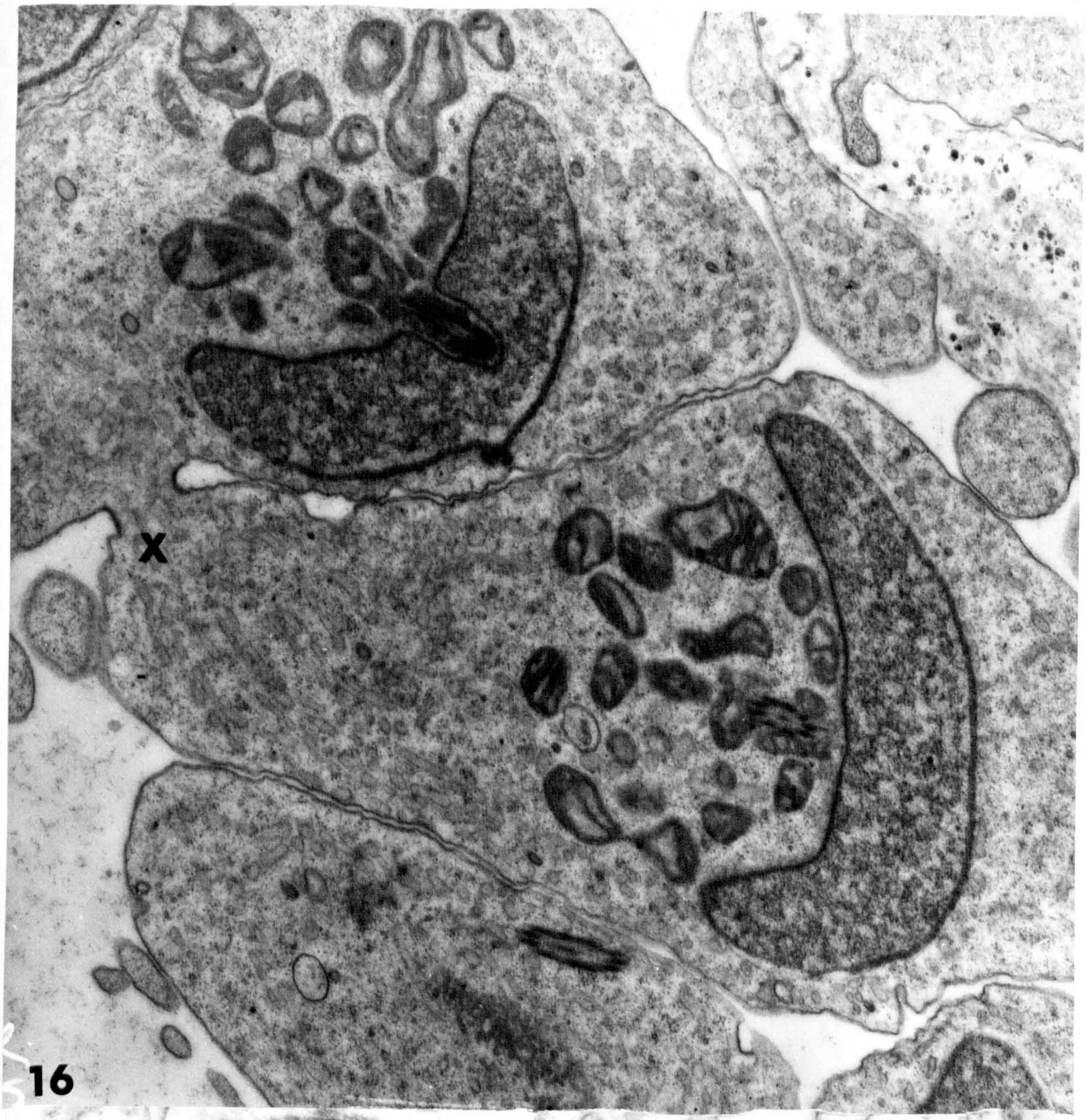
Fig 16 Spermatids showing cytoplasmic connection (X). X 20,000.

Fig 17 and 18 Transverse Sections of Spermatid head showing condensing D.N.A. protein fibers. X 40,000.



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apex of the spermatid head and at its base (Fig 13). This phenomenon seemsto be the same as that described by Watts (1952) in Arion subfuscus where this thickening was found to be Feulgen positive.

The dense nucleus becomes positioned near the part of the cell in contact with the nutritive cell and starts to change its shape. The side of the nucleus furthest from the nutritive cell cytoplasm invaginates becoming hemispherical and finally "mushroom" shaped (Fig 16 etc.). At this time the centrosomes become apparent towards the centre of the cell. The mitochondria become grouped around and between the centrosomes to form a "stalk" to the mushroom shaped nucleus. At the centrosome, the typical 9 + 2 arrangement of the fibres of the developing sperm tail become apparent. The rim of the mushroom shaped nucleus folds further back finally almost enclosing one centrosome and some of the mitochondria. At this stage the flagellum becomes elongated while the nuclear contents form a lattice of dense interconnecting fibres (Figs 18 - 19). These fibres run in a longitudinal direction in the developing spermatid head and are probably condensing D.N.A. protein fibres. The residual cytoplasm of the cell, now devoid of most of its inclusions, passes down the spermatid tail in "droplets" (Fig 15) leaving a system of tubules and membranes enclosed in cytoplasm around the tail. Finally, the

nuclear contents which have assumed an elongated ellipsoid shape, further condense and take on the final dense spiral shape of the mature spermatozoon head (Fig 12). The formation of the acrosome was not followed. A study of the mature spermatozoon of A.reticulatus has been made by Bayne (1970).

In the later stages of acinus development, the developing sperm stages are often found to have a highly vacuolated cytoplasm (Fig 13). Quattrini & Lanza (1965) describe a similar type of vacuolation in studies on the structure of the gonad of Vaginulus borellianus and Laevicaulis alte.

Nutritive cells.

The arrangement of the developing sperm cells from the spermatogonium to the mature spermatozoon is dependent on the arrangement of the nutritive cells and their cytoplasmic processes. These cells presumably develop from undifferentiated germ cells, possibly by spireme karyodiresis (Koshman et al 1967), and migrate around the acinus wall where they enlarge and become polyploid (Quattrini et al 1965, Serra et al 1967).

The Nurse or nutritive cells abut directly onto the basement membrane of the acinus wall over which they form thin cytoplasmic layers with long processes protruding into the centre of the acinus. Very little of their structure can be seen with the light microscope (Fig 1). The nucleus is clearly visible as it is large

with finely granular chromatin. Small areas of cytoplasm can be seen scattered around the acinus. These cytoplasmic processes can be seen more clearly in the later stages of acinus differentiation.

With the higher powers of the electron microscope, the extent of these cells can be seen (Figs 13, 20, etc.). Their cytoplasm is rarely more than 2 - 3μ thick and usually much less. The cell surface is often irregular with projections and microvilli. The large nucleus is found in the cytoplasm which is in contact with the wall of the acinus (Fig 12). It is often irregular or lobed in shape, containing several nucleoli of various sizes and granular dark staining chromatin. The cytoplasm is very characteristic in its appearance (Fig 19 etc.). It contains vast accumulations of glycogen-like granules which have the same appearance as those in the pigment cells. Mingled with these granules are many lipid droplets. Large multivesicular bodies and myalin bodies are also present, together with mitochondria, golgi bodies, rough reticulum and free ribosomes. Clear vesicles can be seen in the vicinity of the golgi bodies while larger ones can be seen at the cell surface. In the final stages of acinus differentiation the appearance of the nutritive cell cytoplasm is somewhat different. It takes on a highly vacuolar appearance, and partly broken down sperm heads and

Fig 19 Cytoplasmic detail of Immature Oocyte from stage B hermaphrodite gland. X 20,000.

Fig 20 Part of Immature oocyte from stage C hermaphrodite gland. X 3,000.

Fig 21 Previtellogenic oocyte from stage F hermaphrodite gland. X 1,500.

Fig 22 Detail from fig 21. X 35,000.

F = follicle cell.

M = Mitochondria.

N = Nucleus.

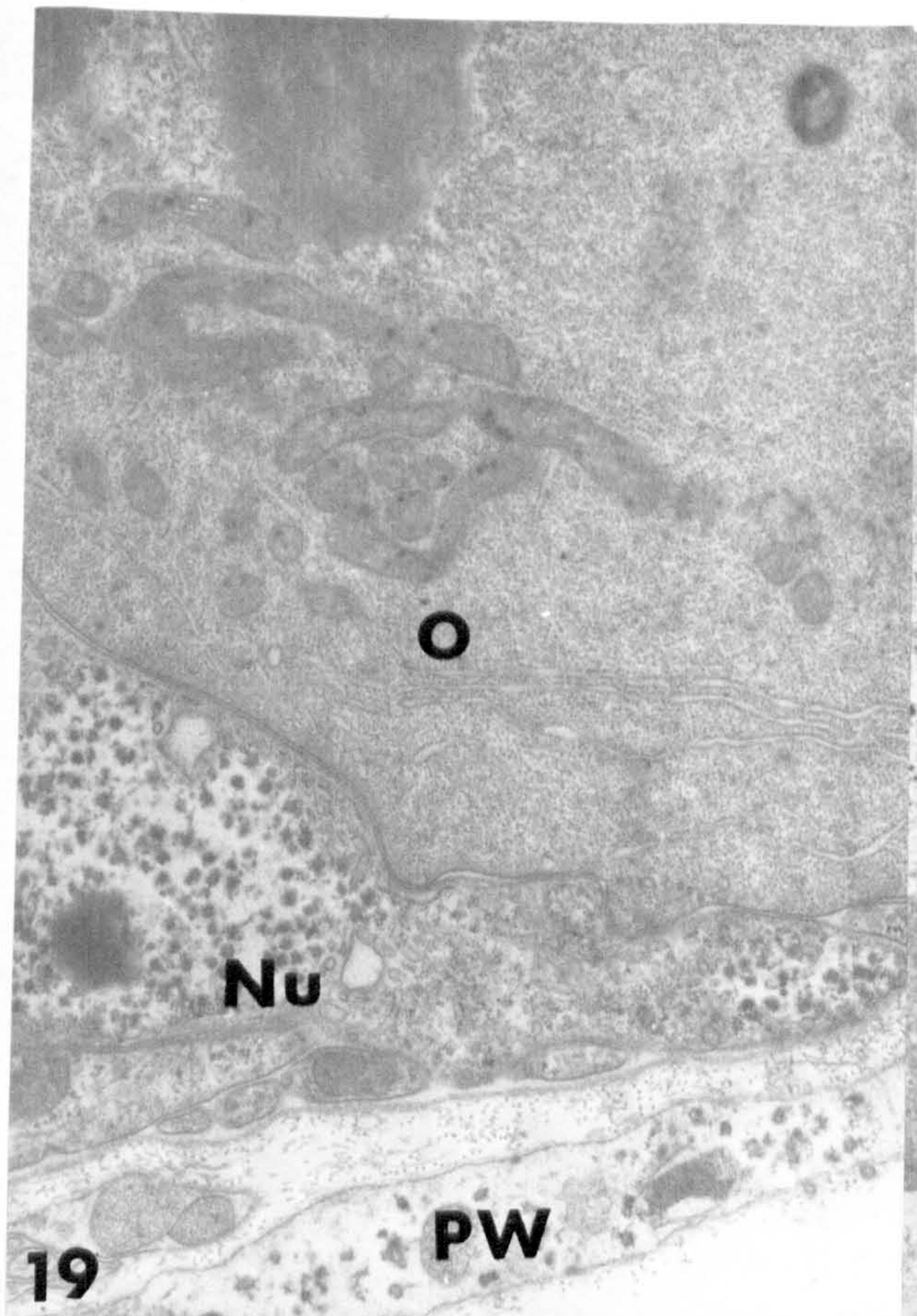
Neo = Nucleolus.

Nu = Nutritive cell cytoplasm.

P.W. = Pigment Wall.

Scl = Primary Spermatocyte.

St. = Spermatid.



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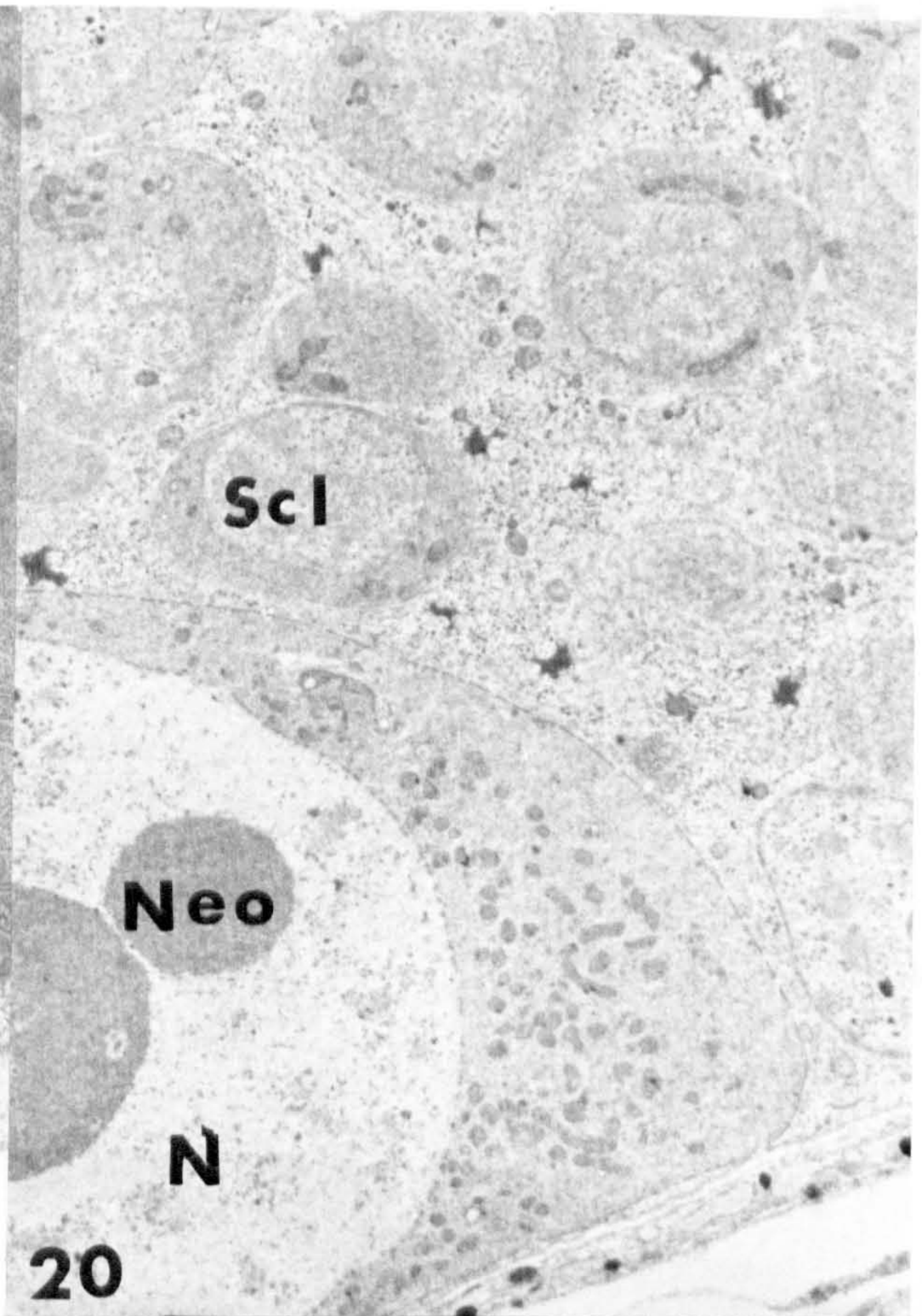

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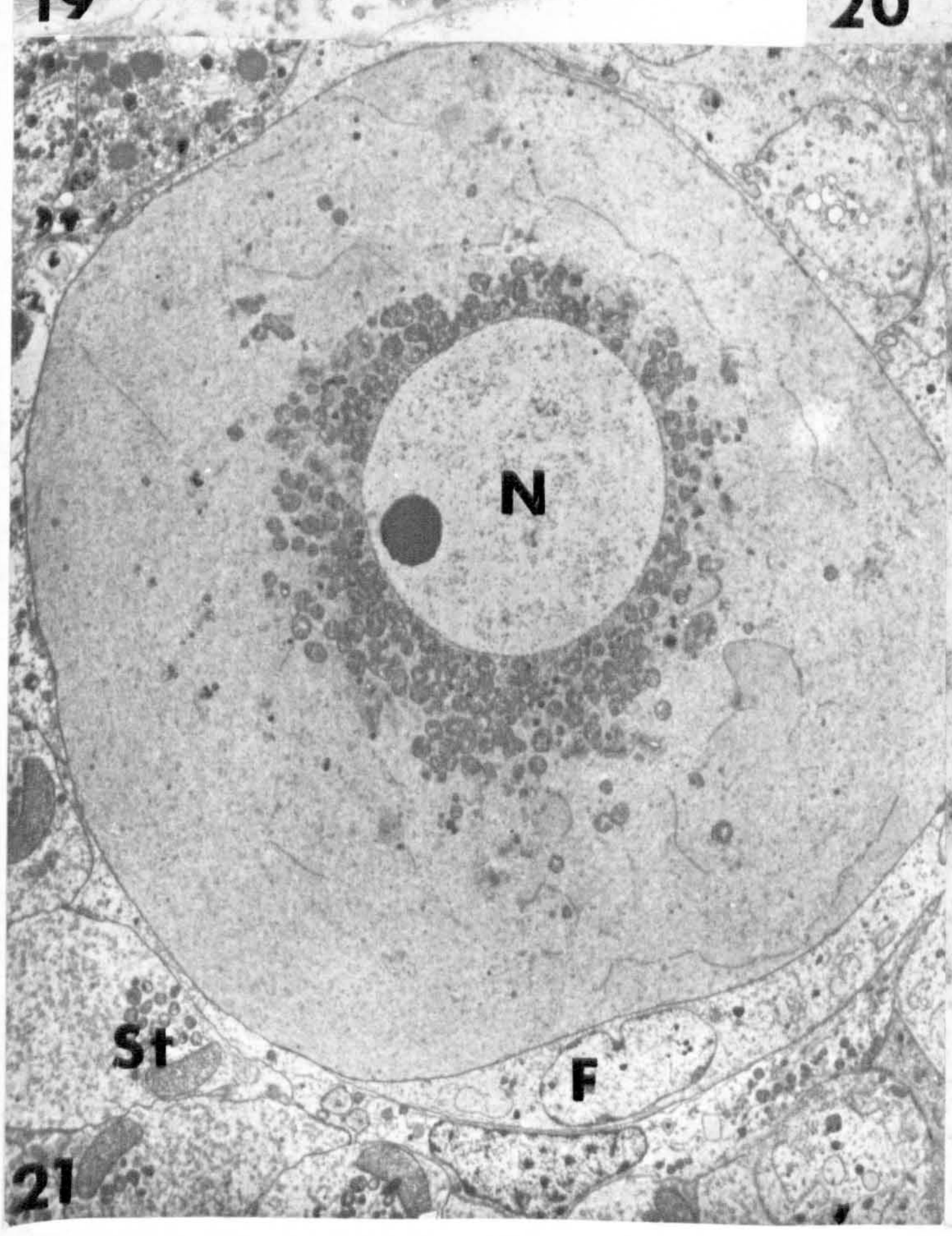

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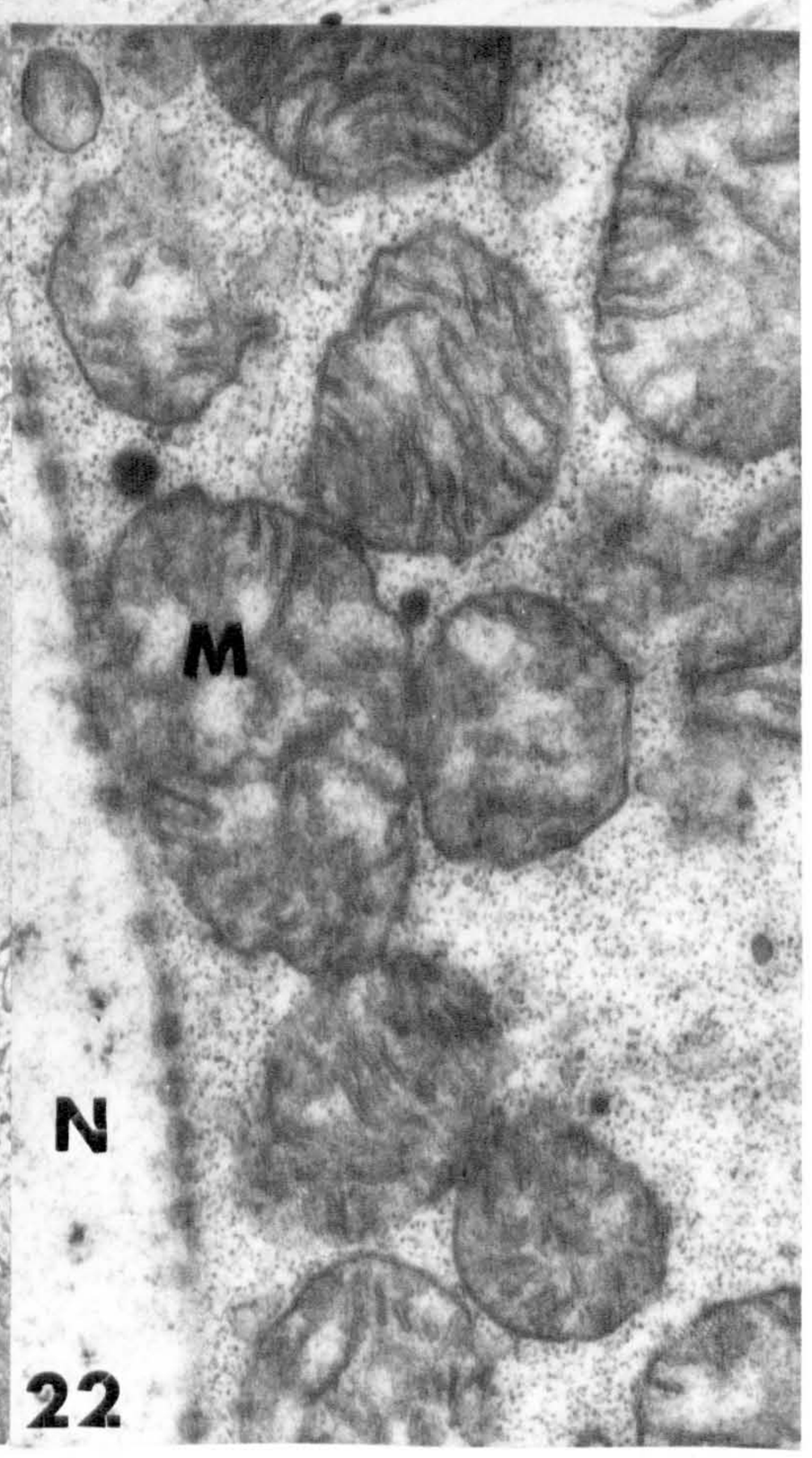

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other sperm stages can be seen in these vacuoles (Fig 31). It appears that the cells are absorbing unwanted material from the lumen of the acinus. This absorption seems to result in the accumulation of vast amounts of lipid throughout the cytoplasm.

The junction between the nutritive cells and the developing sperm cells is small (Fig 14), in the region of 100Å, but this close contact is not continuous and gaps which may be as wide as 1μ are found at intervals. There does not seem to be any specialisation of either cytoplasm in this area. The cytoplasmic processes of the nutritive cells sometimes overlay one another around the wall of the acinus especially in the region of the oocytes. However there does not seem to be any difference between the nutritive cells around the small oocytes in the early stages of acinus development, and those supporting the spermatogenic stages. Indeed the same nutritive cell will often be found in contact with both male and immature female germ cells.

In the latter stages of acinus development, the maturing oocytes are always surrounded by a nutritive cytoplasmic layer that is slightly different to that in association with the male cells (Fig 29). It is rather denser in appearance with fewer vacuoles. Since this female nutritive cell process is usually covered by a male type nutritive cell process and is thus not in contact with

the lumen of the acinus, it is possible that this difference is due only to the inability of the cytoplasm to absorb any of the contents of the acinus. On the other hand, these female nutritive cells may arise from the undeveloped cells of the cell cluster which seems to give rise to an oocyte. The cytoplasmic processes of these female nutritive cells are often extremely thin, less than 100Å at times although more normally around 5000Å.

Complex processes are visible between this cytoplasm and that of the oocytes and structures are sometimes visible in the oocyte cytoplasm apparently associated with these processes (Fig 28 etc.). The gap between these two cell types is around 5000Å but in the vicinity of these processes it may be as little as 50Å or they may even appear in direct contact. The nuclei of these female nutritive cells ressemble those of the other nutritive cells and they are usually to be found in the angle between the oocyte and the wall of the acinus (Fig 28).

Female germ cells.

The mature oocytes are by far the largest of the cell types to be found in the acinus, and when mature have an average diameter of 100μ (Figs 20, 23, etc.). The large clear nucleus has a diameter of 40μ and is apparently empty when viewed with the light microscope save one or occasionally more large nucleoli

Fig 23 Part of Post vitellogenic Oocyte.

X 1,500.

Cytoplasmic detail from above.

Fig 24 Mitochondria. X 30,000.

Fig 25 Annulate Lamellae. X 15,000.

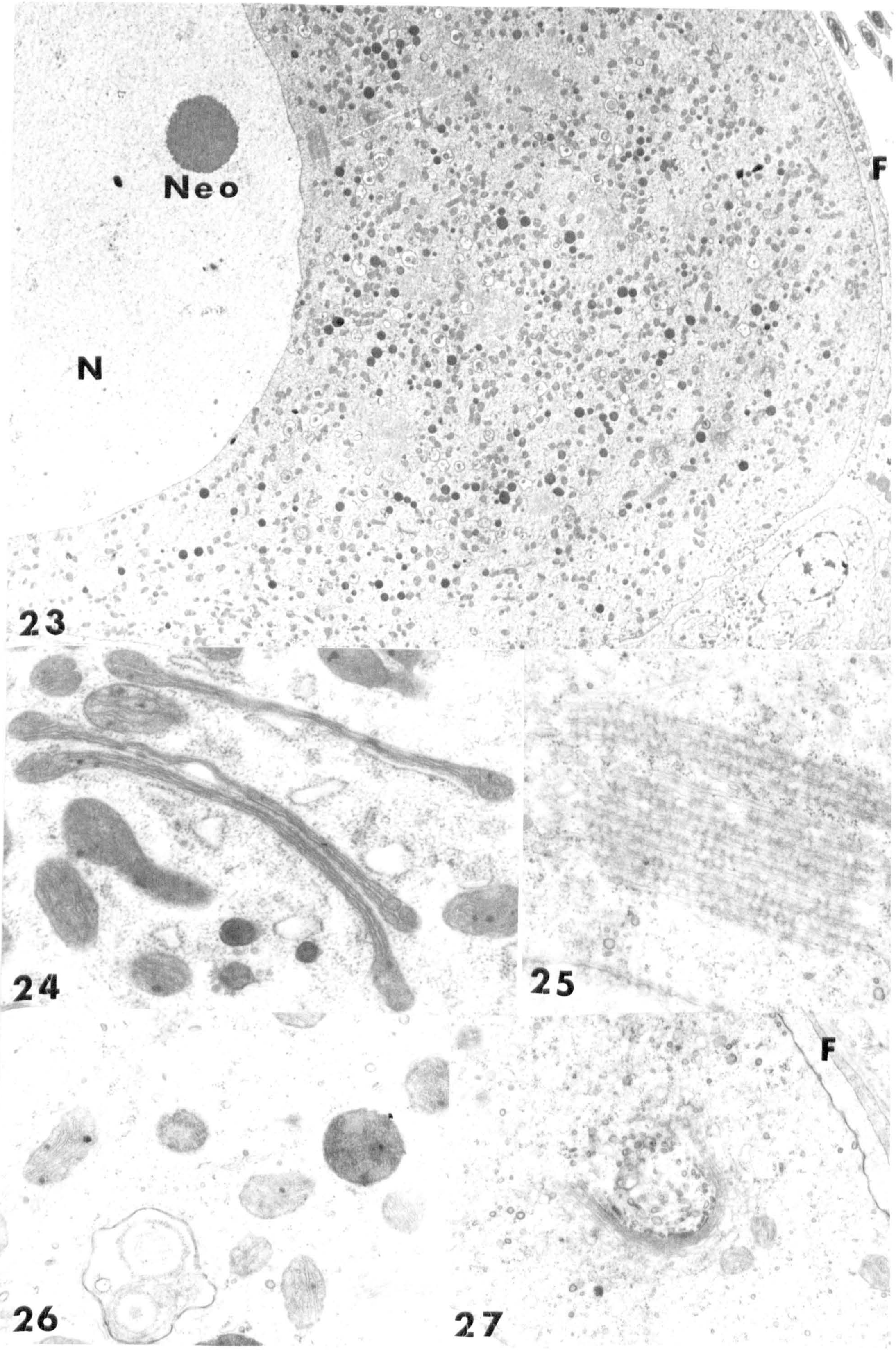
Fig 26 Vesicles in cytoplasm. X 15,000.

Fig 27 Golgi body. X 10,000.

F = Oocyte folliclo.

N = Nucleus.

Neo = Nucleolus.


Neo
F
N
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F
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(Fig 20). These nucleoli may be up to 15μ in diameter. Using the electron microscope, the nucleus appears to contain, in addition to the nucleoli, a very fine reticulum of indistinct fibres 50 - 100Å across (Fig. 22).

The small immature oocytes appear to be in groups of 2 or 3 cells surrounded by nutritive cell processes. Early in their growth one of these cells grows, possibly at the expense of the others, and the others remain small. In the early stages the nucleus contains a mass of granular material in some cases aggregated into larger irregular electron dense areas (Fig 19). The cytoplasm of these small immature oocytes is typical of the other immature cells, containing many mitochondria and a mass of free ribosomes, together with golgi complexes and a limited amount of rough reticulum. Some vesicles may also be found, especially in the vicinity of the golgi bodies, while vesicular myelin bodies and lipid droplets are also present.

In large previtellogenic oocytes the mitochondria are often dumbell shaped (Fig 24) and may be undergoing division. Large concentric rings of lamellae are found which are densely covered with ribosomes (Fig 32). Lipid droplets or rarely mitochondria are found at the centre of these rings. Other flattened lamellae are shaped like golgi bodies (Fig 33) but their membranes are covered with ribosomes. These lamellae seem

to be budding off ribosome covered vesicles.

Just prior to the onset of vitellogenesis, the mitochondria become aggregated around the nuclear membrane in a layer 5 or 6 deep (Fig 21). The nuclear membrane, continuous up until this time, becomes perforated and apparently breaks down. The nuclear membrane now takes on the appearance of a series of small tubules, about 800Å in diameter (Fig 22), containing some internal structure. When the picture of the tubules is rotated and viewed with a stroboscope so that an effect similar to photographic reinforcement is produced, Maximum reinforcement occurs at period 6 suggesting six-fold symmetry in these tubules.

With the onset of vitellogenesis, the mitochondrial layer disperses and the tubules of the nuclear membrane become indistinct, but the membrane remains perforated (Fig 23). The oocyte rapidly enlarges and the cytoplasm becomes filled with granules of four main types (Fig 26):-

a) electron dense multivesicular bodies,

b) granules with one or more concretions which show a laminate structure,

c) small granules which are probably precursors of the multivesicular bodies,

d) large pale staining vesicles.

In addition to these granules, numerous annulate lamellae,

consisting of 4 to 12 smooth perforated membranes piled upon one another, are found throughout the cytoplasm. In addition to these "new" cytoplasmic inclusions, those found in the previtellogenic oocyte are still present. The only exceptions are the concentric rings of rough reticulum and the lamellate golgi shaped structures also of rough reticulum which are now absent. There does not appear to be any rough reticulum in the cytoplasm of the post-vitellogenic oocyte.

Epithelial Cells

The last cell type found in the normal acinus is the cuboidal epithelial cell which is characteristic of the post reproductive stage of hermaphrodite gland development (Fig 30). These cells form a single layer of cells directly on the basement membrane of the acinus wall. They vary in thickness from 2 to 5μ and have a length of about 12μ. The surface of these cells is covered with a thin layer of branching microvilli, up to 1μ in length, which project into the lumen of the acinus. Their cytoplasm contains numerous mitochondria and ribosomes, some of these latter being attached to the small amount of cytoplasmic membranes also present. Near the surface of many of these cells are found small vacuoles and scattered throughout the cytoplasm are numerous tubules or fibers. The nucleus is flattened with peripheral chromatin. The junctions between the cells are

Fig 28 Follicle cell surrounding oocyte in Fig 23. X 3,000.

Fig 29 Detail of oocyte follicle in Fig 23. X 20,000.

Fig 30 Pigment Wall (PW), folded basement membrane and lining of acinus of stage II hermaphrodite gland. X 3,000.

Fig 31 Detail of male nutritive cell cytoplasm showing partly digested spermatozoa heads. X 40,000.

B.M. = Basement membrane.

L = lumen of acinus.

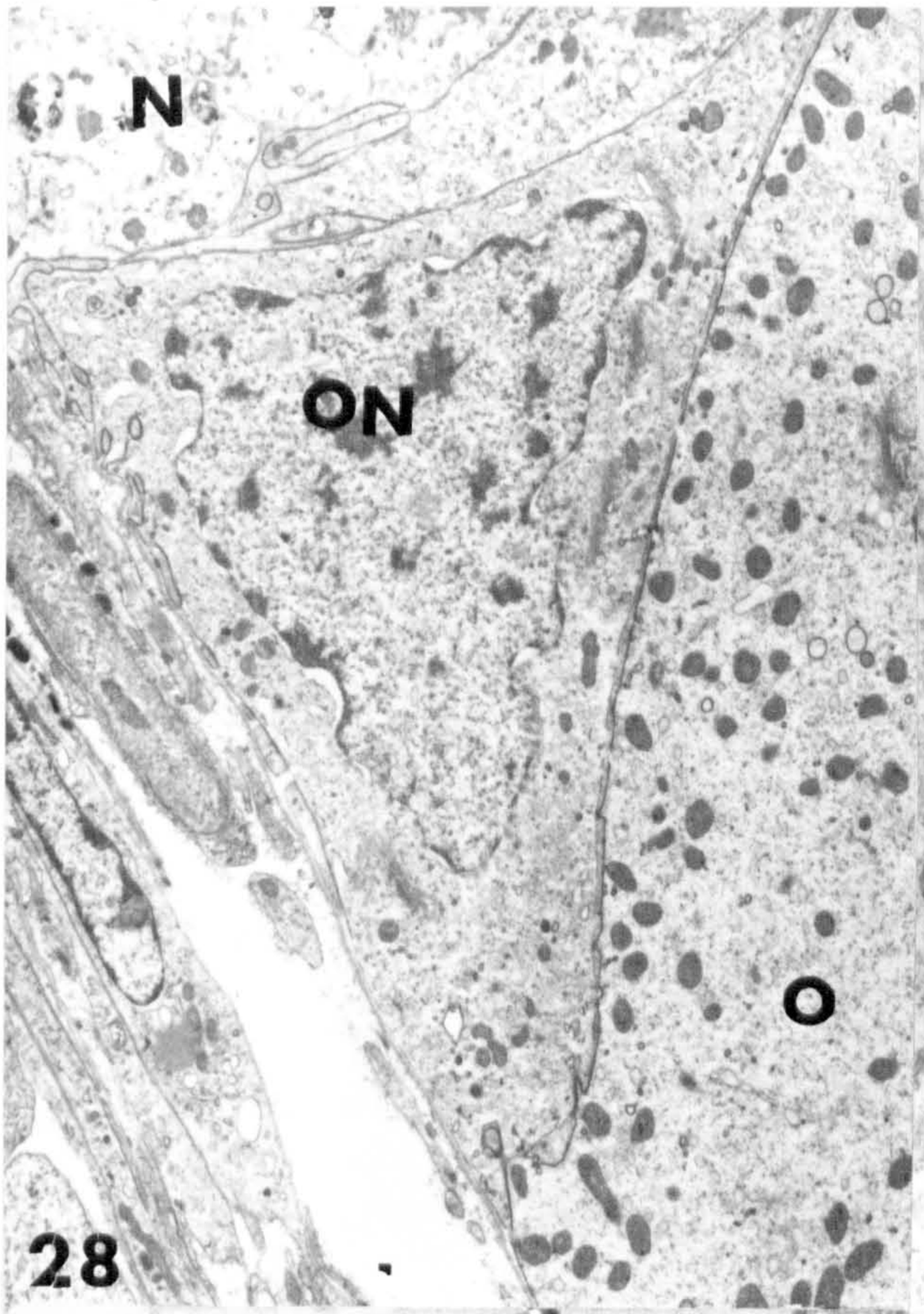
N = Male nutritive cell.

O = Oocyte cytoplasm.

ON = Oocyte nutritive cell cytoplasm.

PW = Pigment wall.

St = Spermatid.



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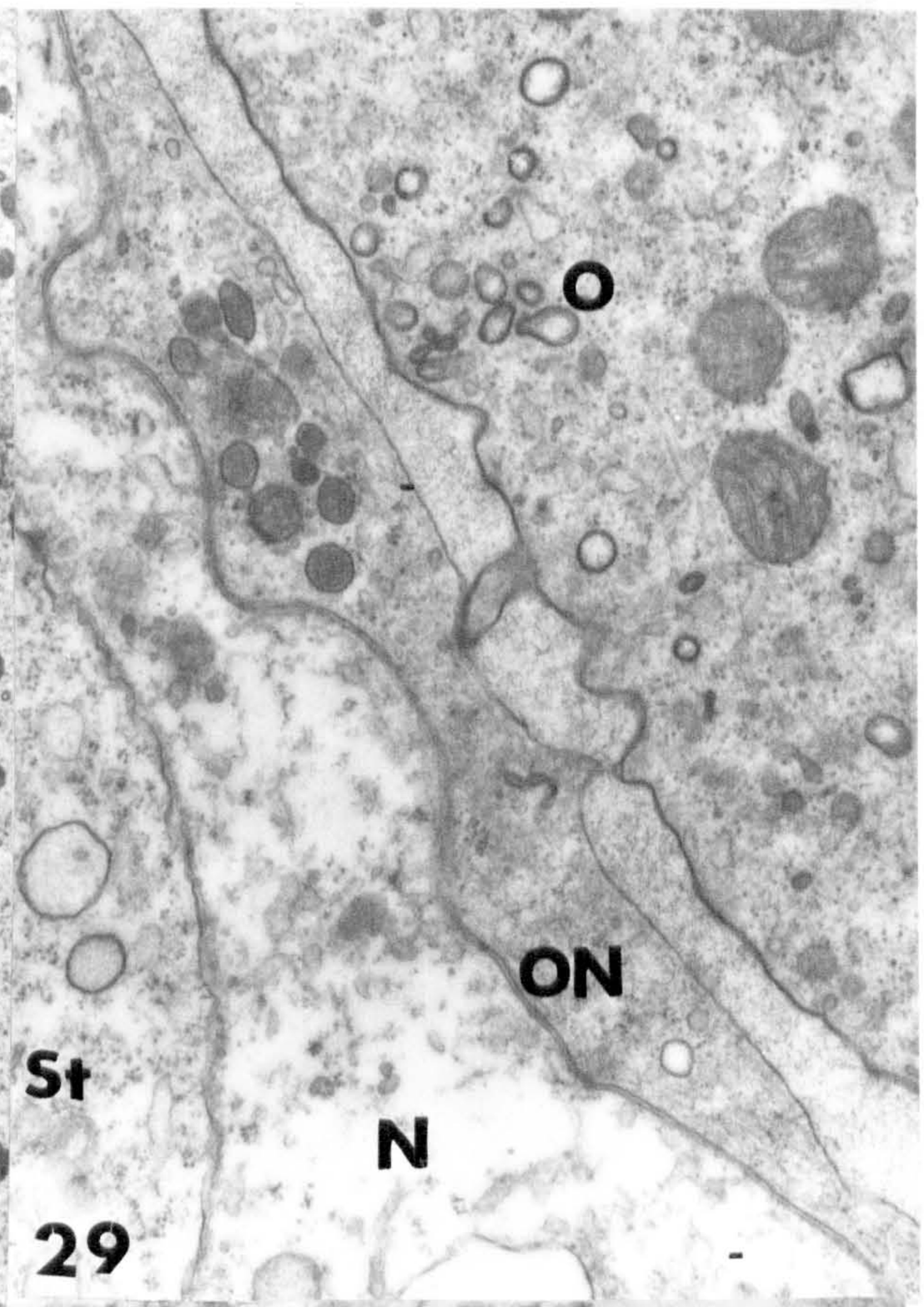

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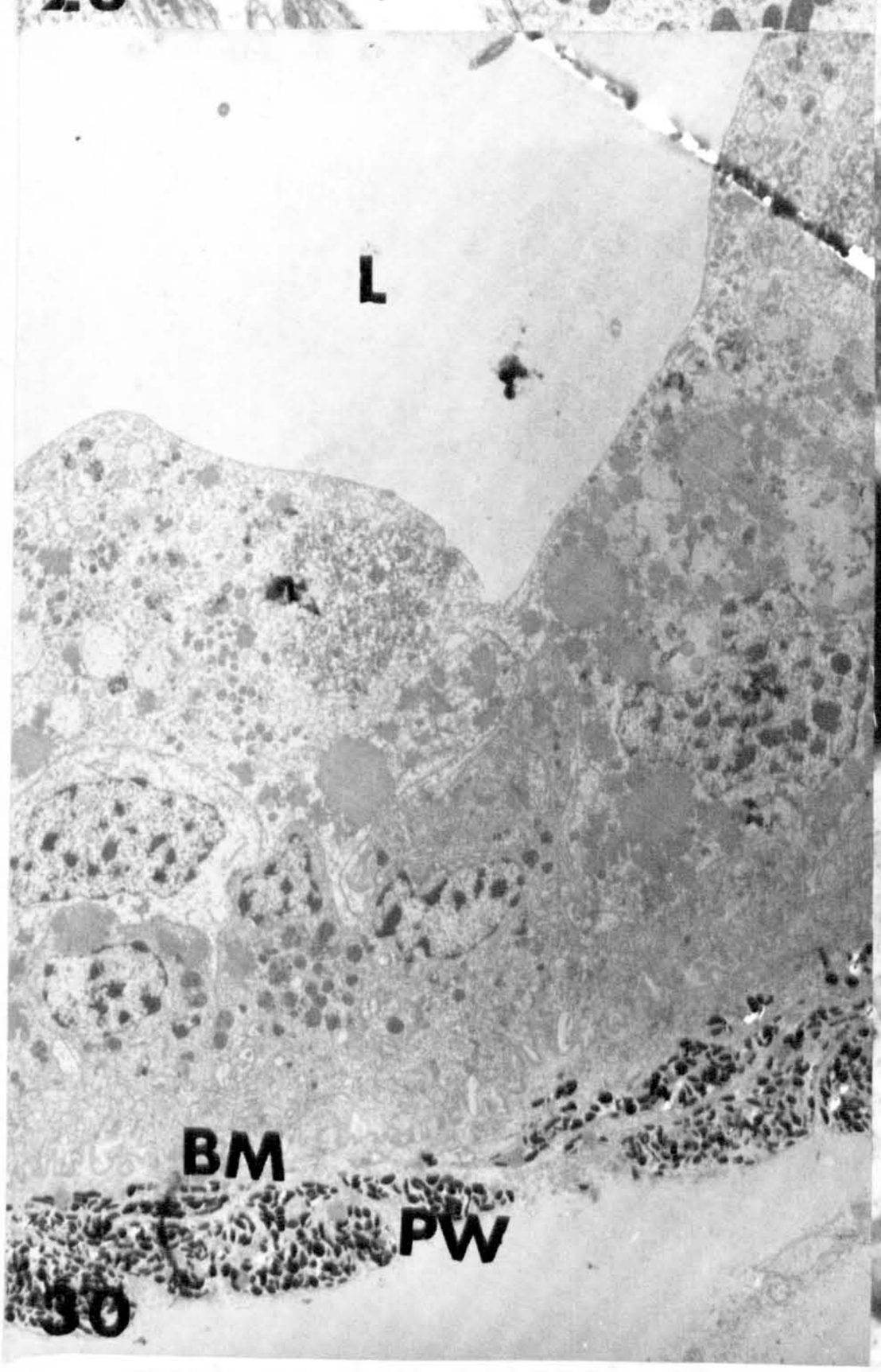

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convoluted and a large desmosome is present at the apex. At the bases of these cells can be found what appear to be processes from neighbouring cells so that there is a certain amount of interdigitation.

The origin of this epithelium is not clear. In some cases the old nutritive cells, whose cytoplasm is full of lipid droplets, appear to develop a surface layer of microvilli and at the same time bud off large droplets of cytoplasm (apocrine secretion) into the lumen of the acinus. Any germ cells which overlay this type of activity are pushed into the lumen of the acinus. This apocrine secretion and subsequent sloughing of the germinal cells is found in the two oocyte stages of hermaphrodite gland differentiation. Thus the nutritive cells normally lining ~~of~~ the late oocyte stage acinus could give rise to this epithelium.

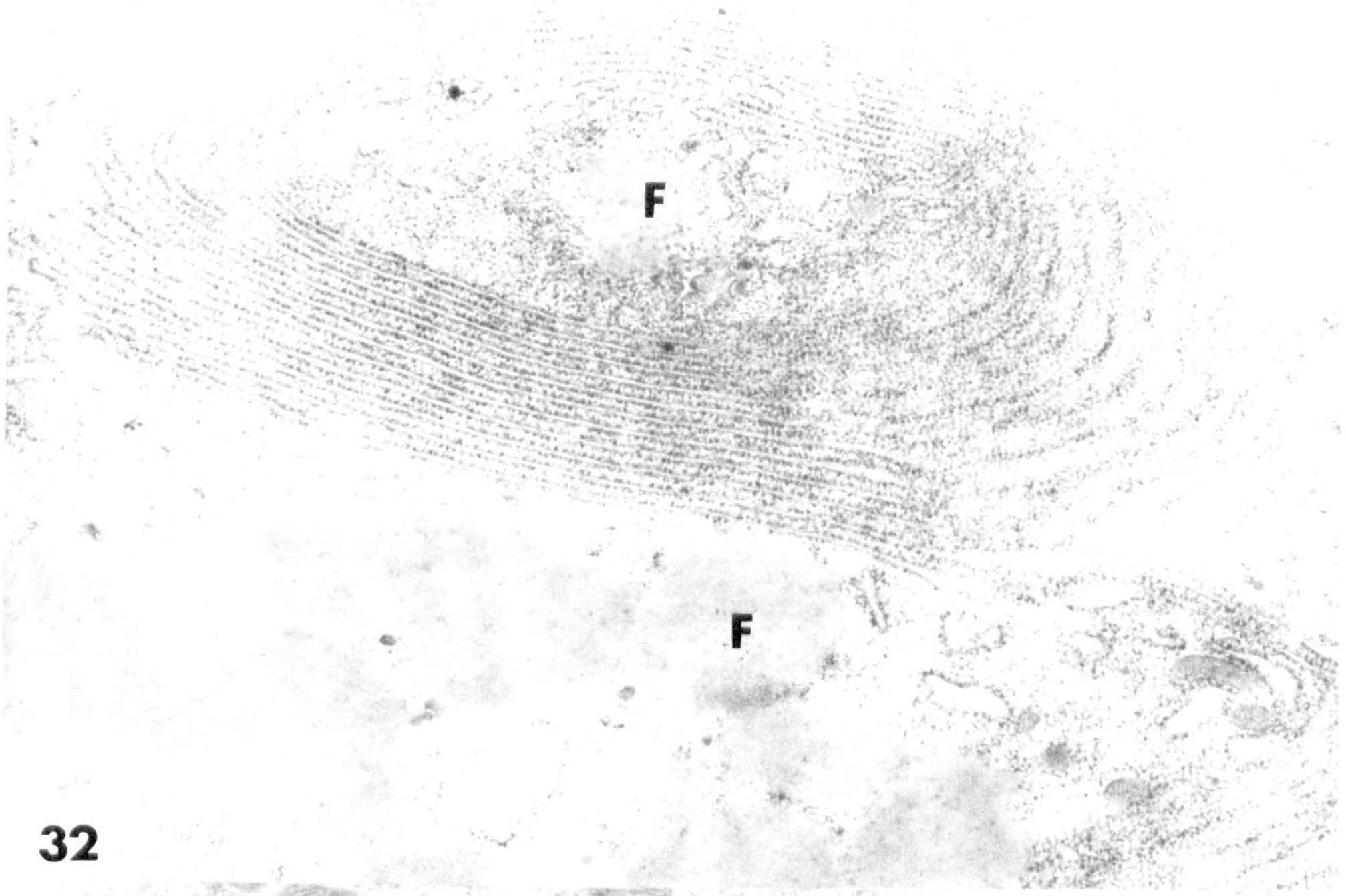
On the other hand, an epithelial layer is first seen in the acinus at the neck region (Fig 34). This epithelium is continuous with the lining of the hermaphrodite duct and very like it in appearance. The cells in this neck region differ from those in the post reproductive epithelium in that their cytoplasm appears more like that found in undifferentiated cells. There is an abundance of free ribosomes so that the cytoplasm appears more dense. It is the opinion of many authors that this neck region epithelium is germinative, giving rise to secondary

Fig 32 Concentric rings of ribosome covered reticulum from oocyte cytoplasm.

X 30,000.

Fig 33 Ribosome covered lamellae from oocyte cytoplasm. X 40,000.

F = Fat bodies.



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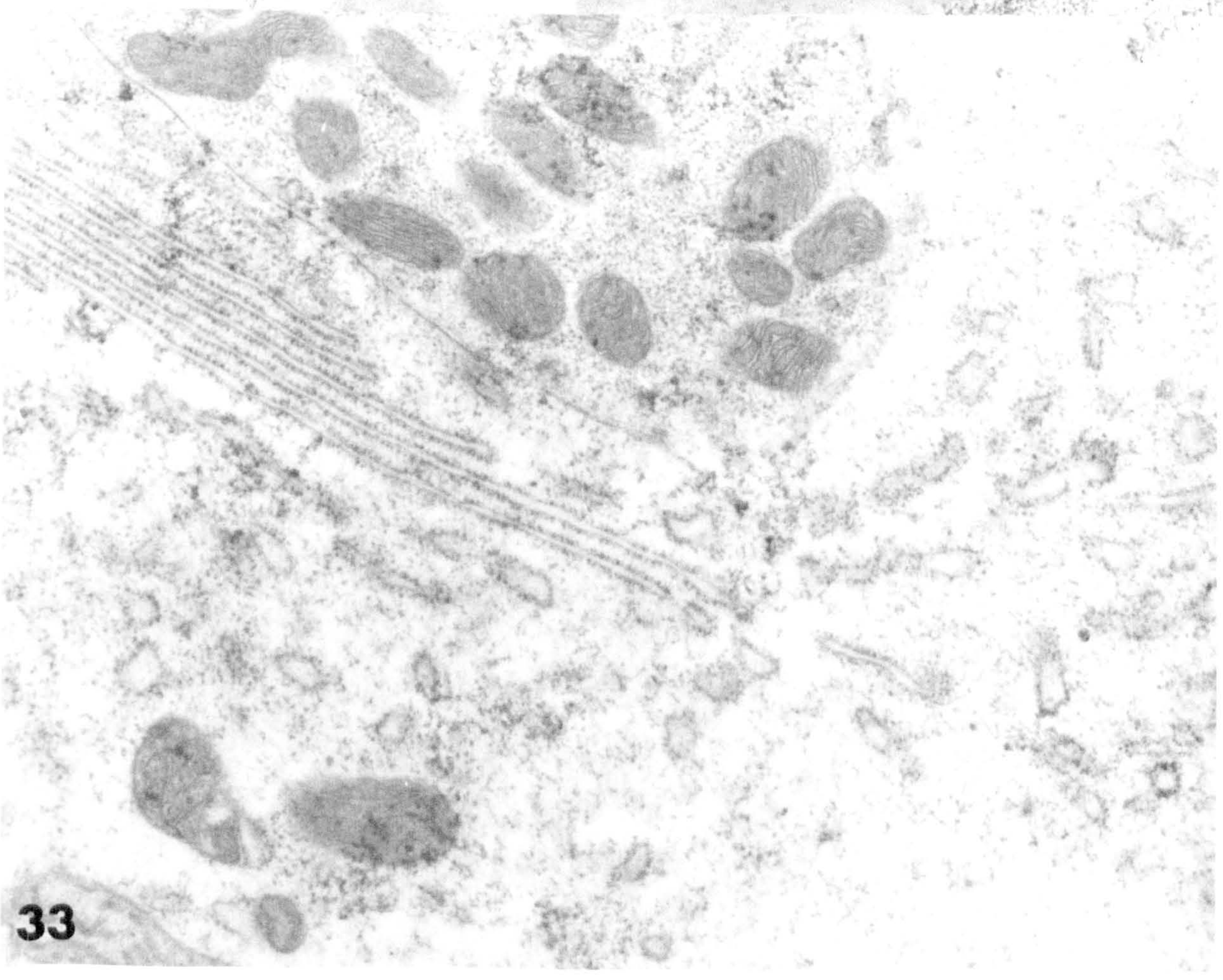

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Fig 34 Neck region of stage B hermaphrodite gland. X 2,000.

Fig 35 Epithelium of hermaphrodite duct. X 7,000.

Fig 36 Detail from fig 35. X 30,000.

A = Acinus.

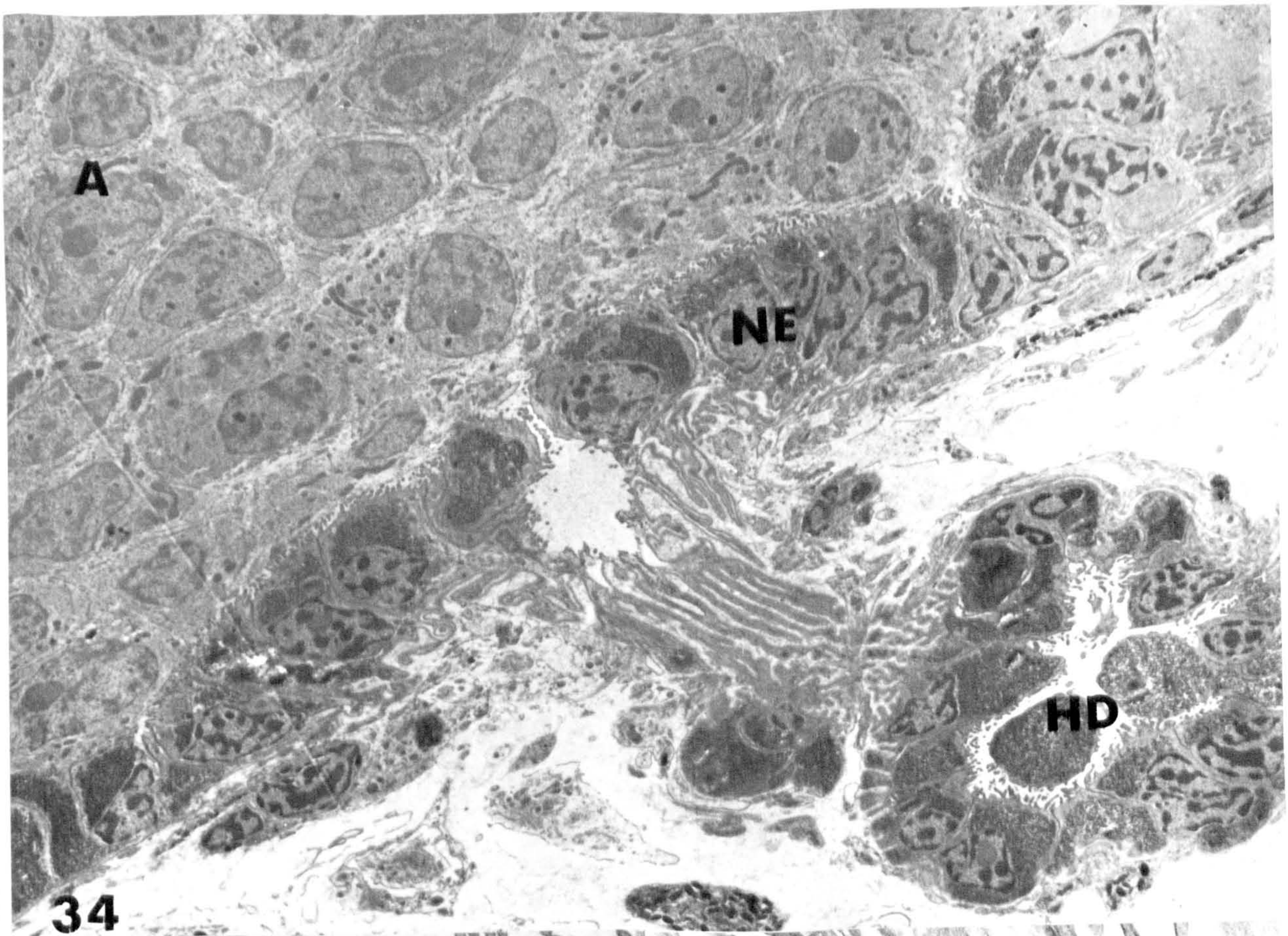
BM = Basement membrane.

Cil = Cilia.

D = Septate dermosome.

HD = Hermaphrodite ductule.

NE = Neck epithelium.



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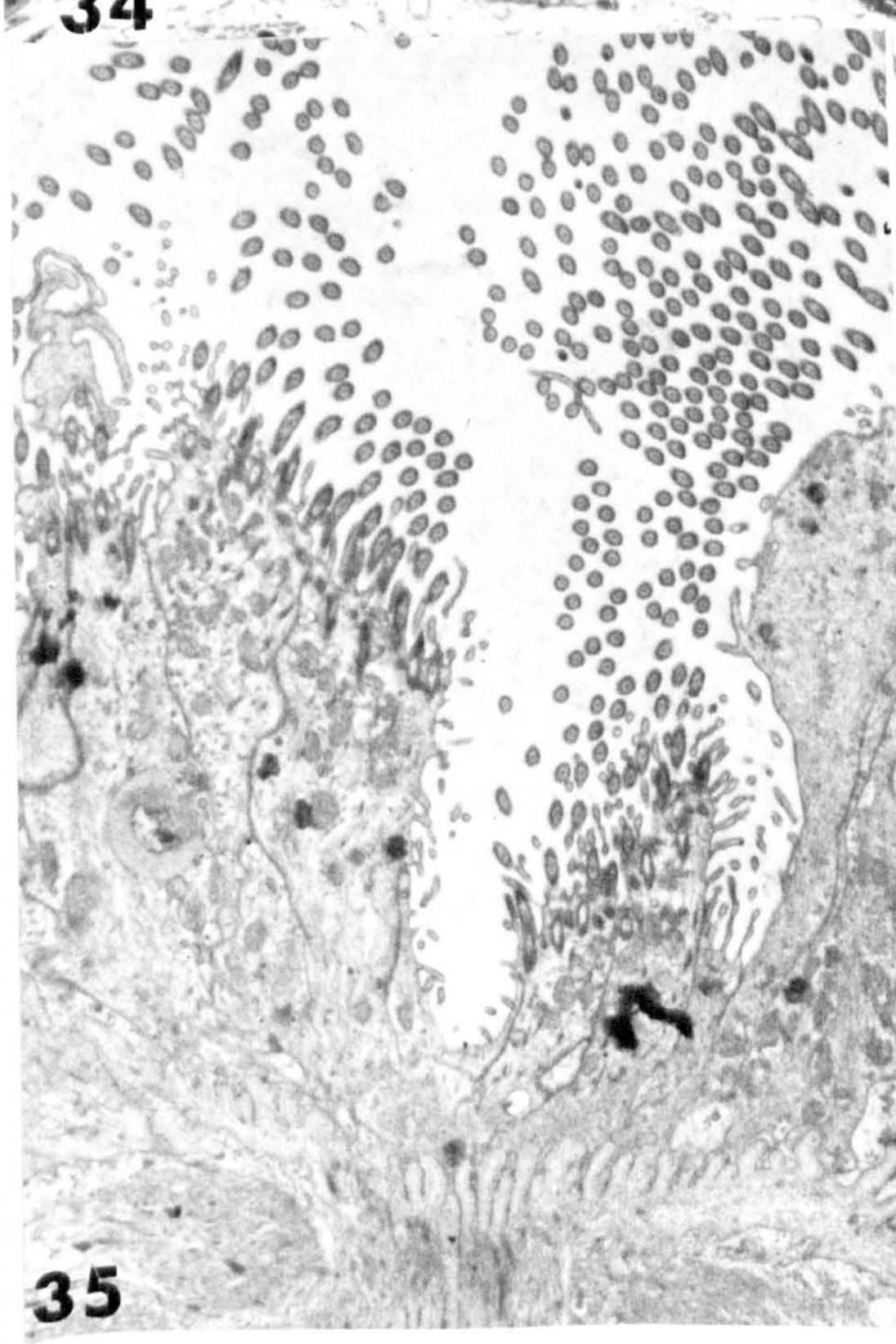

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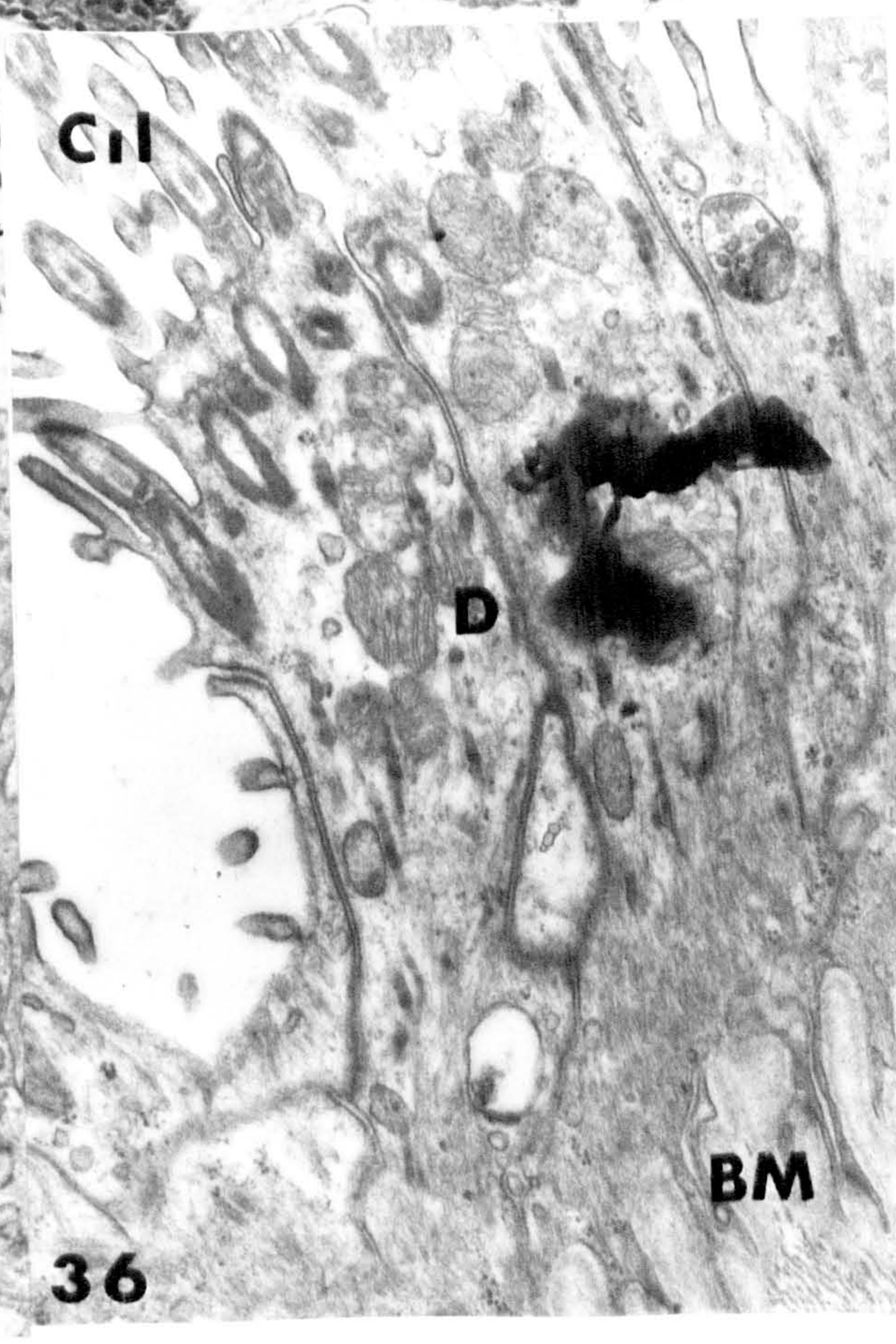

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development. Since this neck region epithelium is present to some extent in all later stages of the development of the hermaphrodite gland such an opinion would seem possible in this case. Thus it would appear that there are two epithelia, one appears early in development and is very limited, and the other is characteristic of late development where it is extensive. It may be that this latter epithelium is produced from the neck region epithelium.

Occasionally other cell types may be found in the acinus. These include mature ova and even young embryos. However, these were not studied in this work.

B.Hermaphrodite ductules (Figs 34, 35 and 36)

In this study only the small ductules (Runham 1970) which join the acinus to the hermaphrodite duct have been examined. Under the light microscope, these small ducts appear as simple ciliated tubes with a rather shrunken appearance. At the higher magnification of the electron microscope the structure of these ducts seem quite complicated. The wall of the duct consists of a collagen layer in which muscle cells and some nerve fibers are embedded, while on its inner face the wall bears a basement membrane. This structure is thus similar to that of the acinus wall but without pigment cells. The cells which line the wall of these ducts all bear microvilli and most of them also

bear cilia. These ciliated cells seem to be grouped to form ridges. Thus the thickness of the ductule wall may vary from 2 to 6μ in the same section. Their cytoplasm also contains many fibres which are usually arranged parallel with the cell surface. These cells are very similar to those found lining the neck region of the acinus, apart from the presence of cilia and fibres. Some of the thickened areas of the wall are simply due to contraction of the muscle cells which compress the cells lining the ductule. Septate desmosomes are found part way down the junctions between the cells (Fig 36) and the associated cytoplasmic fibres are arranged at right angles to the cell surface. Thus these ducts would seem capable of great expansion and this is indeed necessary to allow the mature oocytes to pass out of the acinus.

C. The common duct

The lumen of this duct is sub-divided along most of its length into male and female parts, being known as the prostate and oviducal glands respectively (Fig 40). Studies on the structure and development of this region have been made by Filhol (1938), Martoja (1964) and more recently by Runham and Laryea (1968) on Agriolimax reticulatus. Quattrini (1966a,b,1967) has made a study of the structure and ultrastructure of the mature prostate of a variety of slugs. During this present

description the nomenclature of Runham & Laryea (1968) will again be used.

In newly hatched and very young animals, the common duct has a very simple form. This undifferentiated stage is not distinguished by Runham & Laryea. It consists of an epithelium overlying a thin layer of connective tissue and undifferentiated cells (Fig 37). The whole duct is in the form of a simple rounded tube. Since this stage was not suitable for removing from the animal to form explants, this brief description will not be expanded.

The first stage recognised by Runham and Laryea, and the first stage which may be dissected from the animal satisfactorily, is the Differentiation stage A (Figs 38 & 39). This stage is a long lasting one since it was present in the majority of small animals studied. The duct enlarges slowly, the lumen becoming flattened and crescent shaped. From one end of this crescent shaped lumen, small finger-like diverticulae grow into the wall of the duct. This part of the lumen will be the male groove and the part of the wall containing the diverticulae will become the prostate gland. Many cell divisions can be seen in the epithelial lining of these diverticulae. The diverticulae consist of an epithelial lining bearing microvilli only, and an indefinite number of undifferentiated

Fig 37 Undifferentiated Common duct. X 1500.

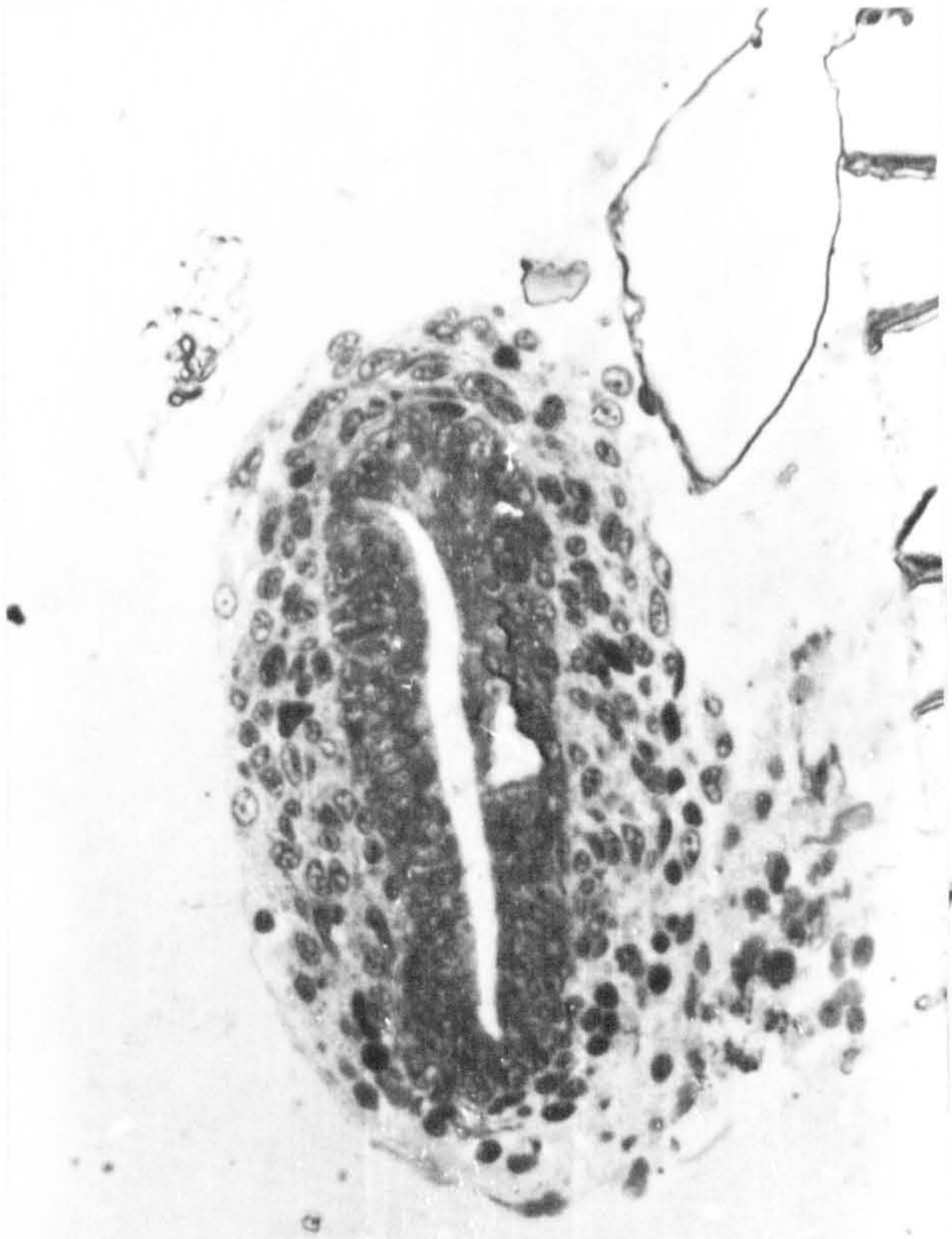
Fig 38 Early stage A common duct. X 800.

Fig 39 Late stage A common duct. X 500.

Fig 40 Stage D common duct. X 50.

O = Oviducal gland.

P = Prostate gland.



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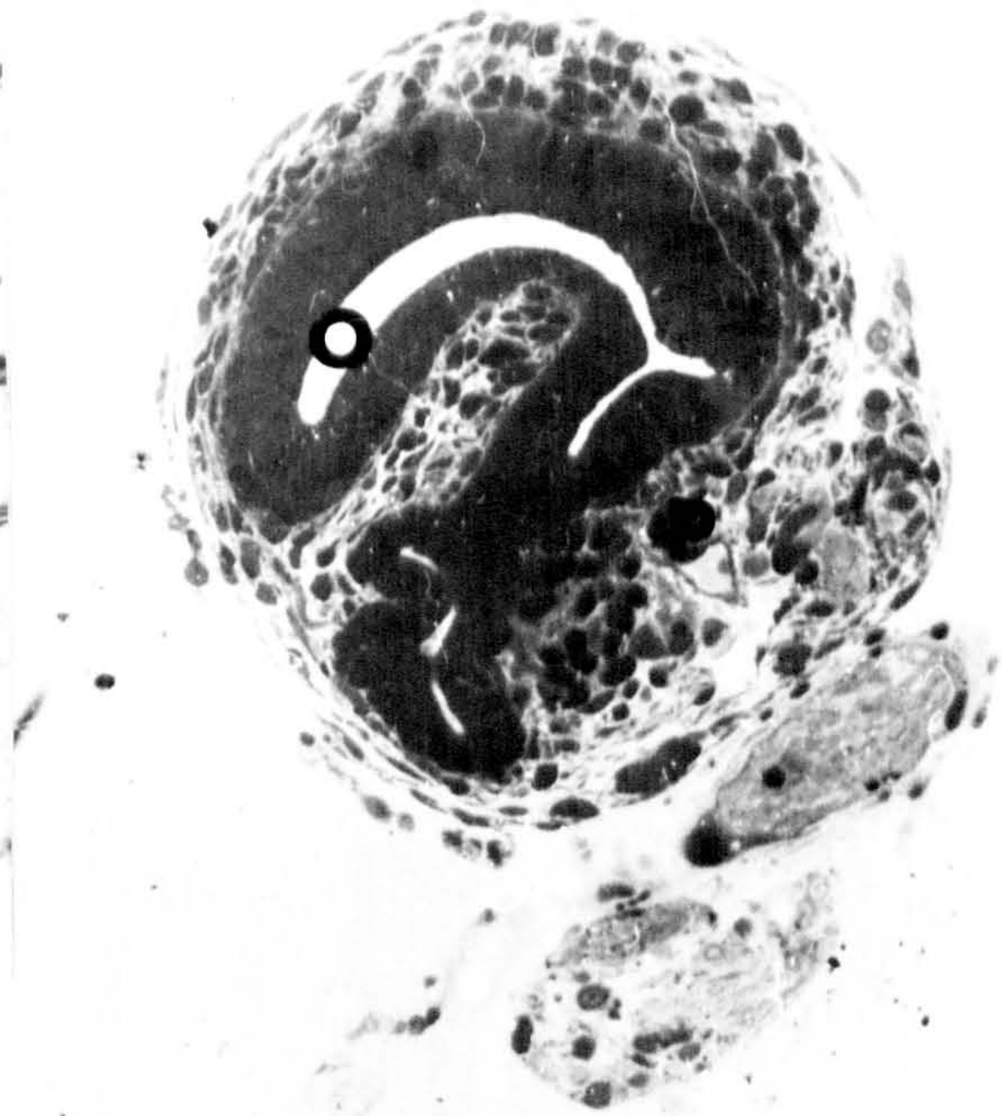

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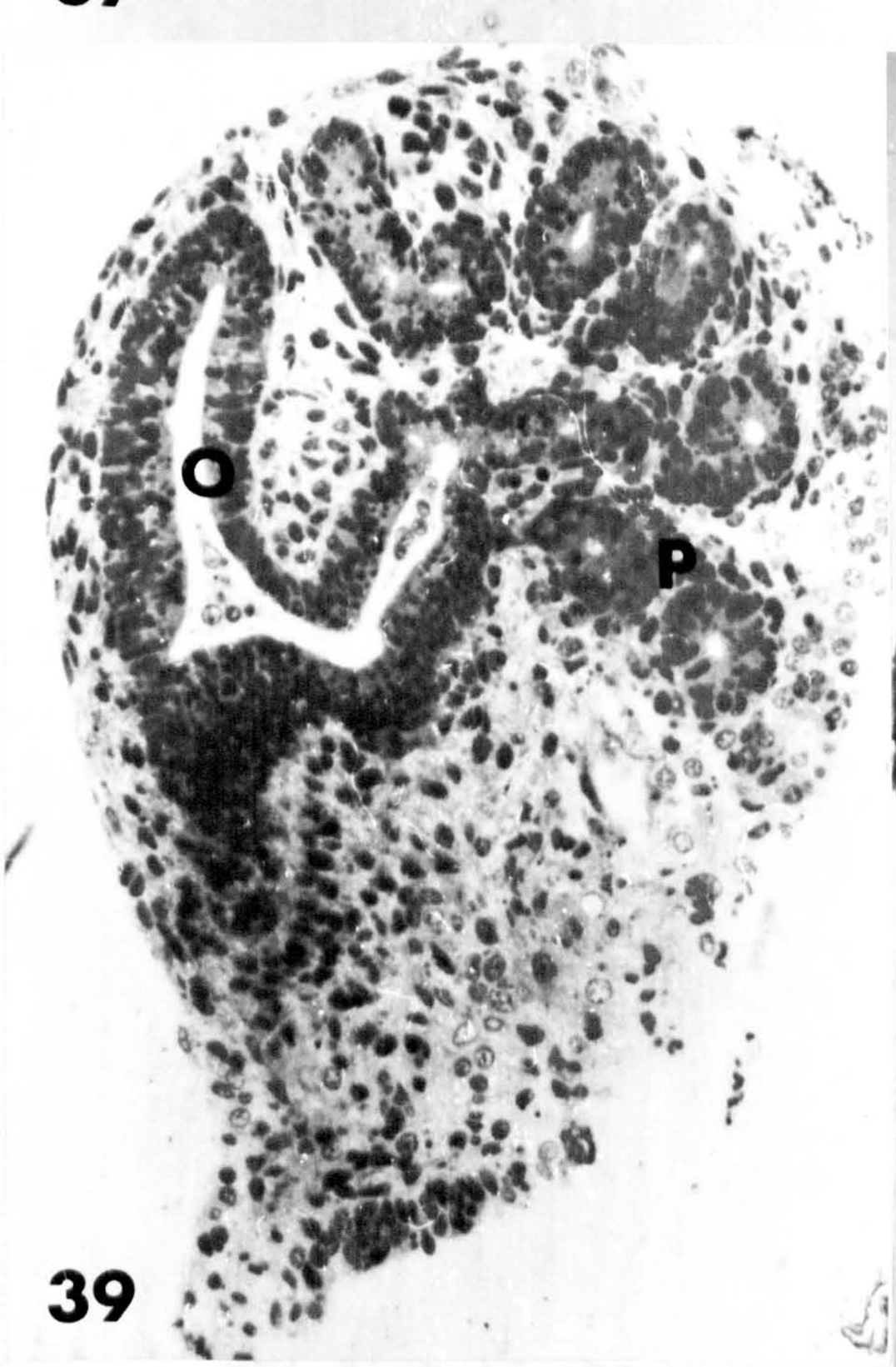

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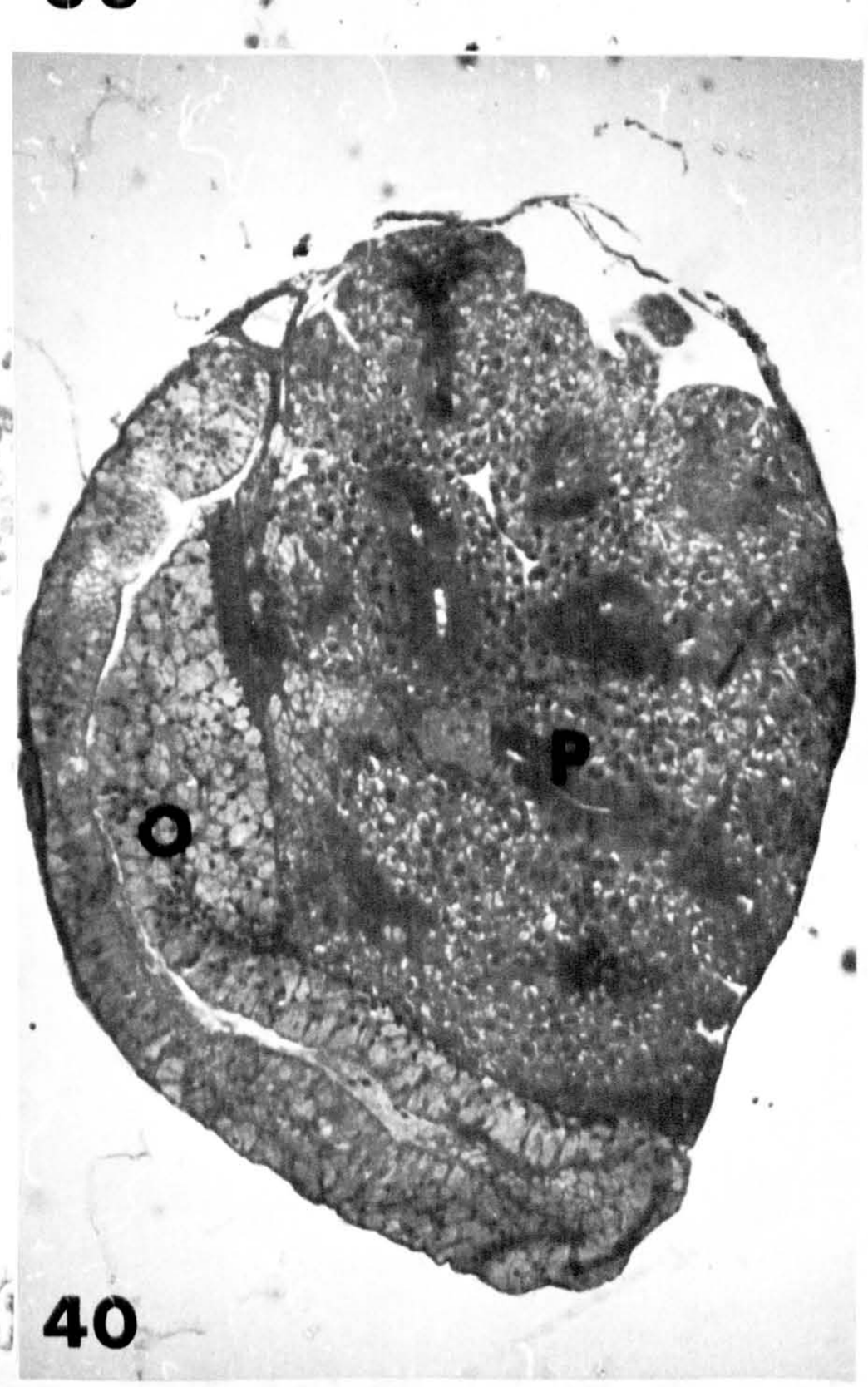


40

cells beneath. The whole structure is contained within a thin layer of connective tissue.

Ultrastructurally, the differentiating prostate is uncomplicated at this stage (Figs 43 & 44). Cells are typically undifferentiated in appearance, and with the exception of the connective tissue cells, can only be distinguished by their position and general shape. Cells which are spherical measure 10μ diameter and are very like spermatogonia in appearance. The nucleus is large in relation to the size of the cell. One or two nucleoli and granular chromatin are present, except when dividing. The cytoplasm is typical of undifferentiated cells and contains, mitochondria, microtubules, golgi bodies, rough endoplasmic reticulum, and many free ribosomes.

Between these undifferentiated elements are various connective tissue cells and processes which form a loose matrix with no apparent pattern.

The diverticulae in this differentiating prostate consist of a single layered epithelium. The cells have microvilli at their luminar surface otherwise they are identical in structure to those undifferentiated cells already described. The male groove is similar in appearance to these diverticulae but its development tends to be a little more advanced.

The part of the common duct destined to become the oviducal gland remains undifferentiated at this time.

Fig 41 Undifferentiated Wall of common duct.

X 6,000.

Fig 42 Undifferentiated cell from SL layer.

X 20,000.

Col = Collagen.

CT = Connective tissue.

Ep = Epithelium.

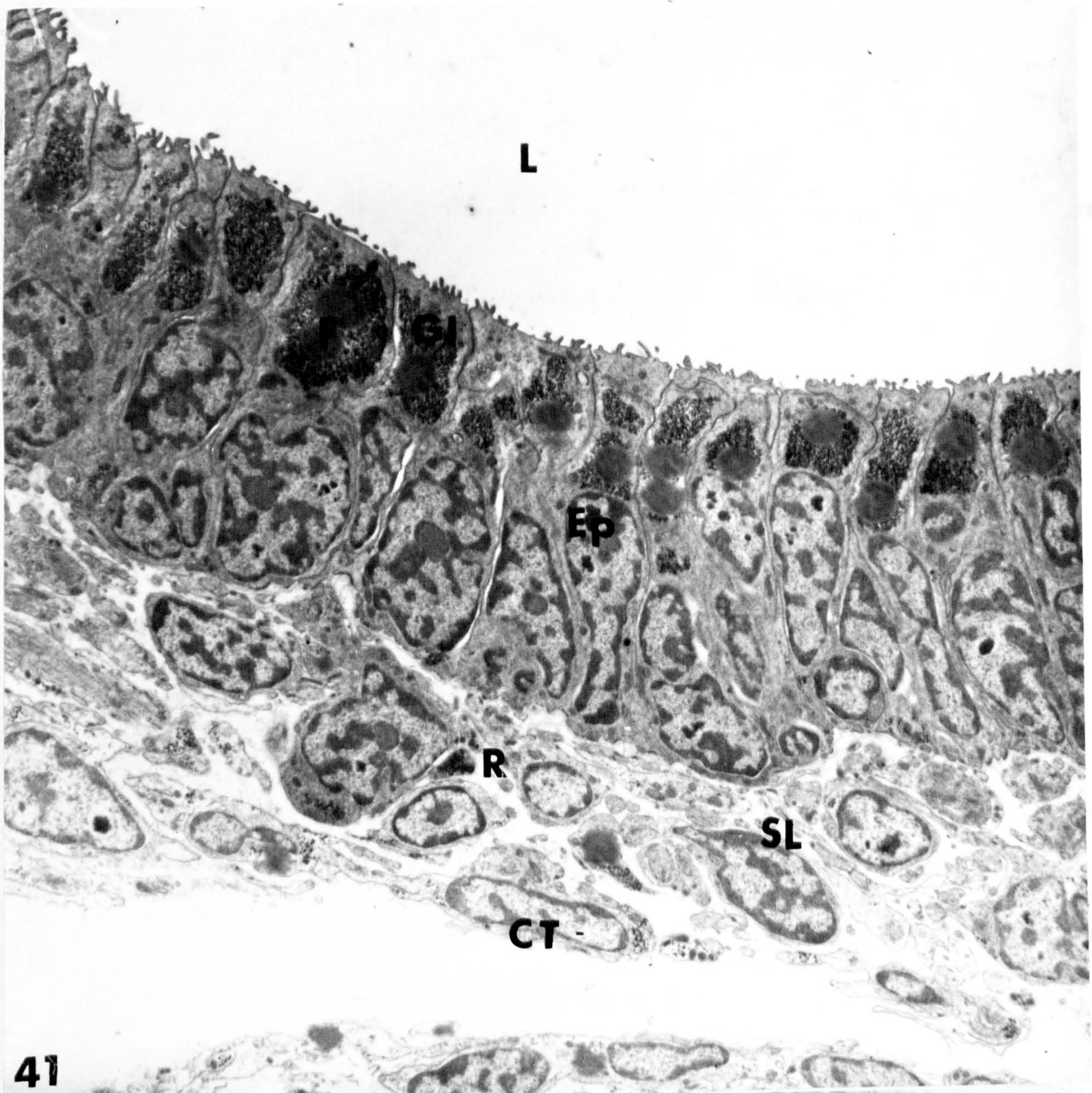
F = Fat globules.

Gl = Glycogen deposits.

L = Lumen.

Pig = Pigment.

SL = Secretory Layer.



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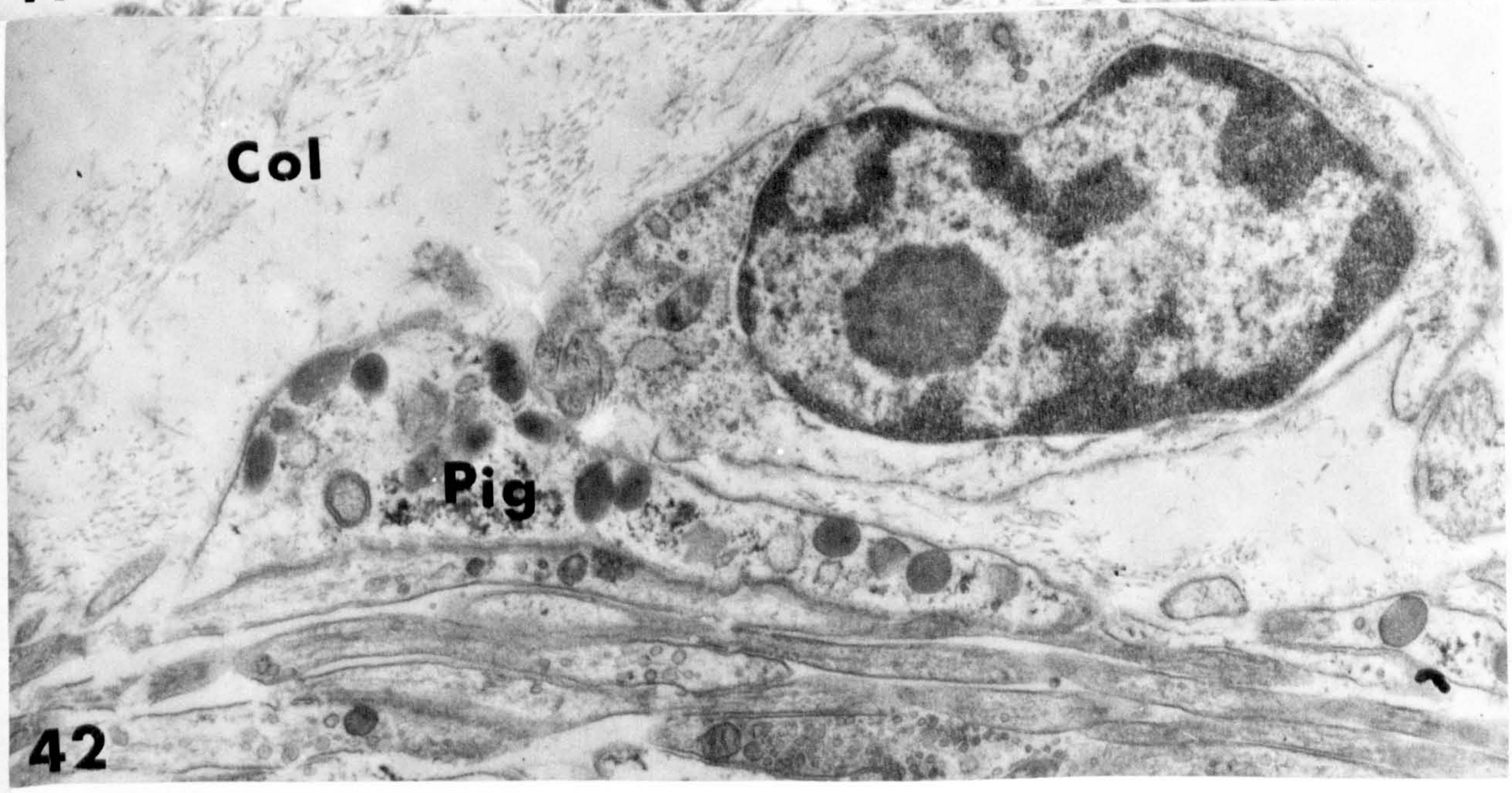

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As the diverticulae of the prostate develop, the secretory cells begin to differentiate. These cells probably move by amoeboid movement since they bear cytoplasmic processes at this time. When positioned beneath the epithelium they send up processes, about 1μ in diameter, between the epithelial cells to the luminar surface of the diverticulum. These processes terminate in microvilli. At this time the cilia are first seen in the prostate, developing first on the epithelium of the male groove and then further up the diverticulae (Fig 43).

The connective tissue (Fig 42) consists of a matrix of collagen fibers in which muscle cells, pigment cells, nerve axons and blood spaces are to be found. This connective tissue is restricted mainly to the bases of the secretory cells. In this way the diverticulae become isolated into partially separate finger-like processes. With the development of this ciliated epithelium, the secretory layer and the connective tissue, the prostate takes on its characteristic appearance.

Prior to the start of male secretion, the male secretory cells form a layer about two cells deep beneath the ciliated epithelium, with the necks passing to the surface of the lumen (Fig 45). The secretory cell nuclei are very large at this time, almost filling the cell 'body'. These nuclei contain many small nucleoli and granular chromatin. Immediately around the nucleus

Fig 43 Transverse section of a diverticulum in the prostate gland at early stage A. X 2,000.

Fig 44 Longitudinal section of fig 43.

Fig 45 Part of diverticulum wall of prostate at late stage A showing neck (Nk) of secretory cells (SC) passing between the Epithelial cells (EpC) to the Lumen (L) of the diverticulum.

X 6,000.

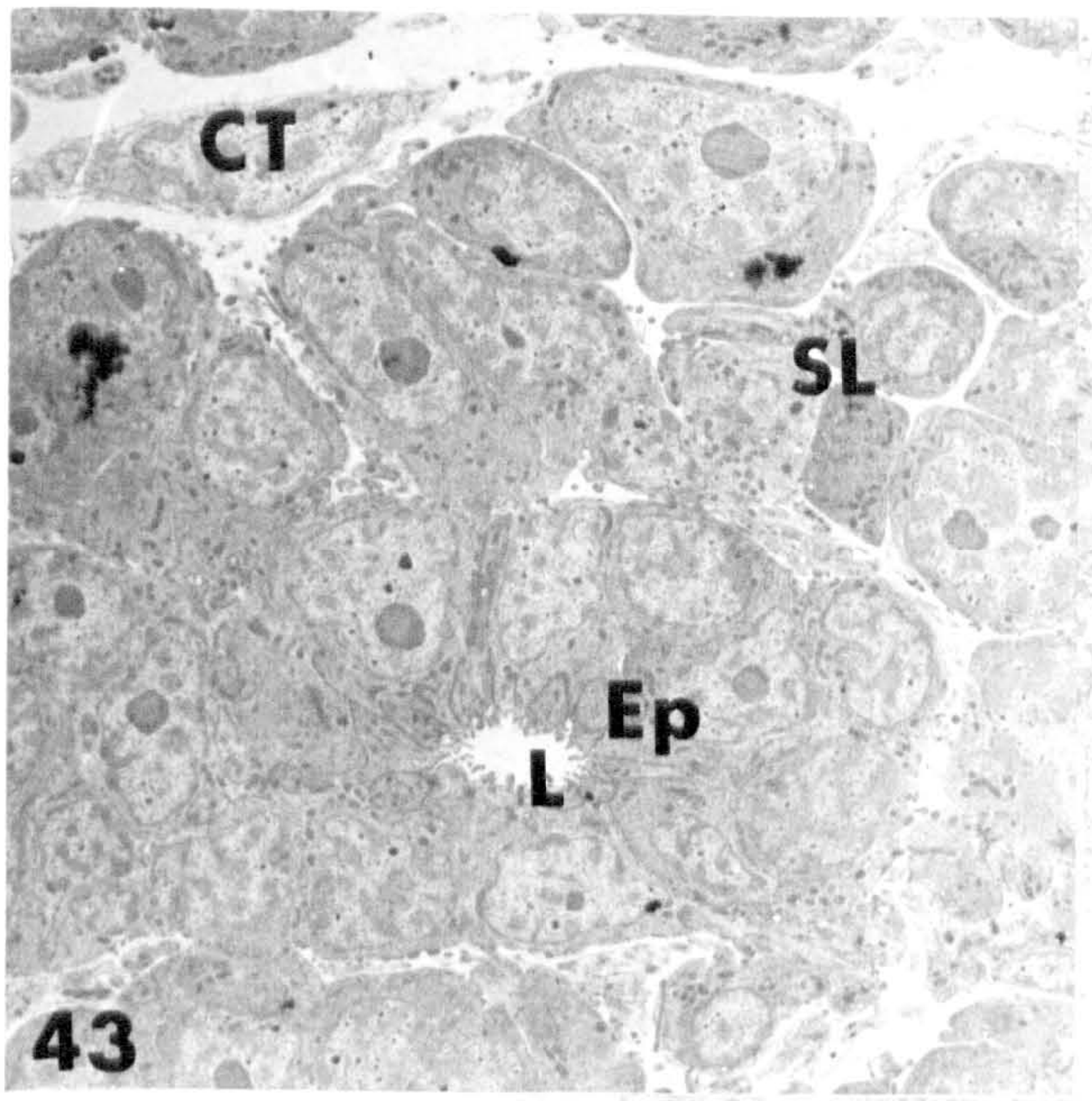
CT = connective tissue.

Ep = Epithelial layer.

L = Lumen.

N = Nucleus.

SL = Secretory cell layer.



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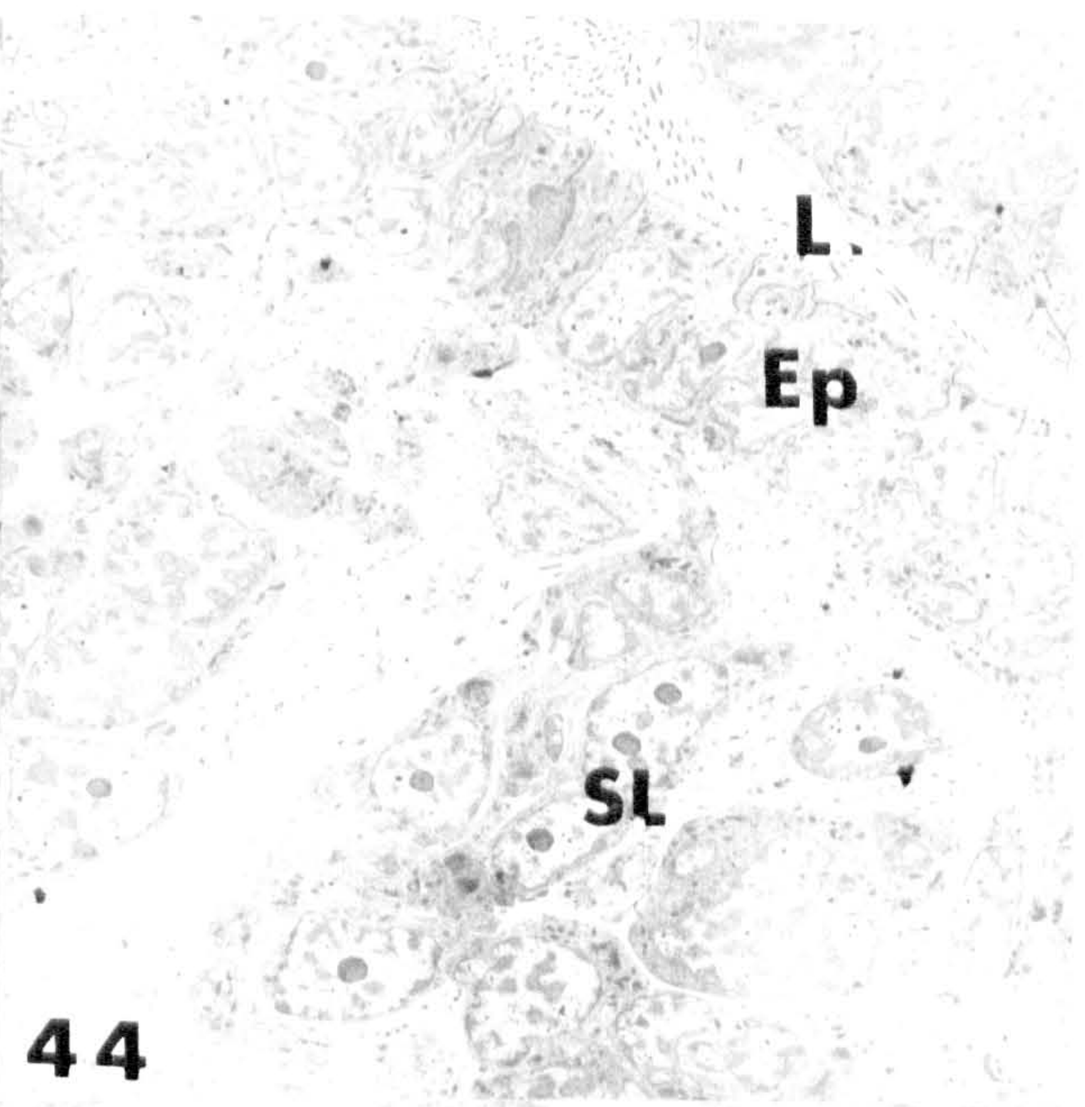

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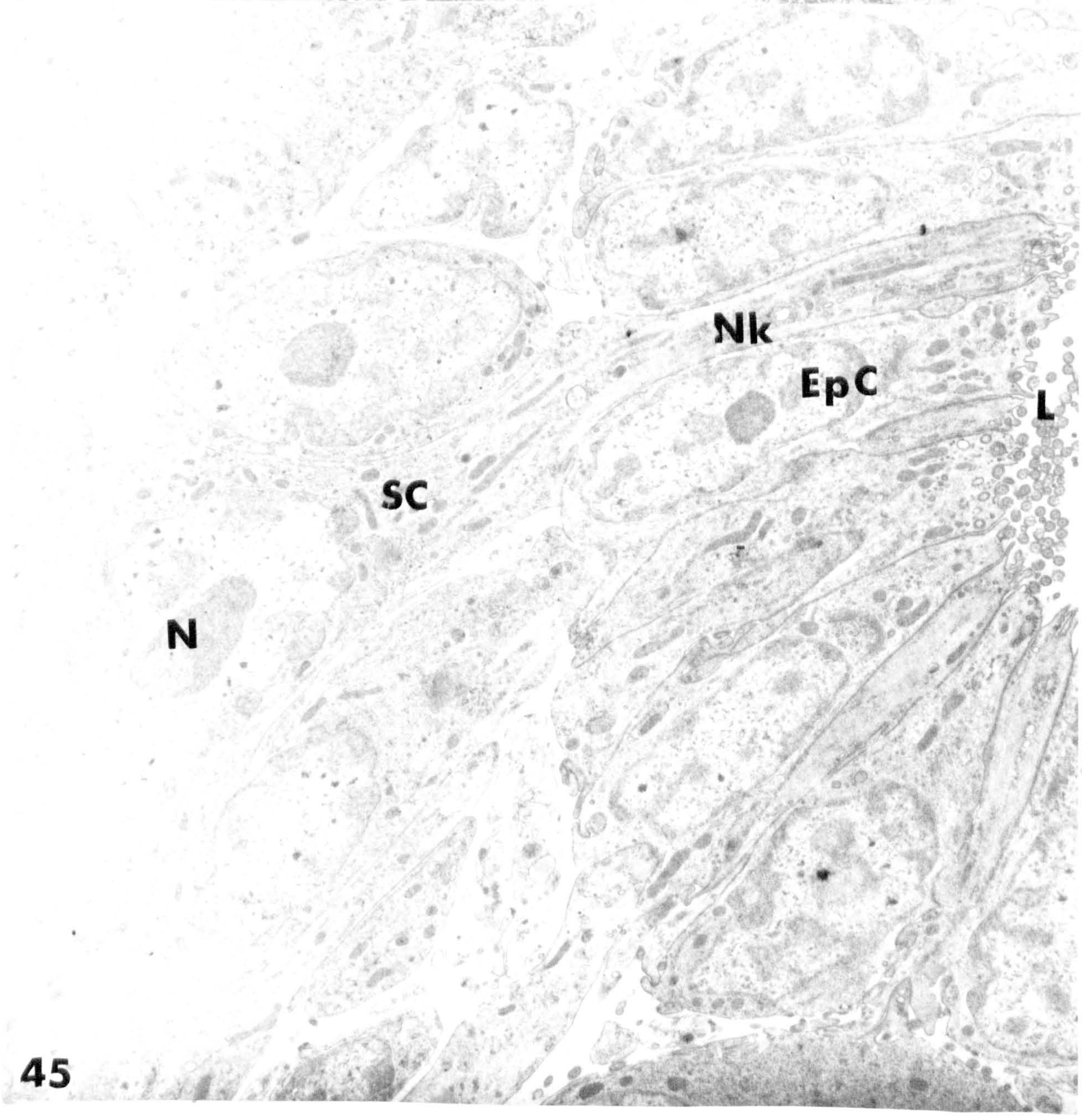

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the cytoplasm contains a mass of ribosomes, some of which are attached to the perinuclear membrane and others to branches from it. Mitochondria are also present. At the apex of the cell body, beneath the base of the neck of the cell, the cytoplasm is rather more complicated. In addition to the mitochondria, ribosomes and membranes like those surrounding the nucleus, several golgi bodies are to be found with vesicles surrounding them. From this region, 200Å dia cytoplasmic fibers or microtubules pass up the neck of the cell to its apex terminating near the bases of the microvilli. In this neck region, the cytoplasm is similar to that of the cell body but the mitochondria are oriented along its length. More vesicles are found and these tend to be larger than those in the vicinity of the golgi bodies. There are also multivesicular bodies present in this region.

At the same time as the differentiation of the secretory cells in the prostate, the oviducal gland begins to differentiate. The number of cells in the epitholium increases and these cells become very columnar in shape (Fig 46). Their contents is very similar to that found in the cells of the prostate epithelium but a mass of glycogen-like granules with some lipid droplets is often found just apical to the nucleus (Fig 41).

Fig 46 Wall of oviducal gland at stage B.

X 6,000.

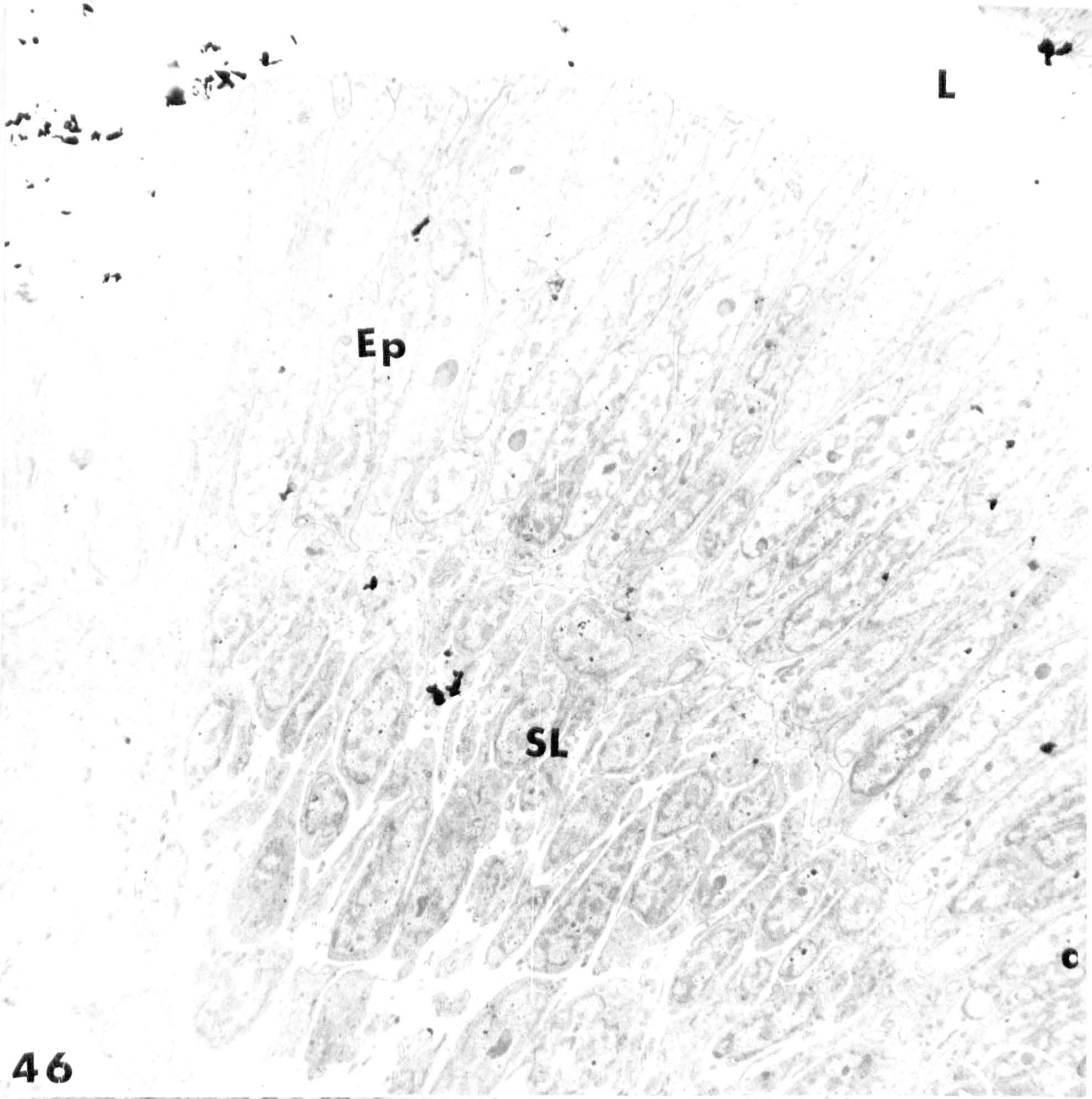
Fig 47 Detail of surface of epithelial cells above. X 16,000.

Ep = Epithelial layer.

L = lumen.

mv = microvilli.

SL = Secretory layer.



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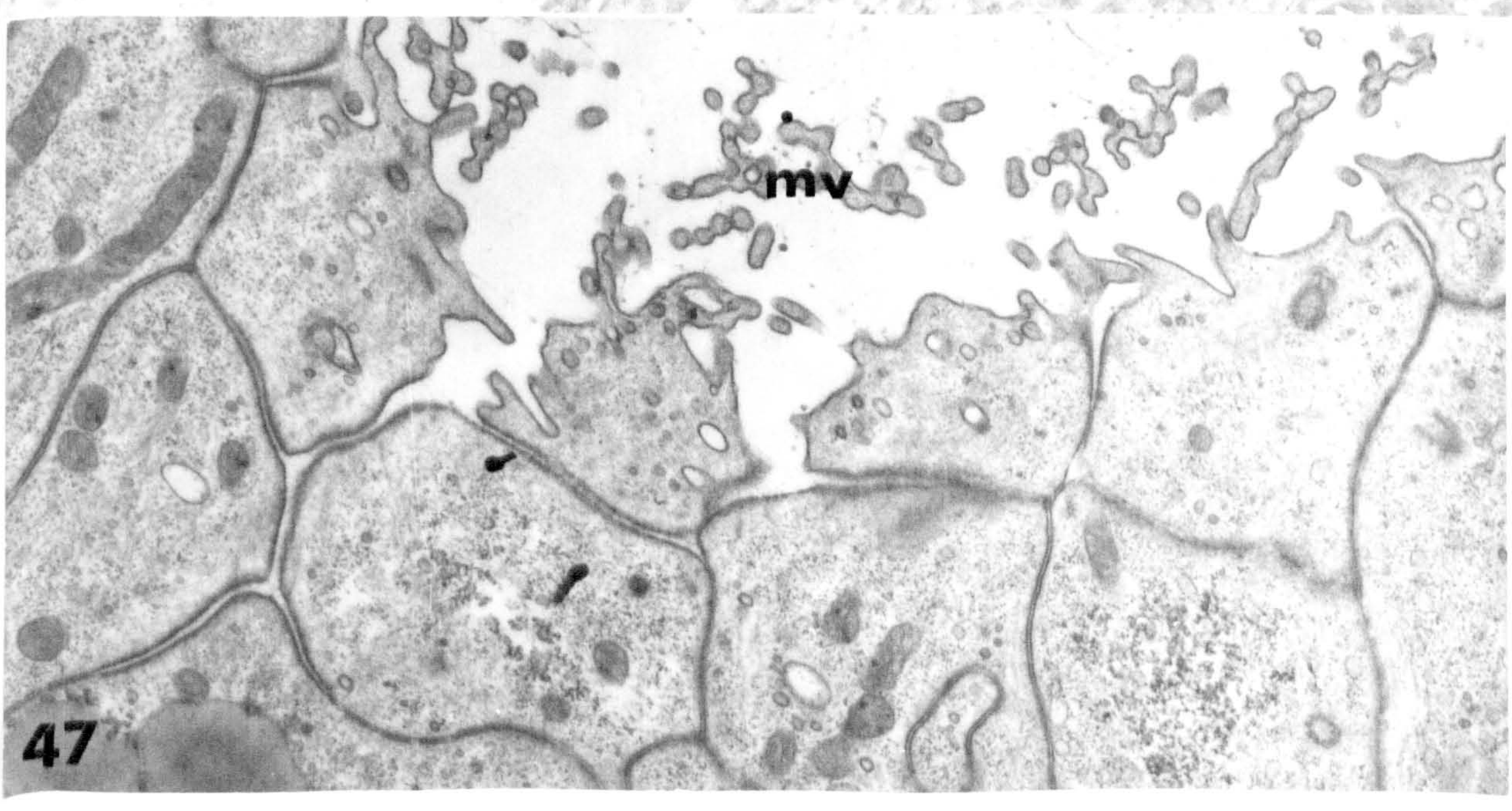

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Stages in mitosis may be seen in this layer.

The underlying tissue layer consists of a loose mass of collagen fibers in which are embedded differentiating smooth muscle cells and a small number of undifferentiated cells. The nucleus of the differentiating smooth muscle cell is often lobed, with a dense peripheral chromatin pattern and it contains a large nucleolus. The cytoplasm characteristically contains a number of swollen vesicles bounded by ribosome coated membranes, possibly specialised endoplasmic reticulum. In addition to these vesicles, the cytoplasm contains muscle fibrils, numerous mitochondria, masses of free ribosomes, normal rough endoplasmic reticulum, some lipid deposits and other vesicles. There is apparently no golgi body. Cells with similar cytoplasmic organelles, but lacking any muscle fibrils, probably represent earlier stages in the differentiation of the smooth muscle cells. The undifferentiated cells are to be found throughout this connective tissue layer but only in small numbers. There is a tendency for them to be concentrated near the bases of the columnar epithelial layer. The nuclei of these cells are very similar to those of the epithelium, That is, they contain a nucleolus, peripherally arranged chromatin and an irregular nuclear membrane. The nucleus fills most of the cell and the amount of cytoplasm is limited. As in other

undifferentiated cells the cytoplasm contains numerous ribosomes, a few mitochondria, rough endoplasmic reticulum and the occasional golgi body.

Cells with oval shaped granules are also present in this connective tissue, although not in great numbers (Fig 42). They are probably a type of pigment cell since the granules they contain are very similar to those found in the pigment cells in the wall of the hermaphrodite gland acini.

The start of male secretion, stage B. The male diverticulae are now fully developed and the secretory cells begin to accumulate secretion.

There are at least 3 types of secretion that can be distinguished with azan staining, and probably many more could be distinguished using histochemical techniques.

Type 1. Situated mainly in the bases of the diverticulae in the upper part of the tract, these cells contain large blue or yellow staining granules. These different staining granules may be restricted to one cell or they may both occur in the same cell.

Type 2. Occurring mainly in the tips of the diverticulae are cells containing red staining granules, often with a dense central region to them. These cells are reduced in number towards the lower end of the tract.

Type 3. Concentrated in the lower part of the tract are cells

Fig 48 Stage B male groove showing secretory granules. X 3,000.

Fig 49 Detail from Fig 48. X 15,000.

cil = cilia.

Ep = Epithelial cell.

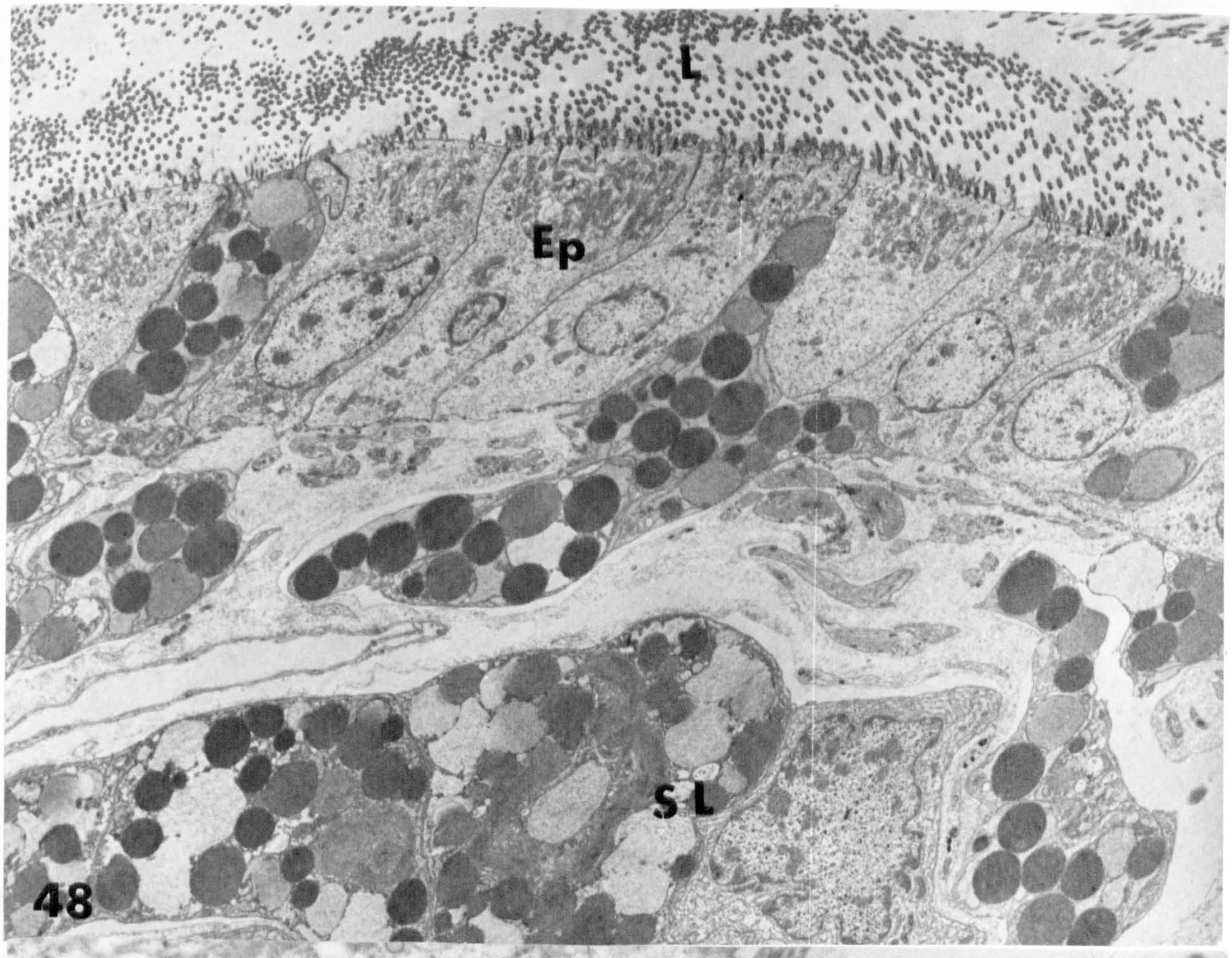
L = lumen.

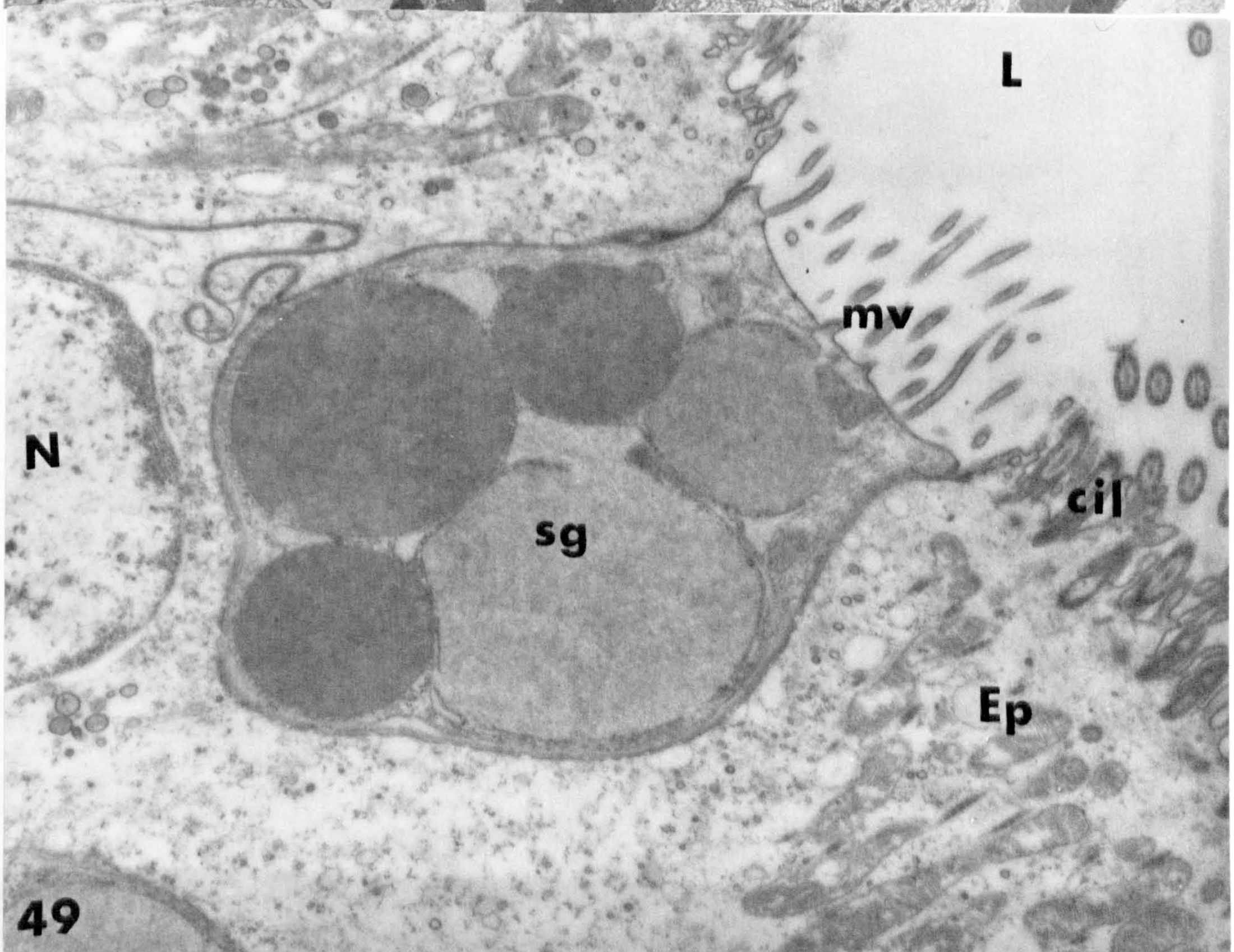
mv = Microvilli.

N = Nucleus.

SL = Secretory layer.

sg = secretory granule.


L
Ep
SL
48


L
mv
N
cil
sg
Ep
49

which contain a blue staining material, which is sometimes granular in appearance although more normally non-granular. In the upper part of the tract, immediately beneath the ciliated epithelium of the male groove, there are some cells containing a similar type of blue staining secretion which may or may not be homologous with this third type of secretion. The cilia lining the male groove are much longer than those lining the diverticulae of the prostate.

Using the electron microscope it is only possible to distinguish two types of secretion and two types of secretory cell, together with a few mucocytes (Fig 55).

The first type of secretory cell (Fig 50) corresponds to type two as seen with the light microscope. The cytoplasm develops masses of swollen endoplasmic reticulum (Fig 52). This is restricted, in the main, to the basal part of the cell. Towards the apex of the cell, at the base of its neck, the cytoplasm contains a large number of golgi bodies. Vesicles, with clear contents, are produced in the region of the endoplasmic reticulum. The secretory granules first appear at the apex of the neck of the cell, accumulating to fill the neck region, and finally the apical part of the cell body. It would appear that the secretory granules from the golgi bodies pass up the neck and then fuse with vesicles from the endoplasmic reticulum to form the cored granules (Fig 51 etc.). These granules are not found in any region of the cell below

Fig 50 Diverticulum of stage B prostate.

X 2,000.

Fig 51 Part of type 2 secretory cell.

X 5,000.

Fig 53 Detail from fig 51. X 15,000.

ER = Endoplasmic reticulum.

G = Golgi bodies.

ILS = Inter lobular space.

L = Lumen of diverticulum.

Mu = Mucocyte.

N = Nucleus.

S = Type 1 secretion.

Sg = Type II secretion.

Sv = secretory vesicle.

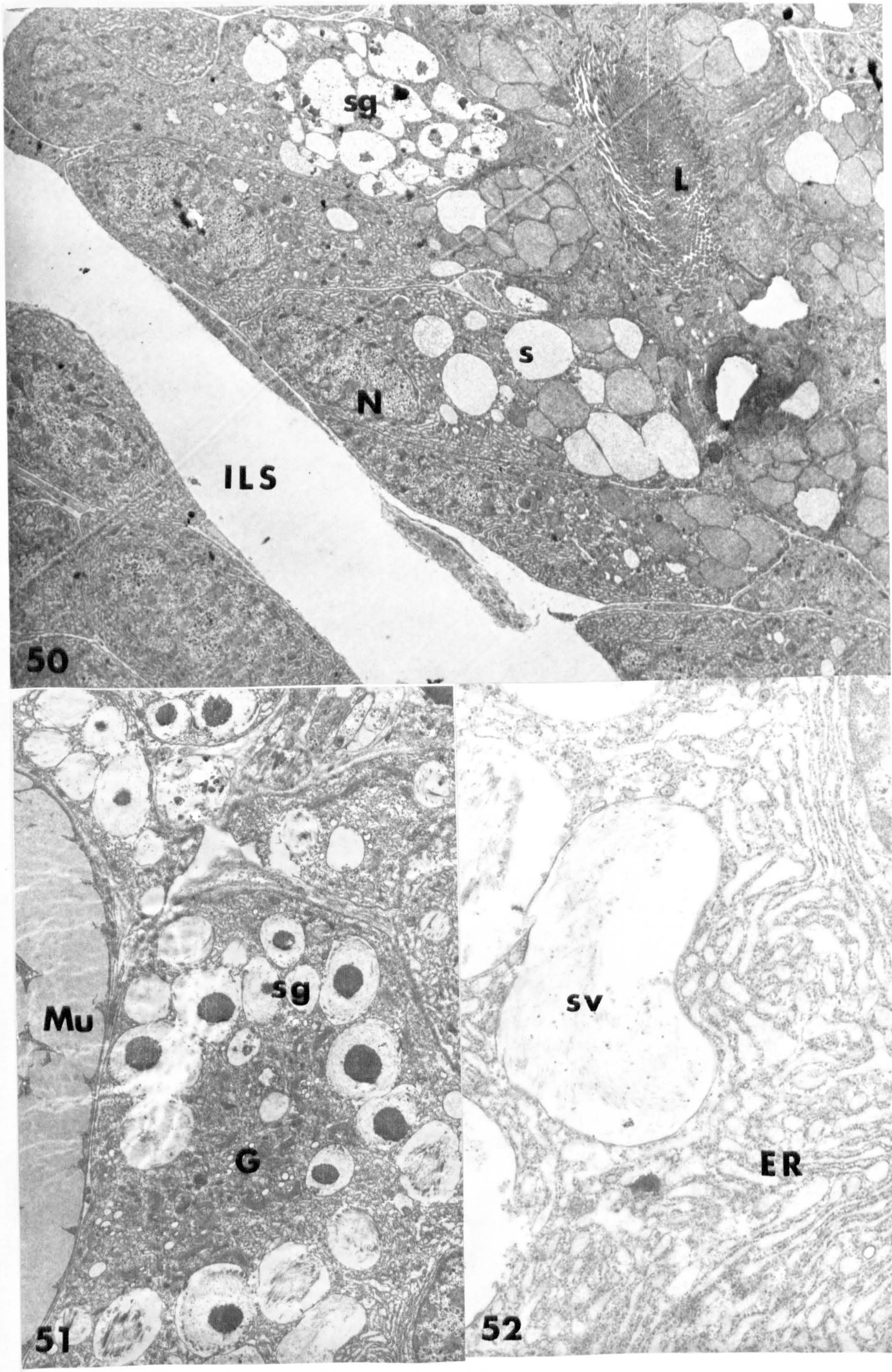

sg
L
S
N
ILS
50
sg
Mu
G
51
sv
ER
52

Fig 53 Stage C. Part of diverticulum showing accumulated secretion.
X 8,000.

Fig 54 Synaptic Junction between epithelial cell process (Ep) and axon (Ax).
X 100,000.

Fig 55 Accumulated secretion in stage C prostate. X 1,500.

cil = cilia.

col = collagen fibres.

Ep = Epithelial cell.

L = Lumen of diverticulum.

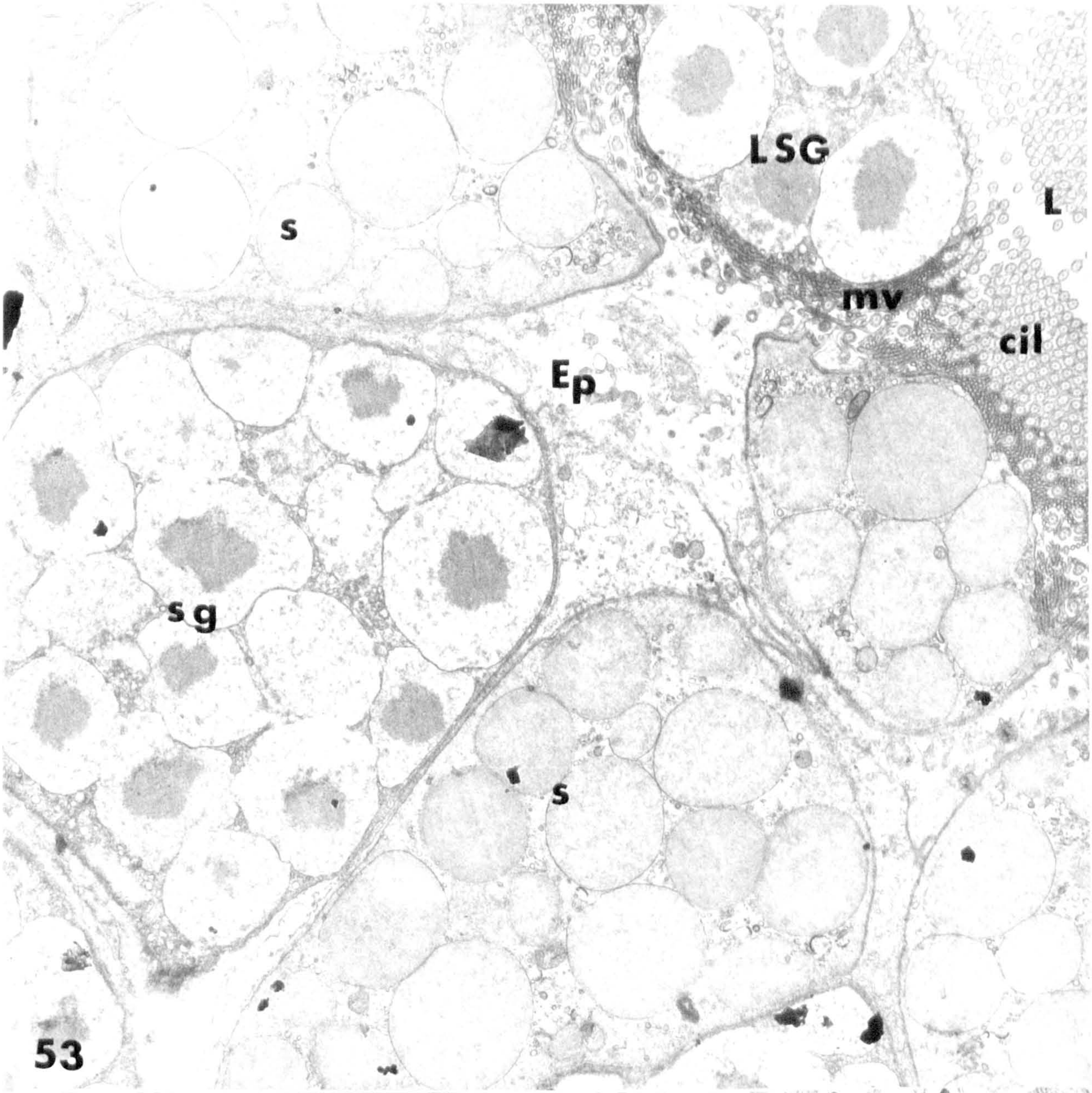
LSG = Liberated type II secretion.

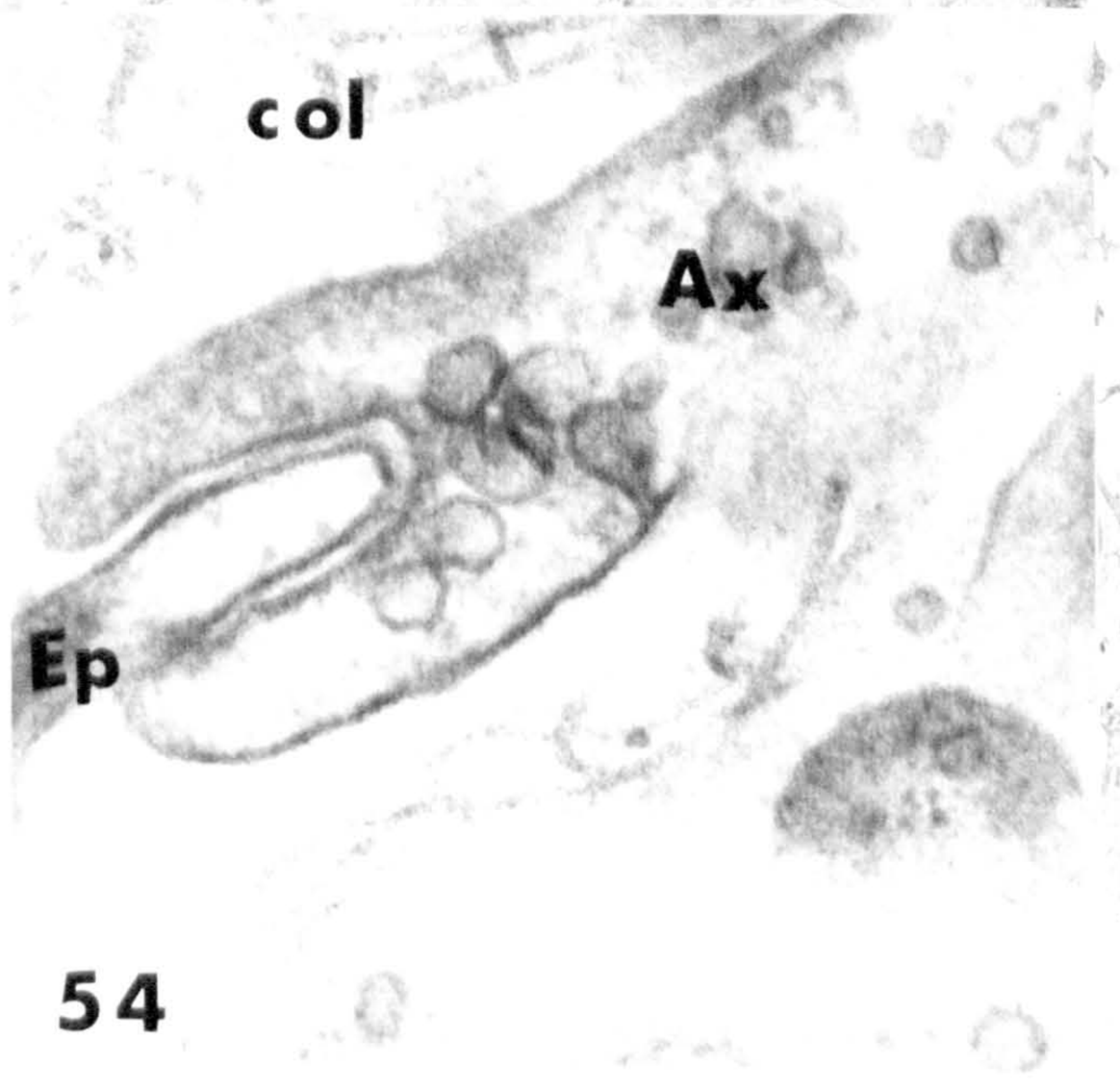
Mu = Mucocyte.

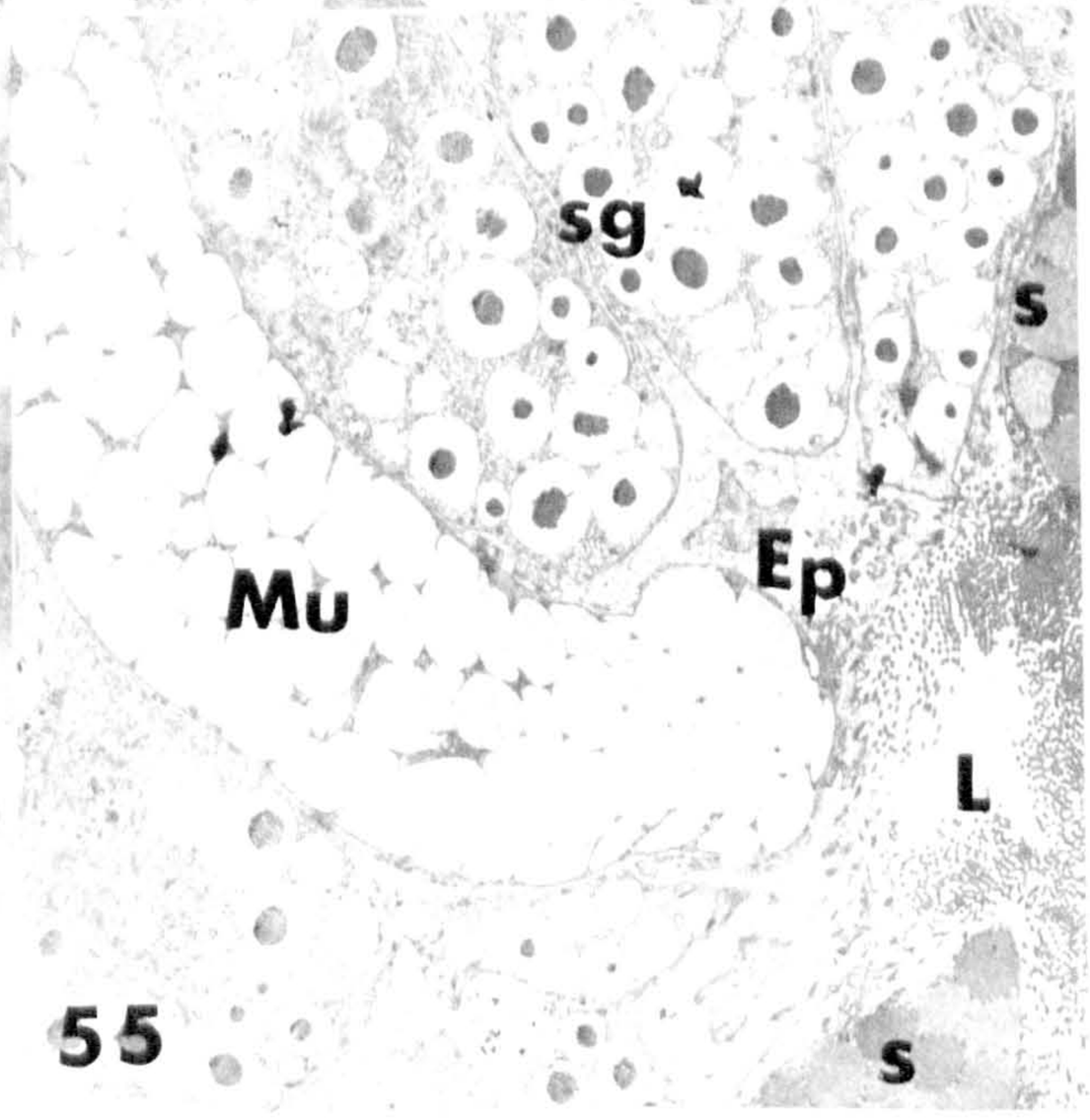
Mv = Microvilli.

S = Type 1 secretion.

Sg = Type II secretion.


LSG
L
S
mv
cil
Ep
sg
s
53


col
Ax
Ep
54


sg
s
Mu
Ep
L
s
55

the area of golgi bodies.

The second type of secretory cell (Fig 50 etc.) distinguished in the electron microscope corresponds to the cell types 1 and 3 as seen with the light microscope. The secretion of these cells is in the form of uniformly dense vesicles (Fig 53 etc.). The staining density of these vesicles varies considerably but the cells producing them appear otherwise identical; indeed, vesicles of different electron densities appear in the same cell. This cell also is seen to develop a large amount of endoplasmic reticulum. This reticulum is found filling the cell body and is covered with a greater number of ribosomes than in the previous cell type. Intermingled with this endoplasmic reticulum are golgi bodies, but far smaller numbers than in the previous type. The endoplasmic reticulum appears to have fewer vesicles and there is a tendency for it to form concentric circular rings. There are considerable amounts of glycogen-like deposits in these cells. The secretory vesicles do not form in the cell body but in the apical region of the neck, as in the previous cell type. The variation in the staining density of the vesicles in this cell type may be due to changes in the vesicles during maturation, or this cell type may consist of a variety of different sub-types.

The remaining features are common in both types of secretory cells in the prostate. The nuclei are large and lobed. They contain many nucleoli or dense chromatin material. The cytoplasm is packed with ribosomes. The few mucocytes, present in the prostate, are very similar to the secretory cells with the uniform vesicles but the secretion is clear and in larger droplets. The secretory cells beneath the ciliated epithelium of the male groove (Figs 48,49) are different to the two types of secretory cell described for the prostate. Each secretory vesicle is uniform in its electron density, but each cell contains vesicles of different densities. These vesicles appear to arise as a result of golgi activity. In other respects these secretory cells are similar to the other secretory cells of the prostate.

In the stage B common duct, differentiation of the oviducal gland has started. The cells of the columnar epithelium are undergoing ciliogenesis and ciliary bases can be seen in the apical zone of the epithelium, together with a small number of longitudinally arranged cytoplasmic fibres or microtubules. In the connective tissue layer there is an increase in the number of muscle cells. The layer has become somewhat thickened. The undifferentiated cells in this layer

are localised in an area beneath the epithelium. These cells are destined to become the secretory cells. At this time they are starting to produce processes or necks which pass between the bases of epithelial cells (Fig 57). These necks do not reach the surface of the epithelium as do those of the secretory cells of the prostate. That part of the common duct which becomes the oviducal gland has become slightly convoluted but discrete diverticulae do not appear to be present.

Stage C is characterised by the start of female secretion. In this and the next stage D, the prostate gland is at its maximum size (Figs 53, 55). The gland now appears to consist of a number of discrete diverticulae, consisting of several zones. There is a certain lumen, which may be up to 10μ at its widest point, surrounded by four zones.

A) A thin single cell layer of ciliated epithelium up to 3μ thick. The bases of these epithelial cells are elongated and pass down between the necks of the secretory cells to make synaptic connections with axons in the spaces between the secretory cell bodies (Fig 54).

B) A large zone which may be up to 30μ thick and contains accumulated secretory material. This layer is derived from the necks of the secretory cells.

C) A zone of nuclei and swollen endoplasmic reticulum, which is derived from the basal parts of the secretory cells. This zone is also up to 30μ in thickness.

Fig 56 Wall of oviducal gland at start of its differentiation. X 3,000.

Fig 57 Differentiation secretory cell from oviducal gland. X 30,000.

col = collagen.

CT = connective tissue.

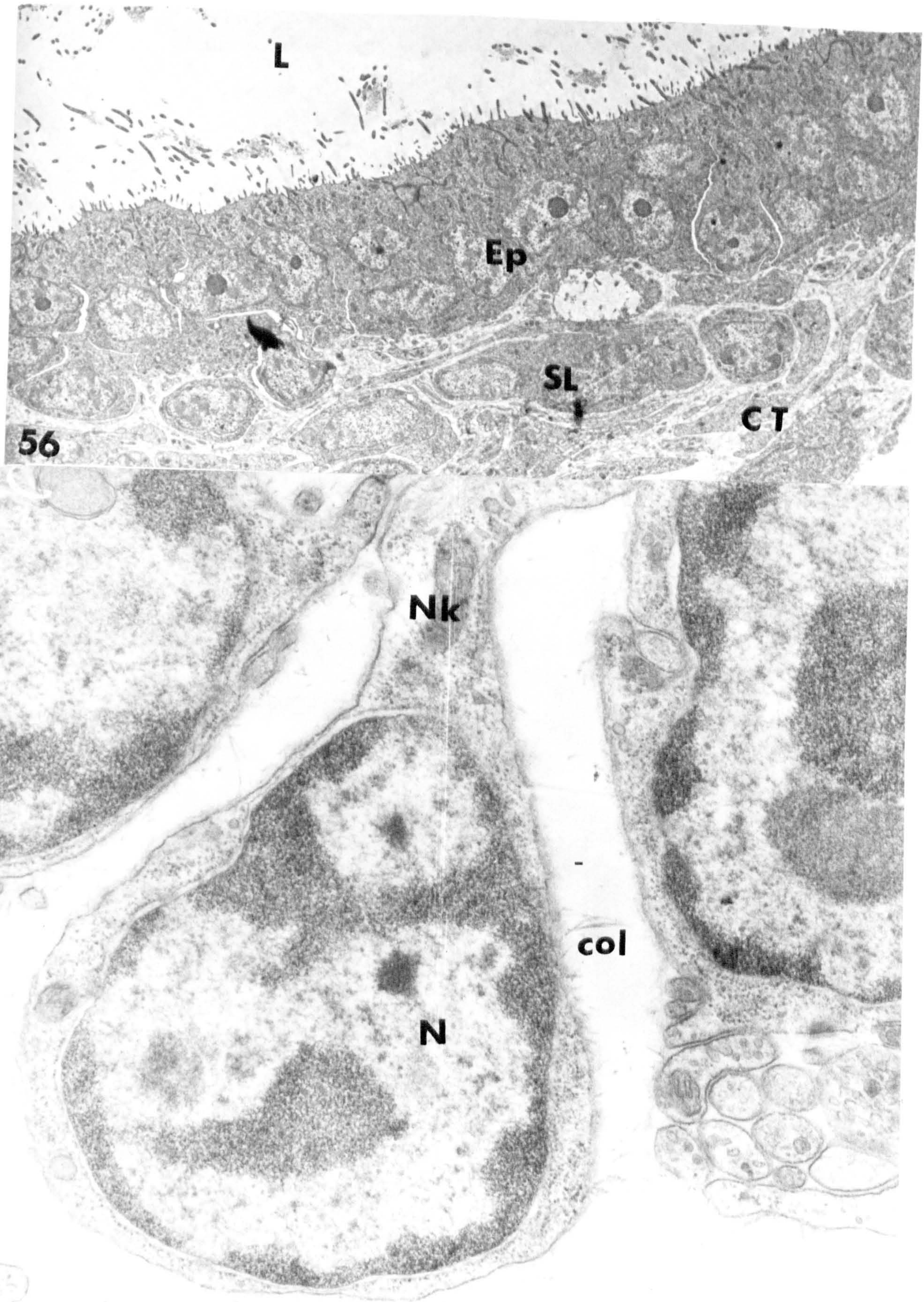
EP = Epithelial layer.

L = Lumen of oviducal gland.

N = Nucleus.

Nk = Neck of secretory cell.

SL = secretory cell layer.


L
Ep
SL
CT
56
Nk
col
N
57

D) Finally a thin layer of connective tissue, mainly collagen, which is less than 1μ in thickness.

Thus the mature diverticulae of the prostate are 120-130μ in diameter.

Secretion takes place during this stage. The secretory droplets and granules which accumulate in the necks of the secretory cells are released, together with a small amount of cytoplasm into the lumen of the prostate (Figs 53, 55). Individual vesicles do not seem to be shed.

The stage C oviducal gland enlarges with the onset of secretion and because of this, its structure is obscured by the distortion which this enlargement produces. The epithelial lining becomes cuboidal and is about 10μ in thickness (Fig 56). This epithelium is richly ciliated and shows the features seen in other ciliated epithelia studied, c.f. that of the prostate. The mitochondria are restricted to the apical region of these cells, forming a zone about 1μ thick, below the surface (Fig 64). Between many of these cells can be seen ductules which are lined by a cytoplasmic layer about 0.2μ in thickness, bearing small microvilli. These ductules are almost always seen in transverse section, indicating that they are probably very convoluted. They open at the surface of the epithelium via what appear as horse-shoe shaped structures

in section. The cytoplasmic lining of these ductules is seen to be continuous with the cytoplasm of the secretory cells (Figs 65,66). These ductules are probably concerned with the release of secretory material. A few isolated mucocytes can be seen in the epithelium. Numerous blind ending pits, of various sizes, some with no epithelial lining and others with a partial lining, are seen projecting into the cytoplasm of the secretory cells. These pits contain sections of cilia but no ciliary bases (Fig 60). Thus the cilia from the lumen of the common duct pass into these pits.

The secretory layer is composed of two types of cell, distinguishable by cytoplasmic characteristics. These cell types differ in the following way:-

Type A. (Figs 58, 59,60). The nucleus of this cell type is very lobed (Fig 59), several sections being visible in each cell. The cytoplasm appears to contain many golgi bodies and free ribosomes, with very few other organelles present.

Type B. (Figs 61, 62, 63). The nucleus of this cell type is more or less rounded. The cytoplasm contains a mass of rough endoplasmic reticulum, together with some golgi bodies and mitochondria. In all other respects these cells are similar. The nucleus is situated basally and contains many nucleoli. The cells are large, about 60 x 15μ , and the secretion

Fig 58 Part of Type A secretory cell in Oviducal gland at stage C. X 3,000.

Fig 59 Nucleus of Type A cell at stage C. X 5,000.

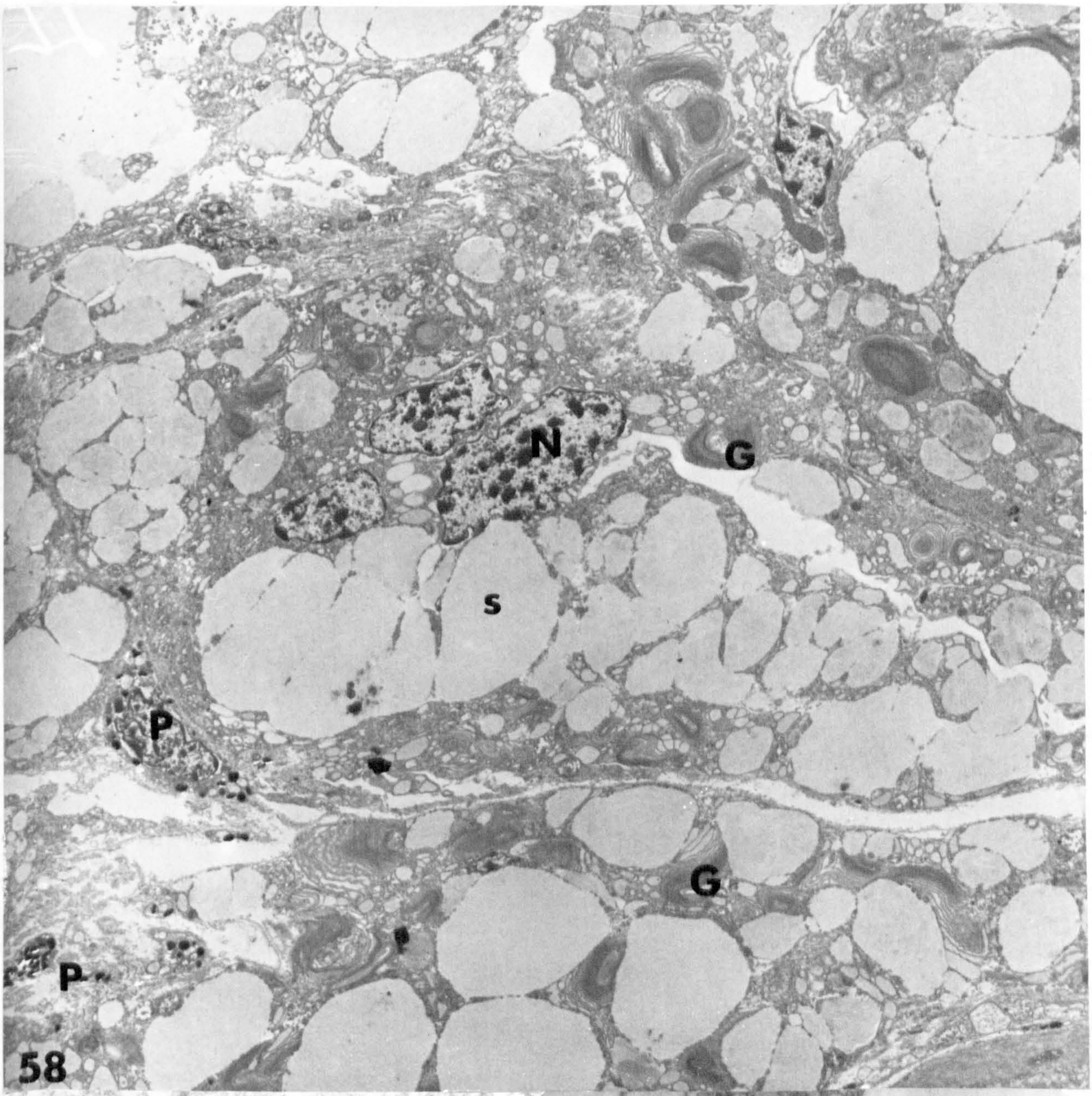
Fig 60 Cytoplasmic detail of Type A cell. X 20,000.

G = Golgi body.

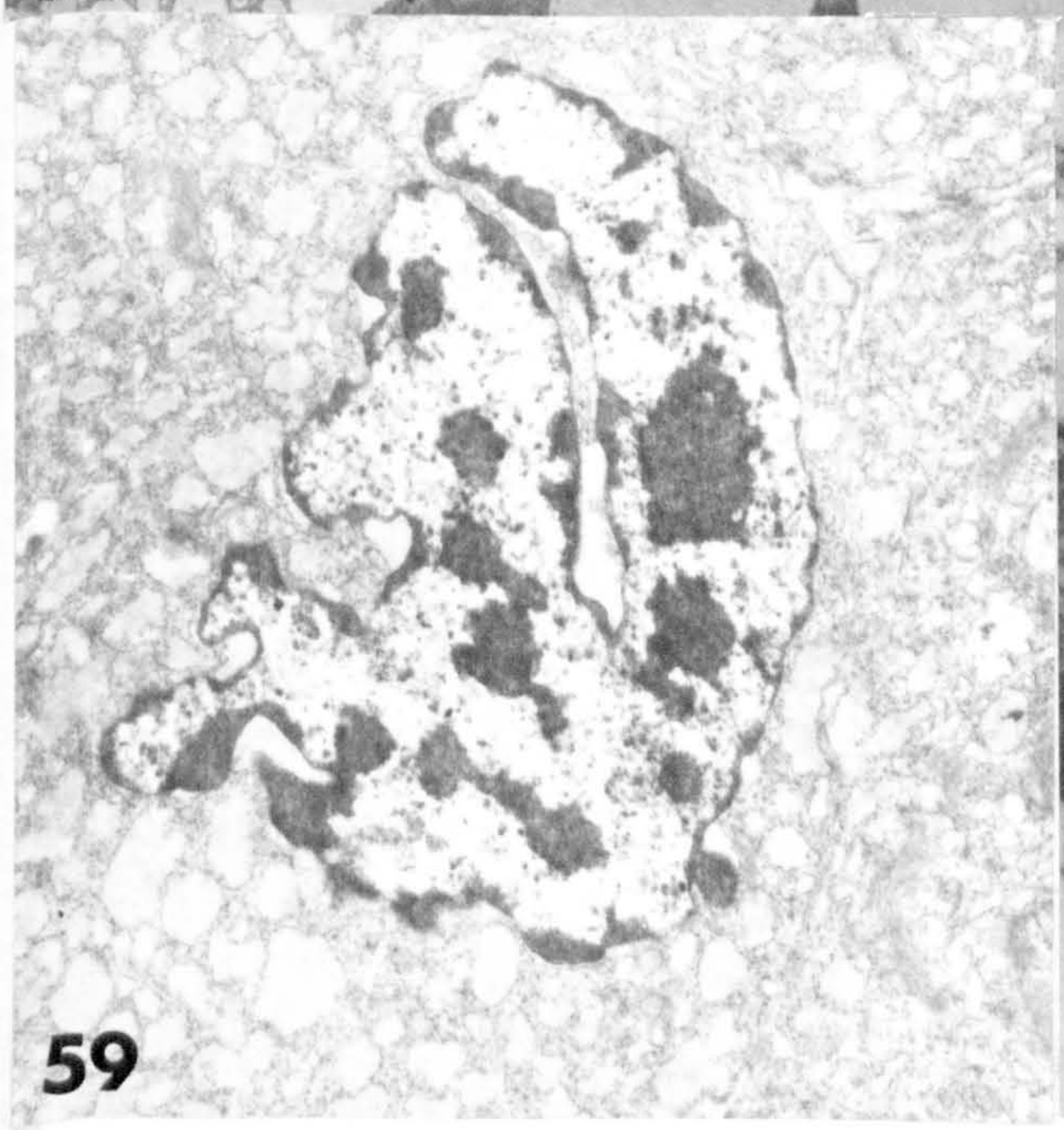
N = Nucleus.

P = Pigment cell.

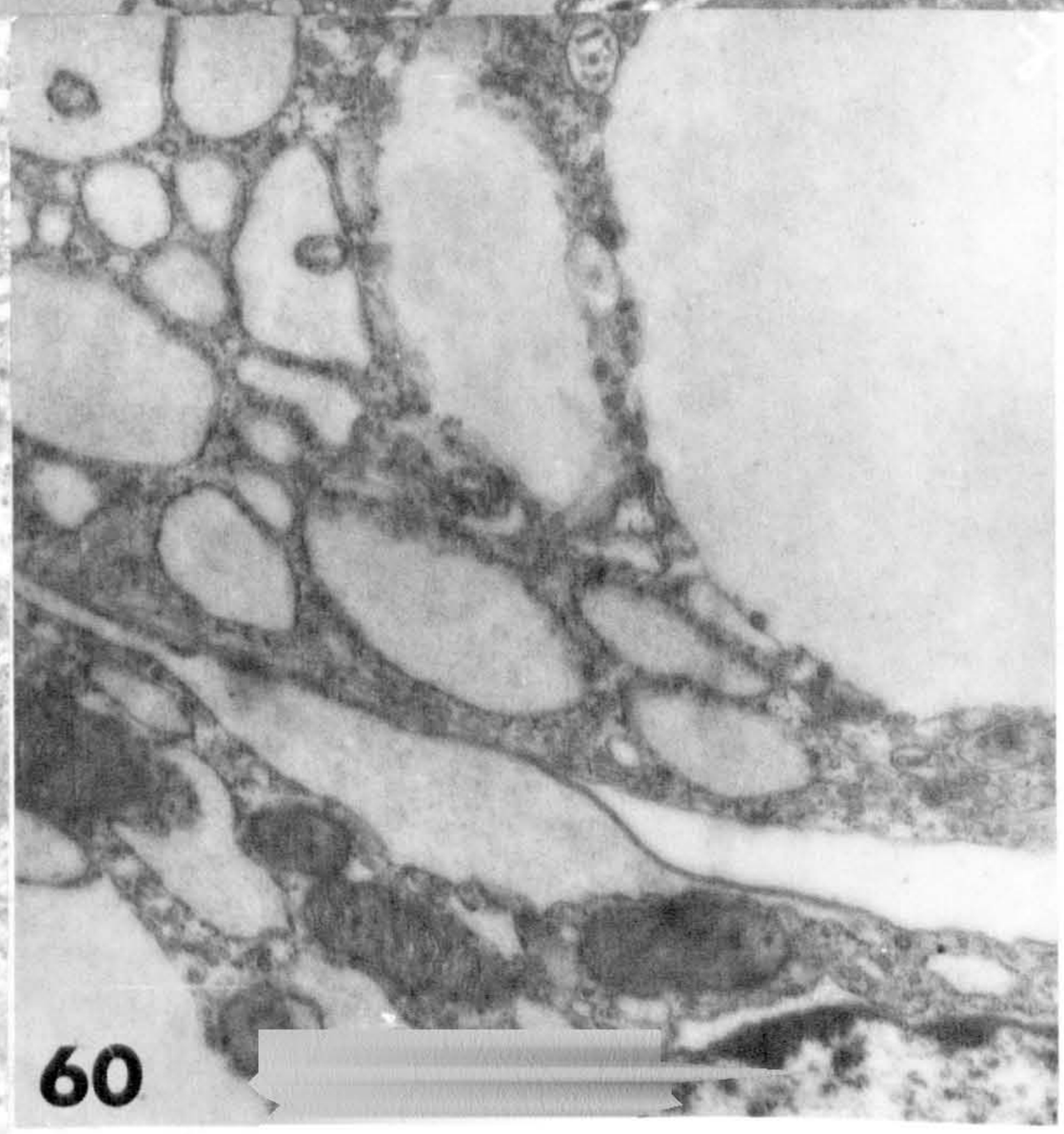
S = Secretory vesicle.



58



59



60

Fig 61 Type B secretory cell in Oviducal gland at stage C. X 3,000.

Fig 62, 63 Cytoplasmic detail from above. X 20,000.

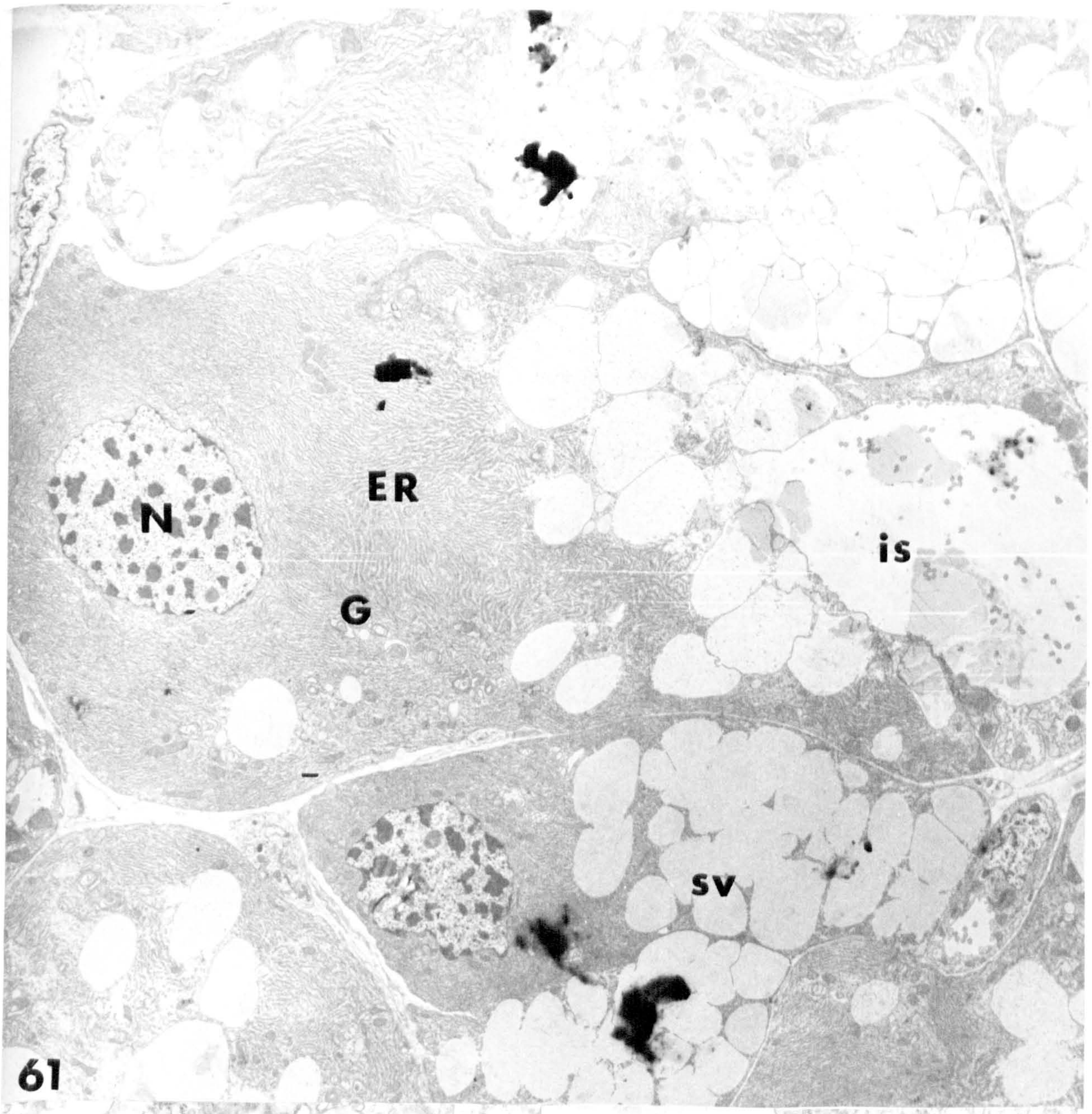
ER = Endoplasmic reticulum.

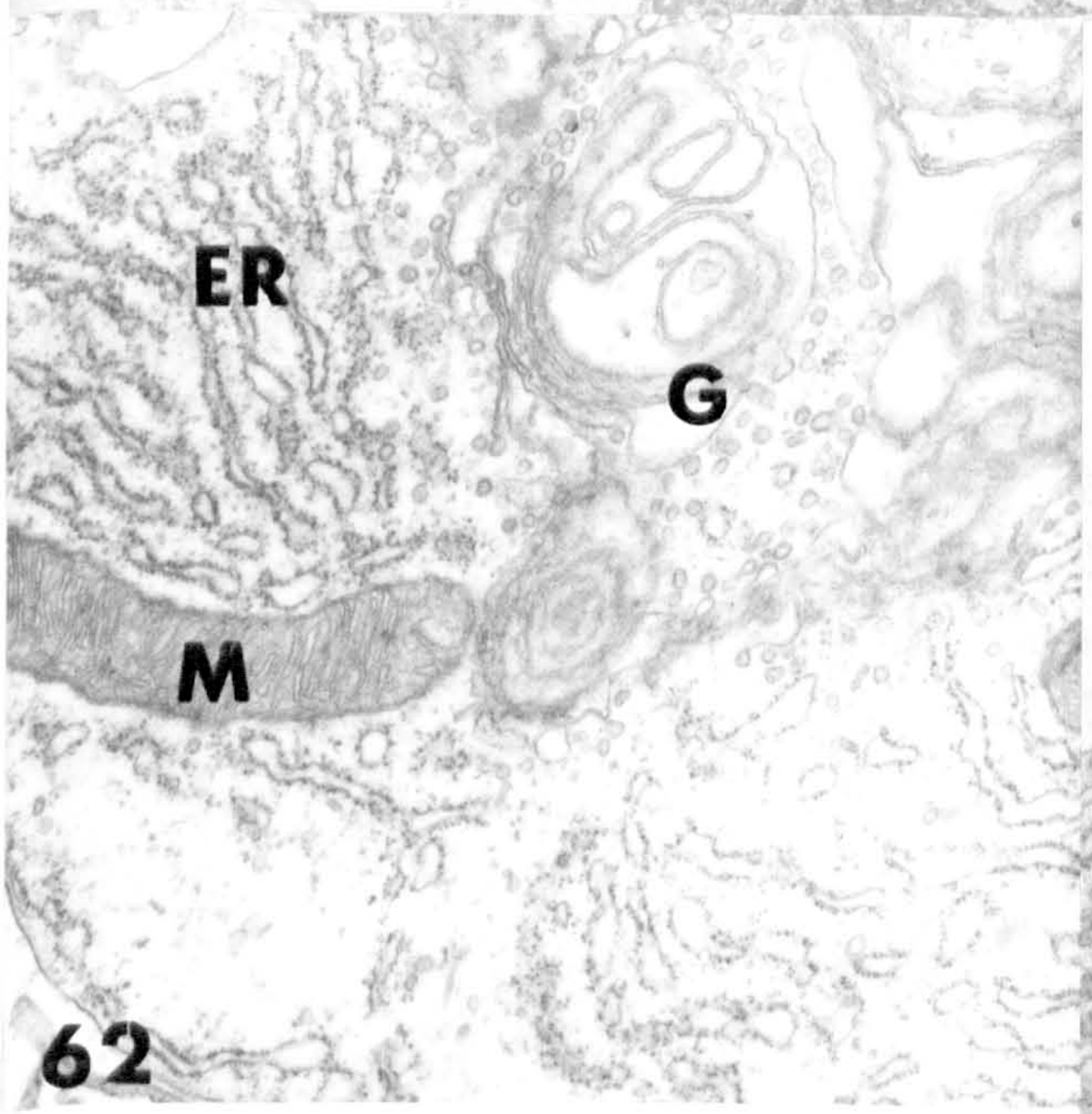
G = Golgi Body.

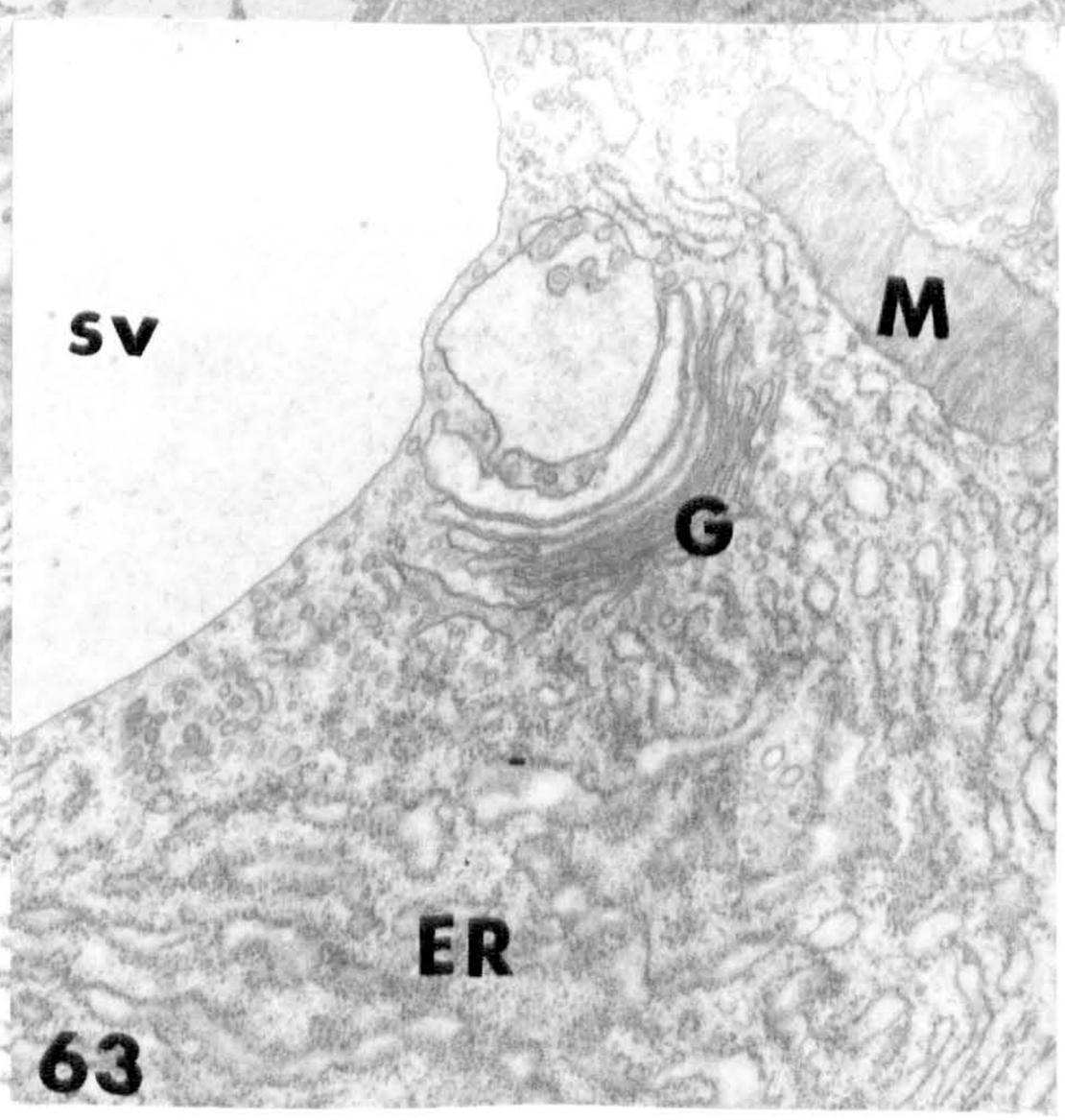
is = Intracellular space.

M = Mitochondria.

sv = secretory vacuoles.


N
ER
G
is
SV
61


ER
G
M
62


SV
M
G
ER
63

accumulates at the apex of the cell. At this stage the accumulated secretion in both types of cell is very similar and almost electronlucent.

The connective tissue layer is found throughout the secretory cell layer and in a layer beneath it. Collagen fibres are found surrounding the secretory cells, presumably providing mechanical support to these large cells. In addition, muscle cells and pigment cells are found randomly arranged throughout this collagen. The smooth muscle cells are similar to those found in earlier stages in the differentiation of the oviducal gland, but as one would expect, they possess far more muscle fibrils.

As secretion accumulates and the secretory cells enlarge, the ciliated epithelium becomes stretched and flattened (Figs 64, 68). The basic appearance of the cells remains the same but the convoluted cell junctions become even more exaggerated. The ductules and pits passing through the epithelium become very flattened and are often only recognisable, when they occur under the epithelium, by the presence of cilia in their compressed lumen.

The secretory cells enlarge rapidly until they become

Fig 64 Epithelium of stage D oviducal gland. X 3,000.

Figs 65, 66 Detail from fig 64. X 10,000.

Fig 67 Intra cellular duct containing spermhead (Sh). X 15,000.

A detail shown in fig 65.

B detail shown in fig 66.

cil = cilia.

Ep = Epithelial cell.

M = mitochondrial layer.

s = secretory vesicles.

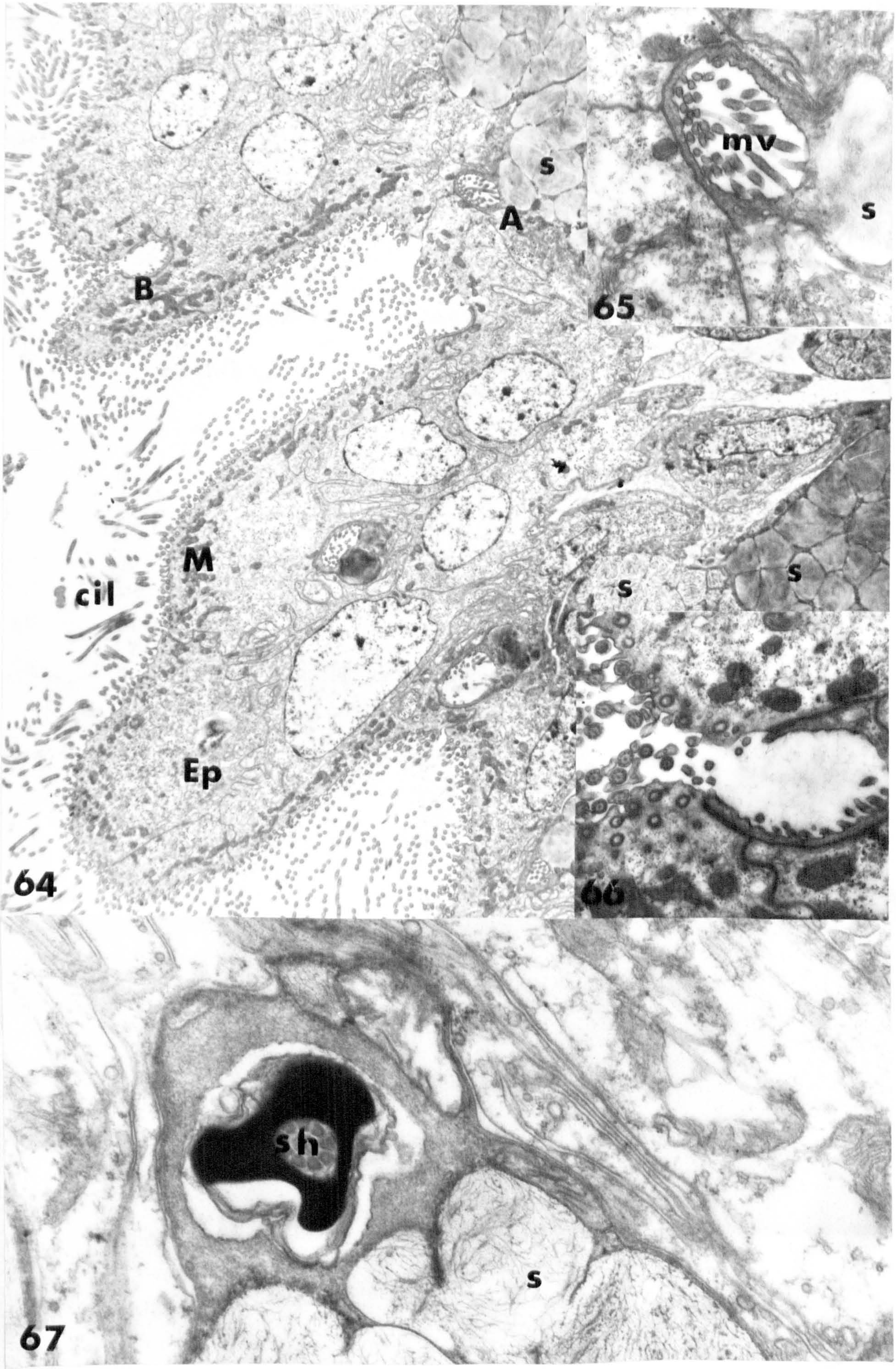

B
A
s
mv
s
65
M
cil
s
s
Ep
64
66
sh
s
67

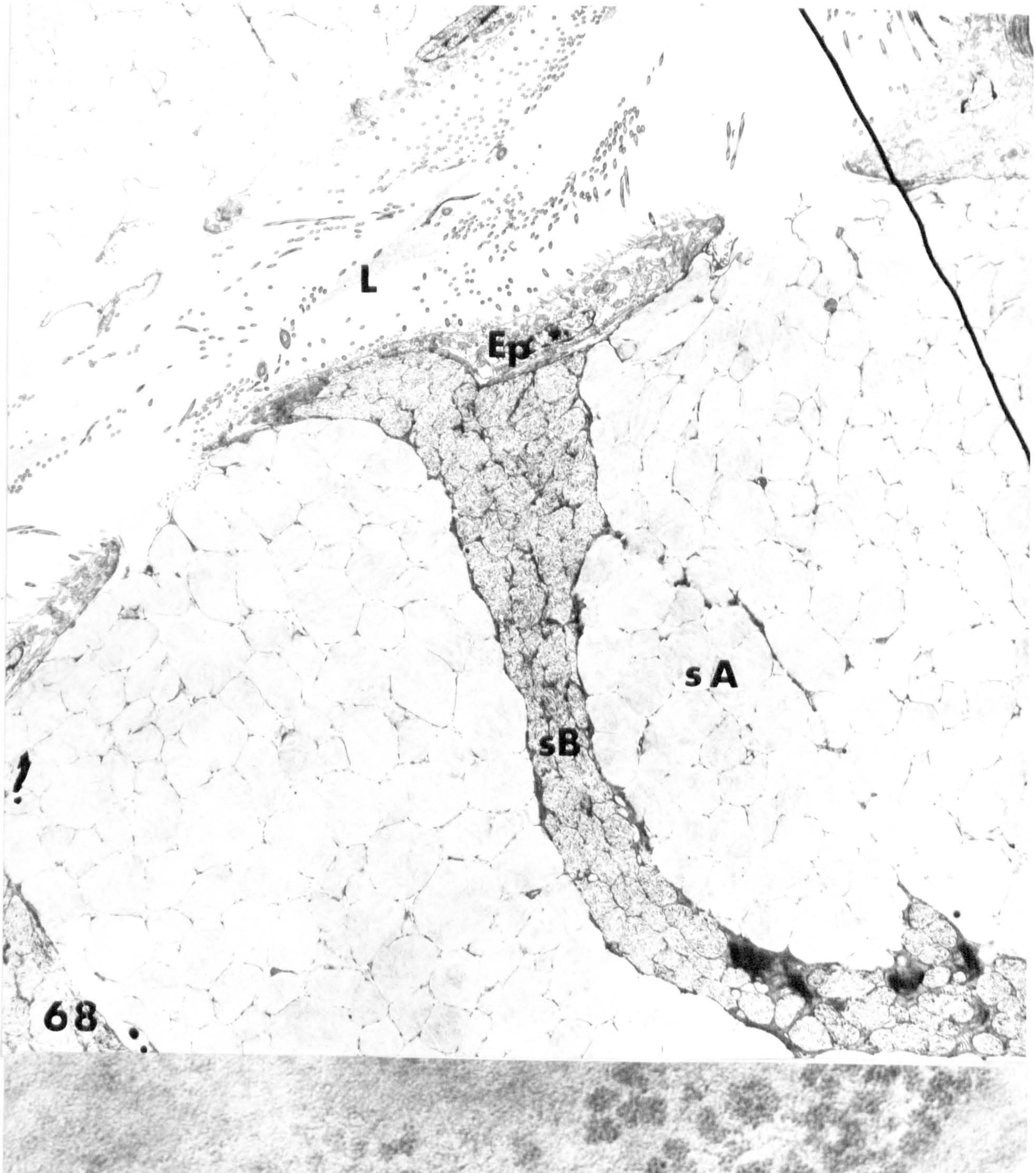
Fig 68 Accumulated secretion of stage D oviducal gland. X 2,000.

Fig 69 Glycogen-like granules in cytoplasm of secretory cells. X 200,000.

Ep = epithelial cell.

L = lumen of oviducal gland.

sA, sB = two types of secretion in oviducal gland.



68

69

vast stores of secretion. Their cytoplasm becomes almost totally obscured and only the occasional piece of nucleus or golgi body can be seen (Fig 70). Small amounts of glycogen-like granules are also present in the cytoplasm of both types of secretory cell (Fig 69). When examined at low magnification two types of secretion vesicles are apparent (Fig 68). The most abundant type is only slightly electron dense, and at higher magnification is seen to consist of a mass of 40Å fibres. This secretion is apparently produced by the type A secretory cells. The secretion vesicles result from the swelling of the lamellae of the numerous golgi bodies present. These golgi bodies may be up to 10μ across. It is possible that substances produced by the other cytoplasmic organelles are added to these vesicles but no evidence of this was seen. The second type of secretion is possibly produced by the type B secretory cells. This secretion is more electron dense and is present in smaller amounts. At higher magnifications the material in the vesicles is seen to have a fibrous nature. Fibres with a diameter of about 100Å are associated with granules which are about 200Å in diameter (Fig 72). This association is random but there is a tendency for the material to become aggregated in the vesicles. These vesicles are again produced by a swelling of the golgi lamellae (Fig 71) and presumably material from the endoplasmic

Fig 70 Base of A secretory cell. X 3,000.

Fig 71 Type A secretion from golgi body.
X 20,000.

Fig 72 Type B secretion from golgi body.
X 20,000.

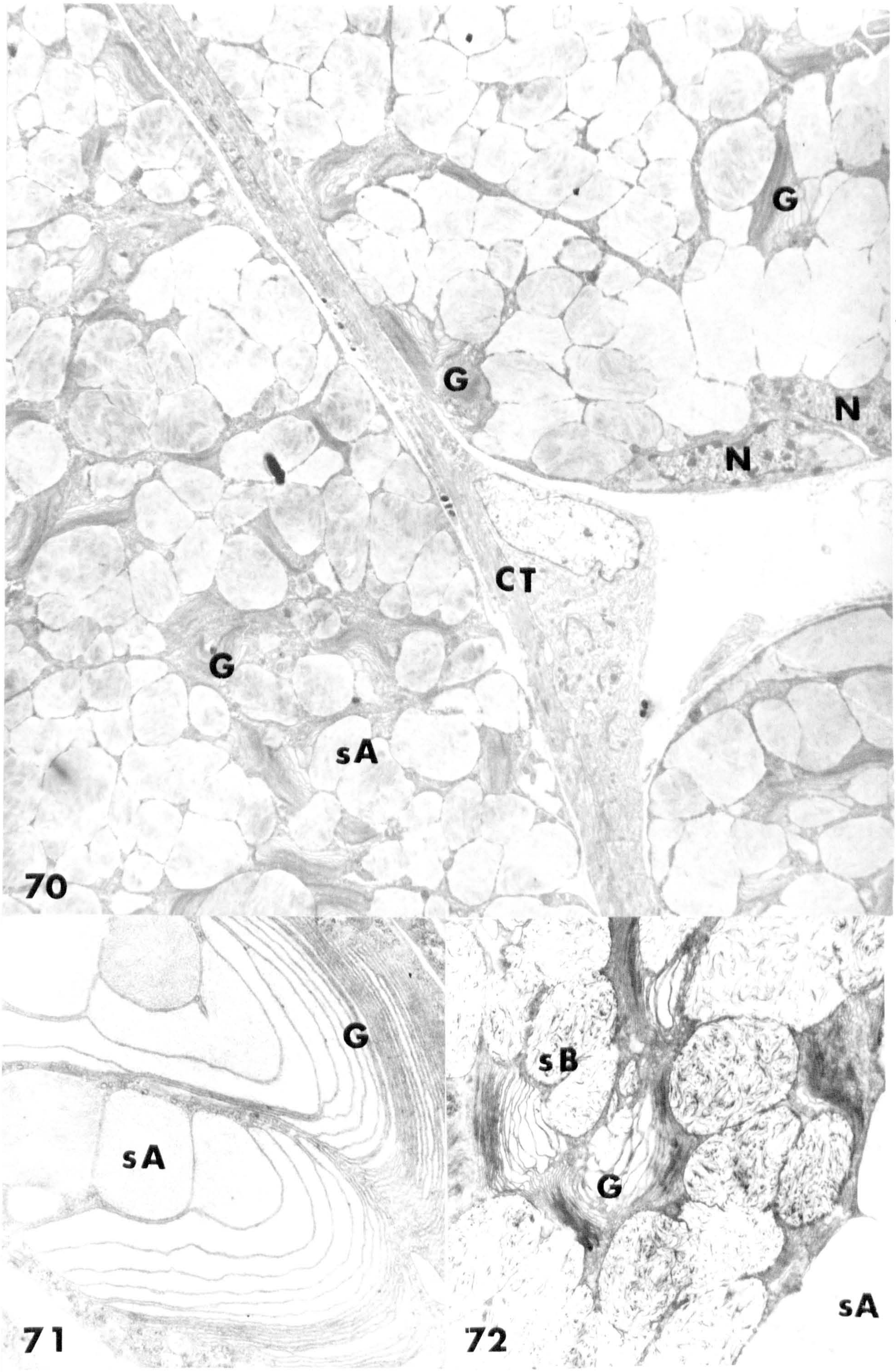
CT = connective tissue.

G = Golgi body.

N = Nucleus.

sA = Type A secretion.

sB = type B secretion.


G
G
N
N
CT
G
sA
70
G
sA
71
sB
G
sA
72

reticulum is also added to these vesicles.

These two typesof secretion are not stages in the maturation of one typo secretory cell because the ratios of the two types of secretion are similar throughout all stages of differentiation in the oviducal gland. The two types of secretion are also present at the apex of the cells where the secretion is presumably about to be released. Both types of secretion are also found in the lumen of the common duct.

The size of the mature secrotory cells is very difficult to assess. However, they would appear to be at least 100μ from base to apex, and 30μ in width. On fixation, the cells of the mature oviducal gland appear to separate slightly into small groups. Each of these groups is surrounded by a connective tissue sheath. The cells within these groups are also separated by a few collagen fibres, but these are very difficult to see except at the highest magnifications. These groups are not apparently present when tissues are fixed in susa. Within each cell, secretion vesicles appear to be arranged in rows possibly representing the products of golgi body. The nucleus of these cells is very lobed and several, apparently separate, sections of nucleus are seen in one cell.

Occasionally a third type of secretory cell is present in the oviducal gland. This cell type is similar to the others already described but the secretion appears electron-lucent.

These are probably large mucocytes, similar to those found in the mature prostate.

Secretion is often seen passing into the lumen of the common duct from the cells of the oviducal gland, via gaps in the epithelium. Whether this is a fixation artifact or not has not been established but with both osmium and gluteraldehyde fixatives the apical secretion vesicles appear to coalesce. When one considers the size of the tissue and the fact that it contains large amounts of carbohydrate (Bayne 1967), it is possible that this effect is a fixation artifact.

Discussion.

The slug Agriolimax reticulatus is a protandric hermaphrodite, but it is not as clearly protandrous as many other slugs studied (e.g. Vaginulus borellianus, Laevicaulis alte).

The hermaphrodite gland contains both male and female germinal elements in all but the first stage of maturation. It is only their relative numbers and state of development that vary. The initial production of gametes is from a mass of undifferentiated germ cells that fill the immature acini of the gland. However, in the early stages of differentiation of the hermaphrodite gland, a small neck region of undifferentiated germ cells is produced. This possibly occurs when a few of the undifferentiated germ cells become attached to the wall of the acinus in the neck region. The cells of this region remain immature in appearance, with a microvillous border, i.e. they have a similar appearance to the undifferentiated germ cells. These cells in the collar region are very similar to those described by Quattrini in Vaginulus borellianus and Laevicaulis alte but they are never ciliated. They contain similar glycogen deposits and in the later stages undergo a similar apocrine secretory activity. In A.reticulatus this neck region remains small in size. In other gastropods there is a continuous production of gametes from the germinal ring in the

neck region of the acinus (Quattrini & Lanza 1965, Runhem & Hunter, 1971, Joosse et al 1968). In A.reticulatus, no evidence was found to suggest that there was a secondary production of male gametes from this neck region, or even that a secondary production occurs at all. In the case of the female gametes, however, groups of young cells, one of which becomes the oocyte, are seen in this area. These groups are seen throughout the development of the acinus and especially in the later steges. These young oocytes seem to migrate away from the neck region. Thus there seems to be a secondary and continuous production of female gametes from the neck region of the acinus. In the post reproductive stage H acinus this germinal region appears to give rise to a cuboidal epithelium. This epithelium migrates around the wall of the acinus causing the remaining germ cells to be sloughed into the lumen of the acinus. The animal then dies. It is possible that under favourable conditions this epithelium could remain undifferentiated, and give rise to another generation of gametes. These cells in the neck region are almost the same as those in the hermaphrodite ductules. Their only difference is that these latter are ciliated. This is interesting because it is from these ductules that a new hermaphrodite gland is regenerated after castration.

The ultrastructure of the oocytes, both before and after vitellogenesis, is similar to that described for the prosobranch Ilyanissa obsoleta (Taylor & Anderson 1969). Organelles such as annulate lamellae and concentric rings of rough reticulum seem to be indicative of vitellogenic activity. The peculiar oocyte/follicle cell attachment and the changes in the volume of the vitelline envelope are also very similar.

In A.reticulatus there seems to be a difference between the male and the female nutritive cells associated with the mature germ cells. In the immature acinus, both male and female germ cells are to be found attached to the same nutritive cell. As the oocyte matures, a small cell at the base of the oocyte grows and a thin cytoplasmic layer is produced which grows over the surface of the oocyte, thus separating it from the nutritive cell supporting the male germ cells, and produces the female follicle. These follicle cells are possibly produced from a cell remaining in the group from which the oocyte developed.

The nutritive cells themselves are very similar to those described by other authors (Quattrini & Lanza 1965). They seem to play an additional role to that of providing nutrients for the developing germ cells. It would appear that they absorb unwanted material from the lumen of the acinus,

including many sperm stages. In the later stages of differentiation, after the majority of spermatogenesis has taken place, the nutritive cells appear to be full of lipid droplets, possibly as a result of their absorbing residual cytoplasm shedi by the maturing sperm tails.

Spermatogenesis is easily followed and most stages may be seen in the acini of the more mature animals. Watts (1952) describes the presence of Feulgen positive areas at the "poles" of the developing spermatid nucleus, using the light microscope. Apparently similar areas have been found in the early spermatid nuclei. These areas appear to arise by fusion of the two layers of the nuclear membrane. In the late stages (G and H) acini studied, the developing sperm shows a great vacuolation of their cytoplasm (cf Quattrini 1965) which is not apparent in the earlier stages. This vacuolation may be due to a hormone inbalance in the "female" animals or it may be simply a sign of senility, since such animals will die within a few weeks.

The cells of the hermaphrodite ductules are, as has been said, very similar to those in the neck region of the acinus. The walls of these ductules appear structurally adapted to be capable of very great expansion. This one would expect if one considers the size of the mature oocytes and the fact that they must pass through the ductules in order to reach the

remaining parts of the reproductive tract.

In the study of the common duct of A.reticulatus a great similarity was noticed between the structure of the oviducal and prostate gland. In their early stages, this similarity is most marked. As the glands develop, the prostate develops diverticulae and the oviducal gland does not, but the basic structure remains the same. It is only when secretion is accumulating causing distortion of the cells that this similarity is apparently lost. This distortion is greatest in the oviducal gland. This fact, combined with the poor araldite penetration in mature oviducal gland, leads to difficulties in the interpretation of its morphology. The glycogen-like deposits found in the secretory cells of the oviducal gland are very similar to the galactogen found in the albumen gland of Helix pomatia by Nieland & Goudsmit. On further investigation these deposits may also be found to be galactogen.

The structure of the prostate of A.reticulatus is also very similar to that described for this same gland in other slugs (Quattrini 1966a, 1966b, 1967), and also similar to the structure of the albumen gland of Helix (Nieland & Goudsmit 1969). However in none of these are synaptic connections described between the cells of the epithelial lining of the diverticulae

of the glands and axons in the underlying connective tissue. This fact possibly indicates some type of nervous control over the release of secretion or its movement by the cilia and hence release from the gland. This would seem likely when one considers the speed with which this secretion is released to form the spermatophore just prior to copulation.

Summary.

1. The structure and development of the hermaphrodite gland, hermaphrodite ductules and common duct have been studied using both light and electron microscopy.

2. The germinal epithelium in the neck region of the acinus was found to be continuous with the epithelium of the hermaphrodite ductules. The cells constituting these two epithelia were very similar.

3. The epithelium which lines the post-reproductive acinus was unlike that of the germinal epithelium.

4. The nutritive cell cytoplasm was found to have a very characteristic appearance.

5. The female follicle cells were only found surrounding the larger oocytes.

6. The arrangement of the tissues in the hermaphrodite ductules suggested a duct capable of great dilation.

7. The basic structure and development of the prostate and oviducal glands was very similar. However, in the mature glands the accumulated secretion obscured these similarities.

8. The secretions of the oviducal gland cells were found to be produced from the large numbers of golgi bodies present in these cells.

References.

ABELOOS, M., 1943, Effets de la castration chez un mollusque Limax maximus. ~~C.R.Acad.Sci.~~ C.r. hebd. séanc. Sci. Paris., 216: 90-92.

ABELOOS, M., 1944, Recherches expérimentales sur la croissance. Bull.Biol.Fr.Belg., 78: 215-256.

ANDERSON, E., 1969, Oocyte-follicle cell differentiation in two species of Amphineurans (Mollusca). Mopalia mucosa and Chaetopleura apiculata. J.Morph., 129: 89-126.

BAILEY, T.G., 1969, A new anaesthetic technique for slugs. Experientia, 25: 1225.

BAYNE, C.J., 1967, Studies on the composition of extracts of the reproductive glands of Agriolimax reticulatus, the grey field slug (Pulmonata, Stylomatophora). Comp.Biochem.Physiol., 23: 761-773.

BAYNE, C.J., 1970, Organization of the spermatozoon of Agriolimax reticulatus, the grey field slug (Pulmonata, Stylomatophora). Z.Zellforsch., 103: 75-89.

BEDFORD, L., 1966, The electron microscopy and cytochemistry of oogenesis and the cytochemistry of embryonic development of the prosobranch gastropod Bembicium nanum. J.Embryol.exp.Morph., 15: 15-37.

FILHOL, J., 1938, Recherches sur la nature des Mélépidosomes et les phénomènes cytologiques de la sécrétion chez Gastéropodes pulmonés. Arch.Anat.Mic., 34: 155-439.

GALANGAU, V., 1964, Le cycle sexual annuel de Milax gagates Drap. (Gastéropodes, Pulmoné) et ses deux pontes. Bull.Soc.Zool.Fr., 89, 510-513.

GATENBY, J.B., 1918, The cytoplasmic inclusions of the germ cells. Part III. The spermatogenesis of some other pulmonates. Q.J.Microsc.Sci., 63: 197-258.

GATENBY, J.B., 1960, Notes on the gametogenesis of a Pulmonate mollusc - An electron microscope study. La Cellule, 60: 289-300.

JOOSE, J., BOER, H.H. & CORNELISSE, C.J., 1968, Gametogenesis and oviposition in Lymnaea stagnalis as influenced by -irradiation and hunger. Symp.Zool.Soc.Lond.,22: 213-235.

KOSHMAN, R.W. & SERRA, J.A., 1967, Spireme karyodiersis: a new type of reductional division. Can.J.Genet.Cytol., 9: 31-37.

KUGLER, O.E., 1965, A morphological and histochemical study of the reproductive system of the slug Philomycus carolinianus. J.Morph., 116: 117-132.

LAVIOLETTE, P., 1954, Étude cytologique et expérimentale de la régénération germinale après castration chez Arion rufus. Ann.Biol. 30: 481-492.

LUSIS, O., 1961, Postembryonic changes in the reproductive system of the slug Arion ater rufus. Proc.Zool.Soc.Lond., 137: 433-468.

LUSIS, O., 1966, Changes induced in the reproductive system of Arion ater rufus by varying environmental conditions. Proc.Malac.Soc.Lond., 37: 19-26.

MARTOJA, M., 1964, Développement de l'appareil reproducteur chez les gastéropodes pulmonés. Ann.Biol.III: 199-232.

NIELAND, M., & GOUDSMIT, E.M., 1969, Ultrastructure of galactogen in the albumen gland of Helix pomatia. J.Ultrastructure.Res., 29: 119-140.

PELLUETT, D., & LANE, N.J., 1961, The relation between neurosecretion and cell differentiation in the ovotestis of slugs. Can.J.Zool. 39: 789-805.

PELLUETT, D., 1964, On the hormonal control of cell differentiation in the ovotestis of slugs (Gasteropoda:Pulmonata). Can.J.Zool., 42: 195-199.

QUATTRINI, D., 1964, Osservazioni sulla spermiogenesi e sugli spermi di Vaginulus borellianus. Bollettino.Zool., XXXI: 1321-1338.

QUATTRINI, D. & LANZA, B., 1965, Ricerche sulla biologia dei Veronicellidae (Gastropoda sokolifera) II.Struttura della gonade, ovogenesi e spermatogenesi in Vaginulus borellianus e Laevicaulis alte. Monitore. Zool.Ital., LXXIII: 3-60.

QUATTRINI, D., 1966, Struttura e ultrastruttura della prostada dei molluschi I. Osservazioni in Vaginulus borellianus. Monitore.Zool.Ital., LXXIV: 3-30.

QUATTRINI, D., 1966, Struttura e ultrastruttura della prostada dei molluschi II. Osservazioni in Laevicaulis alte. Monitore.Zool.Ital., LXXIV: 125-141.

QUATTRINI, D., 1967, Structure and ultrastructure of the molluscan prostate 3. Observations on Milax gagates. Monitore.Zool.Ital.(N.S.) 1: 109-128.

QUATTRINI, D., 1967, Structure and ultrastructure of the molluscan prostate 4. Observations on Aplysia depilans gmelin. Monitore.Zool.Ital.(N.S.), 1: 235-259.

QUATTRINI, D. & FONDI, R., 1969, Structure and ultrastructure of the molluscan prostate. Observations on Veronicella sloanei. Monitore.Zool.Ital.(N.S.), 3: 117-131.

RICHTER, E., 1935, Der bau der zwitterdrusse und die entstehung der geschlechtszellen bei Agriolimax agrestis. Z Naturw. 69: 507-544.

ROSENWALD, K., 1926, Beeinflussung des Gesglechtsmetamorphose von Limax laevis, Z. Indukt Abstammungs - u Vererbungslehre 43 : 238 - 251.

RUNHAM, N.W. & LARYEA, A.A., 1968, Studies on the maturation of the reproductive system of Agriolimax reticulatus. Malacologia, 7: 93-108.

RUNHAM, N.W. & HUNTER, P.J., 1971, The Biology of Slugs. Hutchinsons University Library.

SERRA, J.A. & KOSHMAN, R.W., 1967, Nuclear changes of treptional nature in the differentiation of sperm nurse cells in snails. Can.J.Genet.Cytol., 9: 23-30.

SMITH, B.J., 1966, Maturation of the reproductive tract of Arion ater. Malacologia, 4: 325-349.

TAYLOR, G.T. & ANDERSON, E., 1969, Cytochemical and fine structural analysis of oogenesis in the gastropod Ilyanassa obsoleta, J.Morph., 129: 211-248.

WATTS, A.H.G., 1952, Spermatogenesis in the slug Arion subfuscus, J.Morph. 91: 53-78.

STUDIES ON THE RELATIONSHIPS BETWEEN ORGANS OF THE SLUG Agriolimax reticulatus USING AN in vitro ORGAN CULTURE TECHNIQUE.

Introduction.

The sequential maturation of the reproductive tract of gastropods is well known. Evidence for the control of the development of this tract has been obtained from such phenomena as parasitic castration, anomolies of the tract and multiple sexual cycles (e.g. male phase, female phase, inactive phase and back to the male phase etc.).

The mode of the control of this sequential development of the reproductive tract of pulmonates was first studied experimentally using operative techniques. Castration was first carried out as early as 1938 by Filhol on Helix pomatia and it was observed that secretion in the tract was reduced or stopped. Abeloos (1943) observed a reduction in the size of the reproductive tract in castrated Limax maximus. In the early 1950's Laviolette carried out a series of experiments on various slugs of the families Arionidae and Limacidae. By extirpation or by the grafting of various reproductive organs he concluded that the reproductive tract of Pulmonates was hormone controlled. He stated that the gonad in some way controls the functioning of the reproductive tract but he failed to find any cell type to

which the secretion of such a hormonal factor could be attributed. More recently, Pelluet & Lane (1964) working on Arion ater and Arion subfuscus, have shown a relationship between neurosecretory activity in the optic tentacles and brain, and the sexual state of the gonad. They suggest that the tentacles produce a hormone stimulating spermatogenesis and suppressing oogenesis, and that the brain produces a hormone stimulating oogenesis. The latter reaches its full potency at sexual maturity. Similarly, Berry & Chan (1968) have shown that the removal of the optic tentacles of Achatina fulica causes an increase in oogenesis. Work in progress at this laboratory in Bangor seems to show evidence for a sequence of hormones circulating in the blood of Agriolimax reticulatus (male, then male and female and finally female). This evidence was obtained from a study of the effect of transplanting immature reproductive systems into animals at different stages of reproductive maturation.

An alternative method of studying this problem is the use of in vitro organ culture. This technique has only recently been applied to the study of problems of hormonal relationships between the organs of molluscs. All the workers who have studied this problem have used a technique based on that developed by Wolff & Haffen in 1952.

Streiff (1966a, 1966b, 1967a, 1967b) used this technique to study the relationships between the organs of the marine prosobranch Calyptraea sinensis. This animal shows a definite sex change from male to female during its development and this is directly related to the size of the animal. He was able to show that the normal course of undifferentiated germ cell development was towards the female line ("ovarian autodifferentiation"). Such development occurs if no hormone is present in the culture environment. However, vitellogenesis only occurred if a pulse of hormone was present at the correct stage of oocyte development. Such pulses occur during the female phase of the sexual cycle. Spermatogenic development of the undifferentiated germ cells is under the control of a hormone produced by the brain of animals in the male phase of sexual development. He also found that the reproductive tract was controlled in a similar way. Development of the male tract is determined by two antagonistic hormones. One is produced by the optic tentacle and is responsible for the development and maintainance of the male tract. The other is produced by the brain and causes dedifferentiation of the male tract. Development of the female tract is initiated by a pulse of hormone from the brain of an animal undergoing the sexual change. The further differentiation of the female tract and its maintainance

occurs without hormonal intervention. In the absence of any hormone, the differentiated female tract, the male tract and the immature tract all constitute neutral organs and do not undergo any further development.

Recently, Streiff & Breton (1970a, 1970b) have carried out some similar experiments on two prosobranchs, Littorina littorea and Crepidula fornicata. They found the reproductive tract under similar control to that found in Calyptraea sinensis. The penis when isolated constitutes a 'neutral' organ. Its development is controlled by a hormone from the right optic tentacle while its dedifferentiation is controlled by a substance produced by the pedal-parietal complex of the brain. These active factors are present in all the species they have studied.

Choquet (1965) found that the tentacles of Patella vulgaris produced a spermatogenesis-inhibiting hormone, while the brain produced a spermatogenesis-stimulating hormone.

Gomot & Guyard (1964) and Gomot (1967), working on Helix aspersa, have shown evidence for the phenomenon of ovarian autodifferentiation. They also found that if a brain from an animal in the male stage was cultured with an explant of the hermaphrodite gland then the male cell line was stimulated. If on the other hand a brain from an animal in the female stage was used then the female cell line would be stimulated. This was

found to be true even if the brain and hermaphrodite gland explant were from animals of different species.

Materials and Methods.

Explants of organs of the slug Agriolimax reticulatus were made and cultured using the in vitro organ culture technique previously developed (Bailey, 1969). Various combinations of organs were cultured together in each chamber and their effects upon one another observed. In some cases, extracts of various organs were made and added to the culture medium in the concentration of 5 or 10% of extract to 95 or 90% of medium.

Extracts were made in the following way: The various organs were pooled in clean, weighed glass tubes which were cooled in a mixture of dry ice and 70% alcohol. If these organs needed to be stored for any length of time they were placed in a deep freeze at -20°C. Any condensation on the outside of the tubes containing the pooled organs was then removed and the tubes reweighed to give the weight of tissue. 1 ml of culture medium was added for each gm of tissue. The mixture was then sonicated for 2 minutes by subjecting it to the ultrasonic vibrations from an M.S.E. Ultrasonic Power Unit. The mixture was cooled in an ice bath after every 20 seconds of sonication. The resulting homoginate was centrifuged to remove the tissue debris and the

liquid supernatant was collected in clean McCartney bottles. This procedure was repeated twice more, giving a final weight of liquid of between 3 and 4 times that of the original tissue. This pooled liquid was then passed through a Hemmings filter, containing an E.K.S. grade Seitz filter (Earlson Ford). The resulting sterile liquid was stored in a deep freeze at -20°C. The pH of each extract was adjusted to 7.8 using either N/3 sodium hydroxide or hydrochloric acid.

Sterile haemolymph was similarly prepared for addition to the culture media. Because only small volumes of haemolymph were obtainable from Agriolimax reticulatus it was decided to pool haemolymph from the slug Arion ater, since relatively large volumes are readily obtainable. Haemolymph was collected from a median pedal incision after the slugs had been anaesthetised in carbon dioxide (Bailey 1969). The viscera were carefully pulled through the incision so that the body wall formed a sac full of haemolymph, with minimal contamination from surface mucus. The reproductive tract was examined in each animal when the viscera were removed from the body cavity to see if the animal's reproductive tracts were mature male or mature female. Pooled haemolymph was then centrifuged either at 250G to remove any cell debris, or at 50,000G to remove cell debris and Haemocyanin. The resulting supernatant was passed through a Hemmings filter

(as in the case of the extracts) and stored. After storage a precipitate forms when the haemolymph is initially thawed out. This dissolves in dilute acetic acid producing bubbles of carbon dioxide and is thought to be calcium carbonate (Speeg & Campbell 1968). The blood is added to the medium at a concentration of 10% and the pH adjusted. Haemolymph, from the pericardium of Helix aspersa, was collected and treated in a similar way for addition to culture media.

Culture of isolated hermaphrodite gland explants.

A total of 193
^^ Explants of hermaphrodite gland were cultured in a medium containing no homologous molluscan protein. Explants consisted of groups of about 5 acini taken from hermaphrodite glands at various stages. Cultured individually these explants show a marked change in appearance after only one week. The overall appearance of the acinus, characteristic of the stage used for the explants, has changed and most of the germinal elements have become randomly arranged. The overall stain uptake with Azan triple stain is different. The cultured tissues always stain more with the yellow component of the triple stain while in the controls the tissues stain more with the blue component. The developing spermatogenic stages are pycnotic after 10 days; only late spermatids and mature stages appear normal after this time. The oocytes seem to survive better, but they stain more heavily

with azan and vacuoles are often to be seen in their cytoplasm. Ultrastructurally, the germinal stages break down rapidly (Fig 1). Vacuoles are visible in the cytoplasm of the various cells and the cell walls disintegrate. The sertoli cells seem to alter their role. Their cytoplasm becomes full of vacuoles and is very extensive. The vacuoles often contain degenerating spermatogenic stages and sometimes fat accumulations are found (Fig 2). The disorganisation of the acinus is apparently due to the change in these nutritive cells and the degeneration of the germ elements. Around the wall of the acinus, especially at the neck region there is usually an epithelium of cuboidal or columnar cells which is far more extensive than in the controls. With the light microscope this epithelium appears to consist of healthy non-ciliated cells often encircling the acinus. There is often a graduation from a columnar type of epithelium near the neck region to a cuboidal type, and finally at the edge of the cell layer a few flattened cells which are free from the wall of the acinus. With the electron microscope the bases of these cells are often seen to be convoluted and there is a tendency for the epithelium to 'lean' one way (Fig 3), suggesting that it is migrating from the neck region. The cells themselves appear relatively undifferentiated but have a microvillous border. Some, however,

Fig 1 Part of Acinus from a stage C hermaphrodite gland explant cultured together with other hermaphrodite gland explants for 15 days. X 3,000.

Fig 2 Cytoplasm of nutritive cell from above acinus. X 6,000.

Ep = Epithelium lining acinus.

F = Fat bodies.

Scl = Primary Spermatocytes.

SpH = Sperm head.

T = Sperm tails.

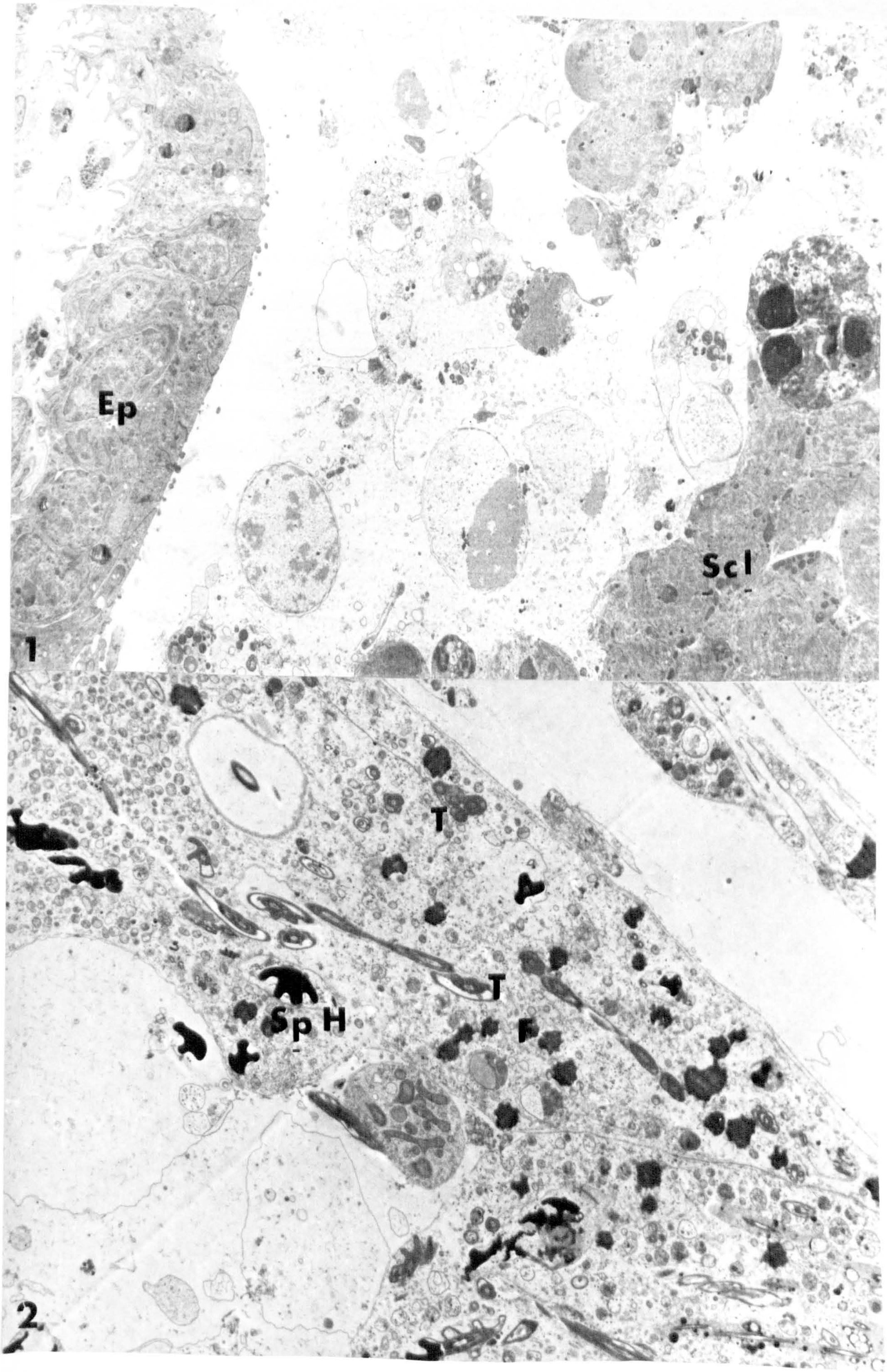

Ep
Scl
T
T
SpH
1
2

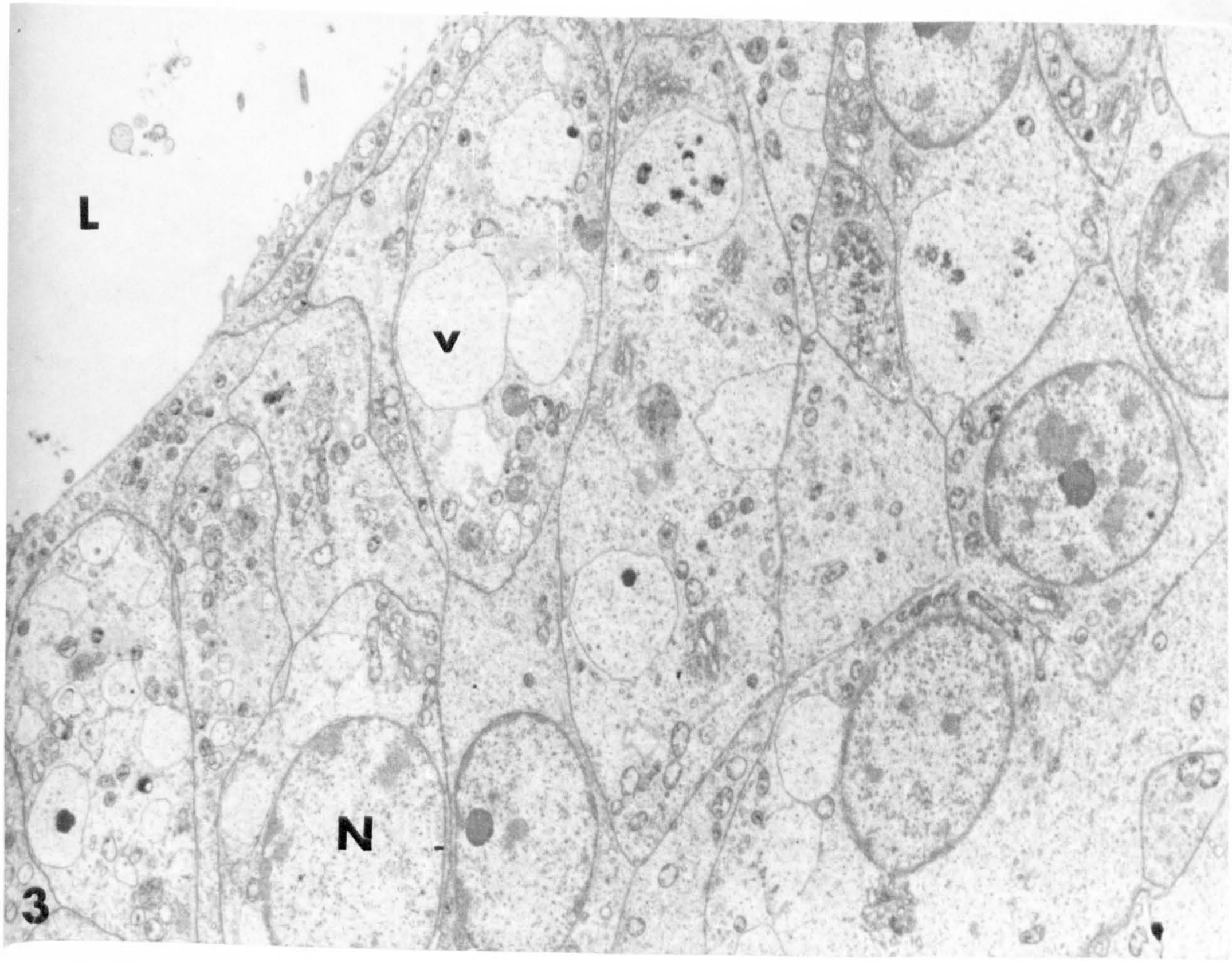
Fig 3 Epithelium of acinus after 21 days of culture "leaning" to the right.

X 6,000.

L = Lumen of acinus.

N = Nucleus.

V = Vesicle.


L
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bear cilia (Fig 5), a phenomenon never seen in the control gonads. The nucleus is basal and contains a mass of chromatin granules (Fig 6) and a nucleolus. Large accumulations of glycogen-like granules are usually found apically to the nucleus, and in some cases completely surround it. Lipid deposits may also be found in this position but not normally in association with the glycogen-like granules. At the apex of the cell a number of small pinocytotic vacuoles are to be found together with a number of multivesicular bodies or residual bodies. The cytoplasm also contains mitochondria, golgi bodies, rough reticulum and free ribosomes. These mitochondria appear to have fewer cristi than those of the controls.

The wall of the acinus, including the pigment layer, characteristically appears thickened after culture. Examination in the electron microscope reveals that this is due to a folding of the wall. The basement membrane appears convoluted and thus this folding is probably due to the loss of the germ cells which filled the acinus. This folding is the same as that found in the normal post-reproductive stage (H) hermaphrodite gland. A certain amount of swelling of the pigment cell's cytoplasm is seen and this seems due to the development of a number of swollen cisterni of rough endoplasmic reticulum. Connective tissue cells, blood vessels and factors of the hermaphrodite duct all appear healthy

Fig 5 Base of cilium from epithelium of acinus after 21 days of culture. X 150,000.

Fig 6 Epithelium from acinus after 21 days of culture. X 6,000.

bb = basal body of cilium.

bf = basal fibre of cilium.

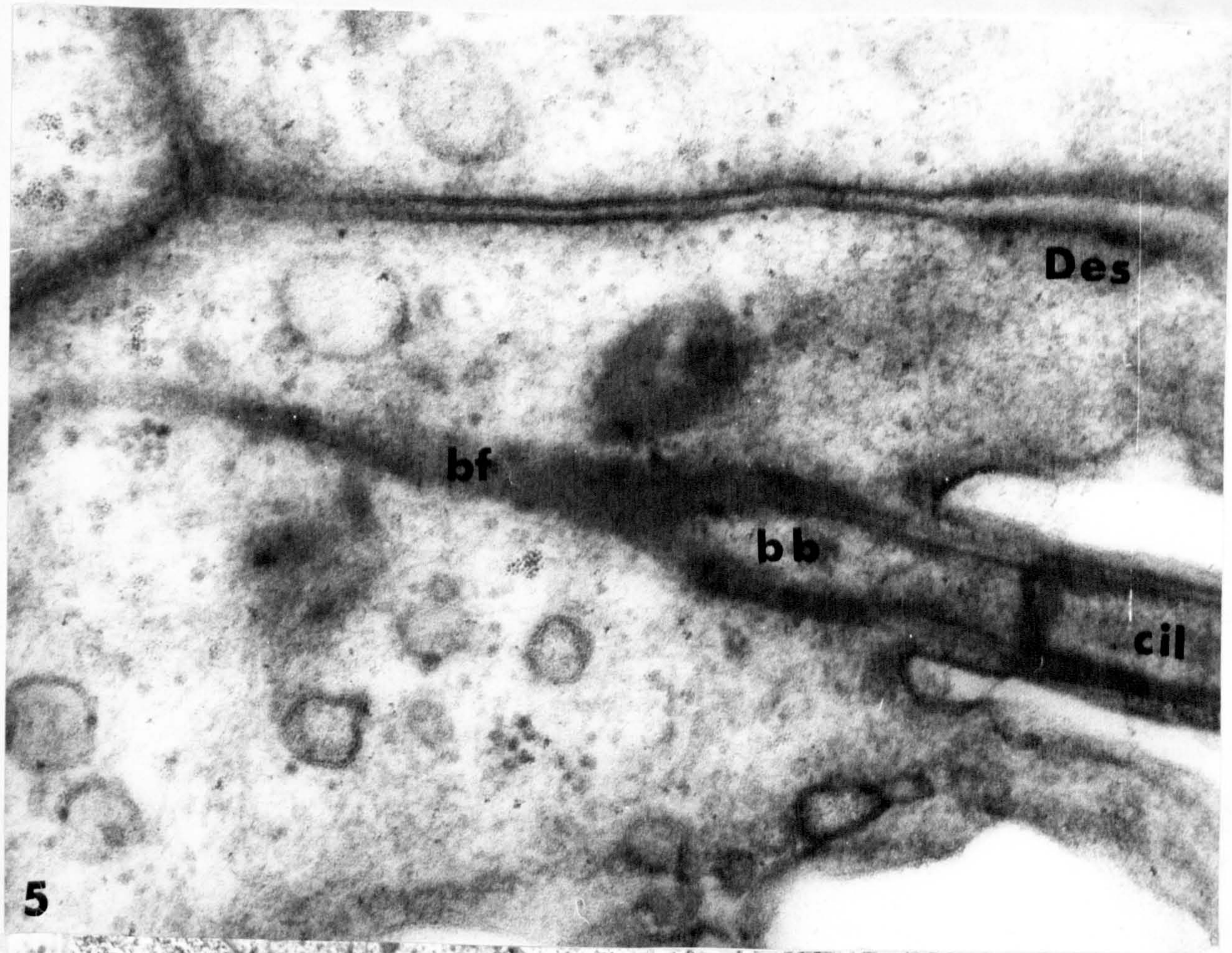
cil = cilium.

Des = septate desmosome.

Gl = Glycogen.

L = lumen of acinus.

PW = pigment wall.



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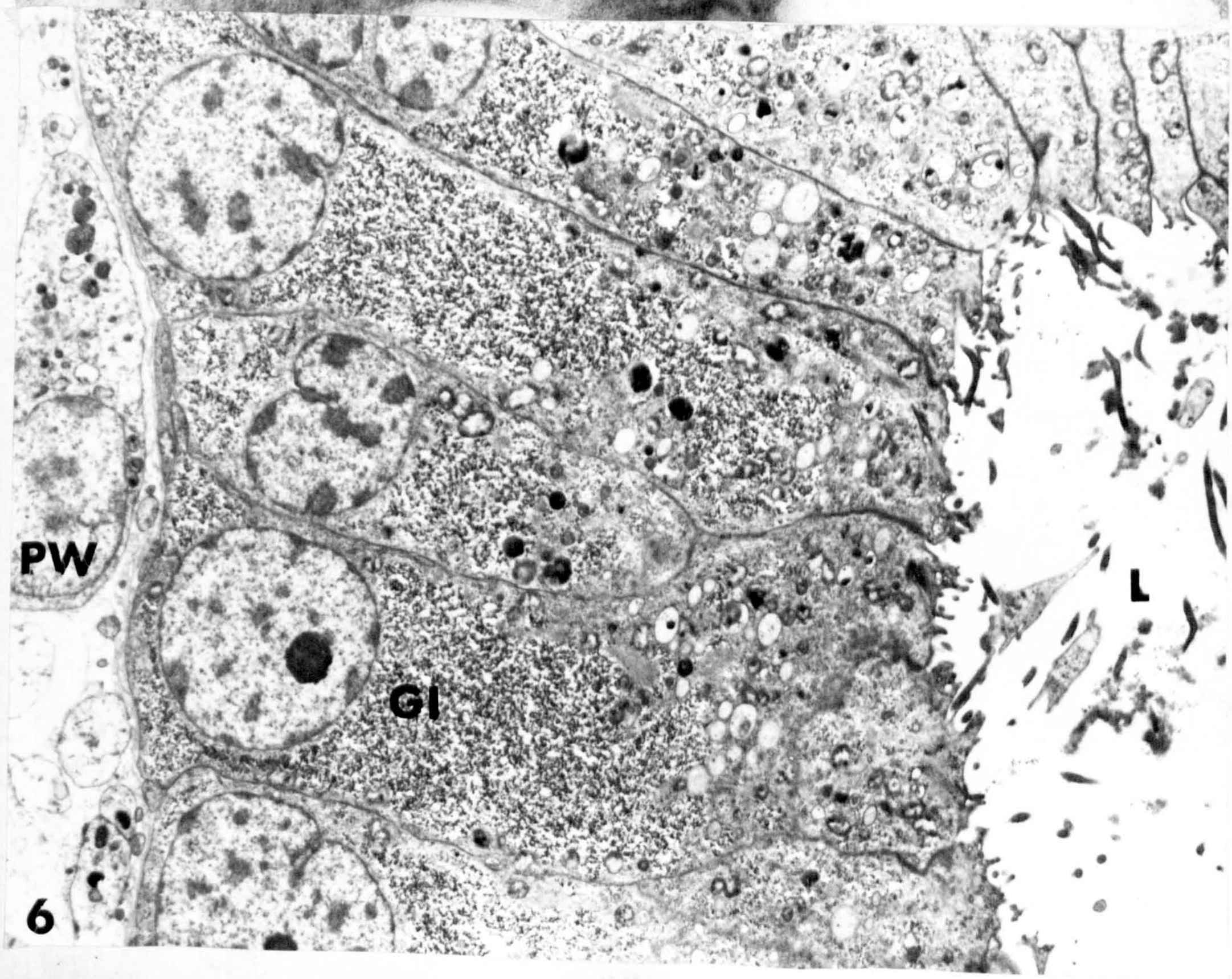

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under the light microscope, while examination in the electron microscope reveals no apparent difference from control sections.

When 20 explants of hermaphrodite gland are cultured together on one millipore filter, the survival of the spermatogenic stages is prolonged to about 15 days. The survival of the other cell and tissue types is as already described.

When cultured alone, the germ cells are incapable of survival. Therefore some factor(s) necessary for germ cell survival is lacking. An increase in the amount of explanted tissue increases the length of survival of the male germ cells. Thus it would appear that something beneficial to spermatogenic survival is carried into the culture system with the explants, probably in the extracellular fluids of the acinus.

Culture of Hermaphrodite gland together with the Brain-Tentacle complex.

Due to the difficulty experienced in successfully culturing the brain and tentacles, it was decided to culture both of these regions as one entity, termed here the Brain-Tentacle Complex. Invariably parts of this complex were found to have become pycnotic. However certain regions were found to be comparatively

healthy and attempts were made to correlate these surviving regions with the state of the associated cultured gonad.

Explants of hermaphrodite glands in various stages of differentiation were cultured with the brain-tentacle complex from the same animal (26 cases) or from other animals in different states of sexual development (31 cases). The following results were taken from experiments lasting between 14 and 42 days.

When the brain-tentacle complex was cultured with the gonad from the same animal there was an improvement in the survival of the germinal elements when compared to that of the isolated gonads. The stage of gonad differentiation and the sexual development of the animal seemed to have little effect on the resulting explants, other than the obvious variation in the ratio of the male and females germ cells. In common with the isolated cultured gonads, these explants showed a marked development of an epithelial layer lining the acinus. This epithelium was identical in appearance with that found in these isolated explants. Oocytes appeared to survive quite well in all cases but, as with the isolated explants of gonad, their cytoplasm was sometimes vacuolated. In cases where the oocytes survived exceptionally well, no correlation could be found with the surviving parts of the brain-tentacle complex, since the surviving elements of the latter

were also to be found when oocyte survival was similar to that in isolated gonads. Oocytes could survive when little of the brain survived and possibly were not influenced by the brain-tentacle complex. In most cases there was some improvement in the survival of the spermatogenic stages when compared to those of the isolated gonads. Only in a few cases was it possible to preserve whole acini in a healthy condition (Fig 20 , Paper 4). In such cases the healthy acini were found to be adjacent to acini in which the survival was not as good. A distinct survival zone could be seen in the majority of this series of experiments. This zonation seemed to centre around the brain-tentacle complex. It would appear that the area that this complex influences was very small, in the region of 2mm. Groups of explants which are spaced more than this distance apart do not have any effect on one another. Therefore, explants cultured in the same chamber at a distance of more than 5mm could be described as isolated.

There were considerable differences between assessments of cell survival made in the light and electron microscopes. Palade's osmium fixative preserved the spermatogenic stages far better than susa did. On examination in the electron microscope spermatocytes, apparently healthy when viewed at low magnification appeared abnormal when examined in detail. Their cytoplasm

appeared to contain extensive flattened vacuoles or inpushings from the cell surface (Fig 7). The plasma membrane of the cell itself appeared indistinct and tended to be discontinuous. This probably accounted for the poor fixation in susa. The granular matrix of the cytoplasm was less dense, the mitochondria had fewer cristi and spiral polyribosomes were seen. However, spermatocytes have been obtained in good condition (Fig 23, Paper 4) and meiotic figures have been observed (Fig 22, Paper 4). The abnormalities found in the spermatocyte cytoplasm were similar to those found in other explants which had been subjected to a pH which was slightly too high. Although parts of the hermaphrodite duct and common duct appeared normal at pH 7.75 $\pm$ 0.05, it would appear that the spermatocytes were more sensitive to pH change and showed signs of deterioration before any other cell types.

An attempt has been made to correlate surviving areas of the brain-tentacle complex with male cell survival. In the experiments where whole acini survived and continued to divide, such areas, common to all experiments, were the procerebrum (Fig 16 ,Paper 4), and some small groups of cells in the other ganglia of the brain. The tentacle did not appear healthy in any of these cases. The dorsal body cells situated over the surface of the cerebral ganglion in this animal (Laryea unpublished), also appeared healthy in such cases. Thus the presence of a brain-

Fig 7 Spermatocyte after 14 days culture.

X 20,000.

Fig 8 Stage A prostate after 28 days culture. X 6,000.

Ep = Epithelial cells.

L = Lumen of diverticulum.

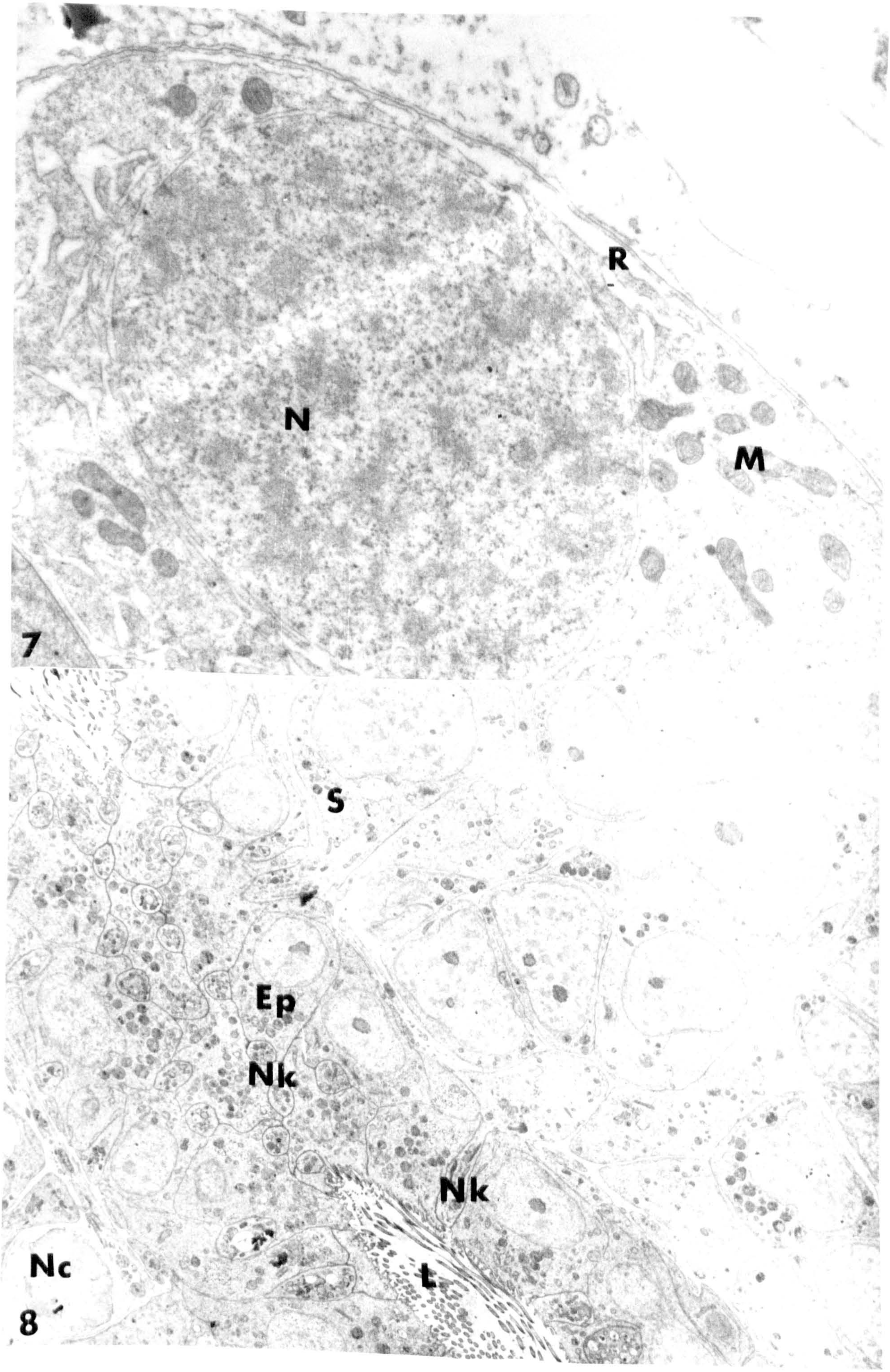
M = Mitochondria.

N = Nucleus.

Nc = Necrotic cell.

Nk - Secretory cell necks.

R = Reticulum of flattened vacuoles.


R
N
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7
S
Ep
Nk
Nk
Nc
L
8

tentacle complex improved the growth of the germinal elements, especially the male cell line.

When gonads were cultured with the brain-tentacle complex of large mature female animals there was a tendency for the survival of the female cell line to improve. The male cell line seemed to gain little benefit from this association and cell numbers reduced considerably. These results were similar to those one would expect and might be due to a factor favouring the female cell line being produced by the associated complex.

If, on the other hand, the brain-tentacle complex from an immature animal was cultured with a gonad there was a favouring of the male line, spermatogenic stages being seen at all stages of the experiment. The female cell line, however, also seems to benefit from this association and oocytes are seen to enlarge and increase in numbers. It therefore seems that there is a factor favouring the male line, and probably a factor favouring the female line. Such results one would expect from an animal which is not truly protandric for the majority of its life cycle.

Culture of explants in media containing extracts of various organs.

Six series of experiments were undertaken using media containing extracts of various organs. Explants of gonad, brain-tentacle complex, and common duct were cultured individually

and in association. Extracts were made from mature "females" and mature "males," the difference between them being the state of the development of the common duct; males having an undeveloped female part or oviducal gland. The organs pooled from each category of animals were gonad, brain, tentacle, common duct and buccal mass. Extracts were used at a concentration of 5%. Gonad, common duct and buccal mass were also used at a concentration of 10%.

The results obtained by culturing explants in medium containing 5% extract were inconclusive. However, when cultured in medium containing 10% extract, considerable improvement was noticed in many of the explanted tissues. This improvement was most marked in the case of oocyte survival (Fig 21 ,Paper 4). Counts were made of the numbers of oocytes which could be seen in one field of view under the microscope, using a X10 objective. No difference could be found in the number of oocytes in the controls and the experimentals. There was, however, an increase in the size of the oocytes of the experimentals. In other respects the gonad explants and the explants of the other organs used, did not show any different pattern of survival, although the amount of the explant which survived was often increased.

It would thus appear that the addition of homologous protein to the culture medium must be in concentrations of at least

10% to be effective. It would also appear that homologous protein from the experimental extracts did not improve any individual developmental stage. The addition did, however, prove beneficial to overall organ survival possibly by supplying some non-specific nutrient lacking in the medium. Similar experiments were carried out using haemolymph from "male" and "female" Arion ater, sex separation being as described above. This haemolymph was added to the medium at a concentration of 10%. No effect was noticed in any of the experiments.

These experiments were repeated using Helix aspersa haemolymph, and again no effect which could be attributed to the addition of the haemolymph was noticed. It would appear that any hormone carried in the blood of the snail or slug species used was either too specific to affect A.reticulatus or its concentration when incorporated into the medium was too low to cause any effect in the explants.

Isolated common duct.

Explants of the reproductive tract (17 cases) were cultured in medium containing no homologous protein. Initially whole tracts from the penial complex to the hermaphrodite duct were cultured. However, the large size of the penial complex, albumin gland and common duct in advanced stages made them

unsuitable for culture. Large areas became pycnotic primarily due to oxygen starvation. The small size of the totally undifferentiated reproductive tract made this stage unsuitable as a source of explants. The early stage of male differentiation was the most suitable source of explants and therefore was used in the majority of the experiments.

The histological appearance of these immature tracts was excellent and no difference could be seen between the controls and these cultured explants when examined with the light microscope. Similarly in the experiments carried out with large mature tracts and albumin glands, no difference could be seen in the surviving areas. These large explants showed zones of survival: an outer zone of dead tissue only a few cells thick, a middle zone of healthy cells and a central core of dead cells (Fig [13] Paper 4). The inner core was almost certainly a result of oxygen starvation. The outer zone was possibly produced as a result of being too far from the surface of the medium, since this outer zone was not present on the underside of the explants.

Cultured tracts at the male stage of differentiation were examined in the electron microscope (Figs 8 & 9). Some slight differences in the cells of these cultured organs were revealed when they were compared to those of the controls. Examination of the epithelium of the male and female parts of the common duct

Fig 9 early Stage B common duct after 21 days culture in vicinity of male groove. X 4,000.

Fig 10 late Stage A prostate after 21 days culture in association with Brain/tentacle complex and gonad of the same animal. X 6,000.

Ep = Epithelial cells.

F = Fat bodies.

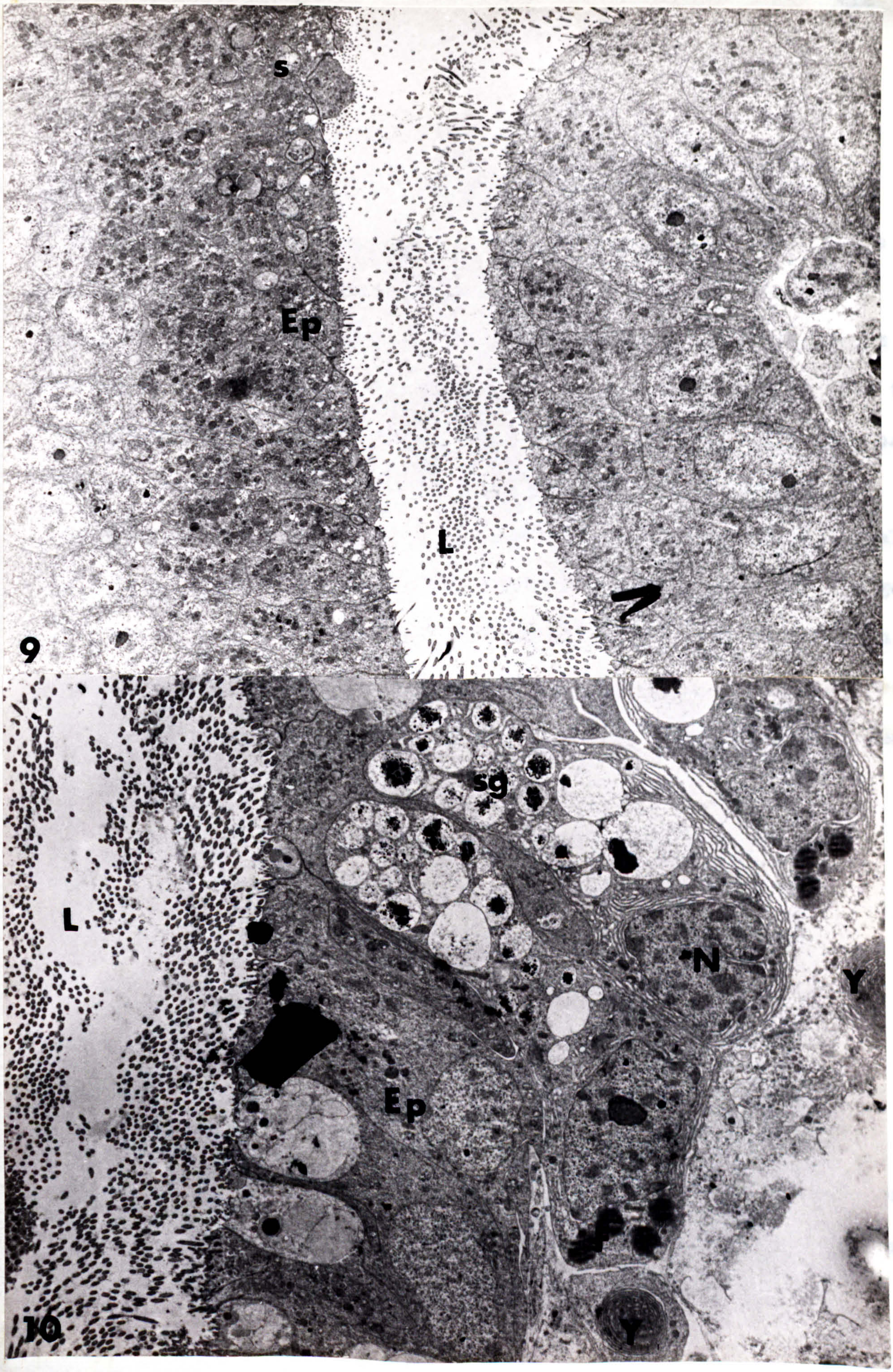
L = Lumen.

N = Nucleus of secretory cell.

S = secretion.

sg = secretory granules.

Y = concentric rings of endoplasmic reticulum.


s
Ep
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9
sg
L
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10
Y

revealed the following differences. The background staining density of the cytoplasm was slightly reduced and in some cases the mitochondria had fewer cristi. Small clear vesicles were usually found at the apex of the cell. Between these vesicles may be found ciliary basal bodies and cilia are sometimes seen to project from the cell surface, and as there were no cilia in some of the controls ciliogenesis appeared to have occurred. Immediately beneath the layer of small vesicles was found a small number of multivesicular bodies. Just above the nucleus, large glycogen deposits were usually found. Although these glycogen deposits were found in the controls their size was always increased in the cultured explants. In all other respects these cultured cells were apparently normal.

Secretory cells of the male and the female parts of the common duct did not appear to alter if cultured in isolation. A few may be found to be necrotic, presumably due to oxygen starvation, but the majority remained completely unchanged, exhibiting the characteristics of the controls. Even if the explants were of secreting tissues the cells remained the same as those of the controls, and no accumulation of secretion was apparent. It appeared that small pieces of the common duct were able to survive perfectly in culture, but it would seem that some other substance was necessary for the continuation of

differentiation and secretion.

Association of the common duct with the gonad.

Association of the common duct with the gonad (31 cases) did not alter its survival pattern when compared to that of the isolated common duct. This observation might be due to the fact that the gonad itself did not produce any substance affecting the common duct, or that it was itself under some hormonal control which must be present in order to produce any substance affecting the common duct. It might also have been due to the poor survival of the gonad when isolated from the brain-tentacle complex.

Association of the common duct with the brain-tentacle complex.

Similarly this association of the common duct with the brain-tentacle complex (15 cases) did not alter the survival pattern of the common duct. The survival of the brain-tentacle complex was, however, quite good, - as good as that which affected the survival of the gonad. Although it was possible that any cells producing a substance which might affect the tract may not be surviving, it would seem that the brain-tentacle complex does not affect the common duct directly.

<u>Association of the common duct with the gonad and the brain-tentacle complex</u> (Figs 10 - 14).

Association of the common duct, the brain-tentacle complex and the gonad of the same animals (35 cases) does cause a change in the secretory cells of the common duct when compared with those of the controls and the isolated explants. The male secretory cells of the prostate appear to enlarge, but not to any great extent, and the nucleus appears more lobed than in the controls (Fig 12). The secretory cell itself develops a neck region if one is not already present and this passes between the cells of the epithelium lining the ducts of the prostate. The golgi bodies in the cytoplasm enlarge, appearing active, and are often seen apparently dividing (Fig 13). The most noticeable feature, however, is the development of enormous onion bodies (Fig 14), up to 12μ in diamter, almost filling the cytoplasm. These onion bodies consist of concentric rings of rough endoplasmic reticulum enclosing a vesicle, or mitochondria, or fat droplets or any combination thereof. The onion body itself is usually closely associated with several golgi bodies. These onion bodies are occasionally found in the control prostate gland but they are always much smaller, being 3 - 4μ in diameter. (They have also been observed in vitellogenic oocytes). The remainder of the cytoplasm of secreting male cells consists of

Fig 11 Secretory cells of stage A prostate gland after 21 days culture in conditions similar to those of fig 10. X 3,000.

Fig 12 Detail from fig 11. X 40,000.

F = fat body

G = Golgi body

N = Nucleus of secretory cell

Neo = Nucleolus

P = Pigment cell

Y = concentric rings of endoplasmic reticulum.

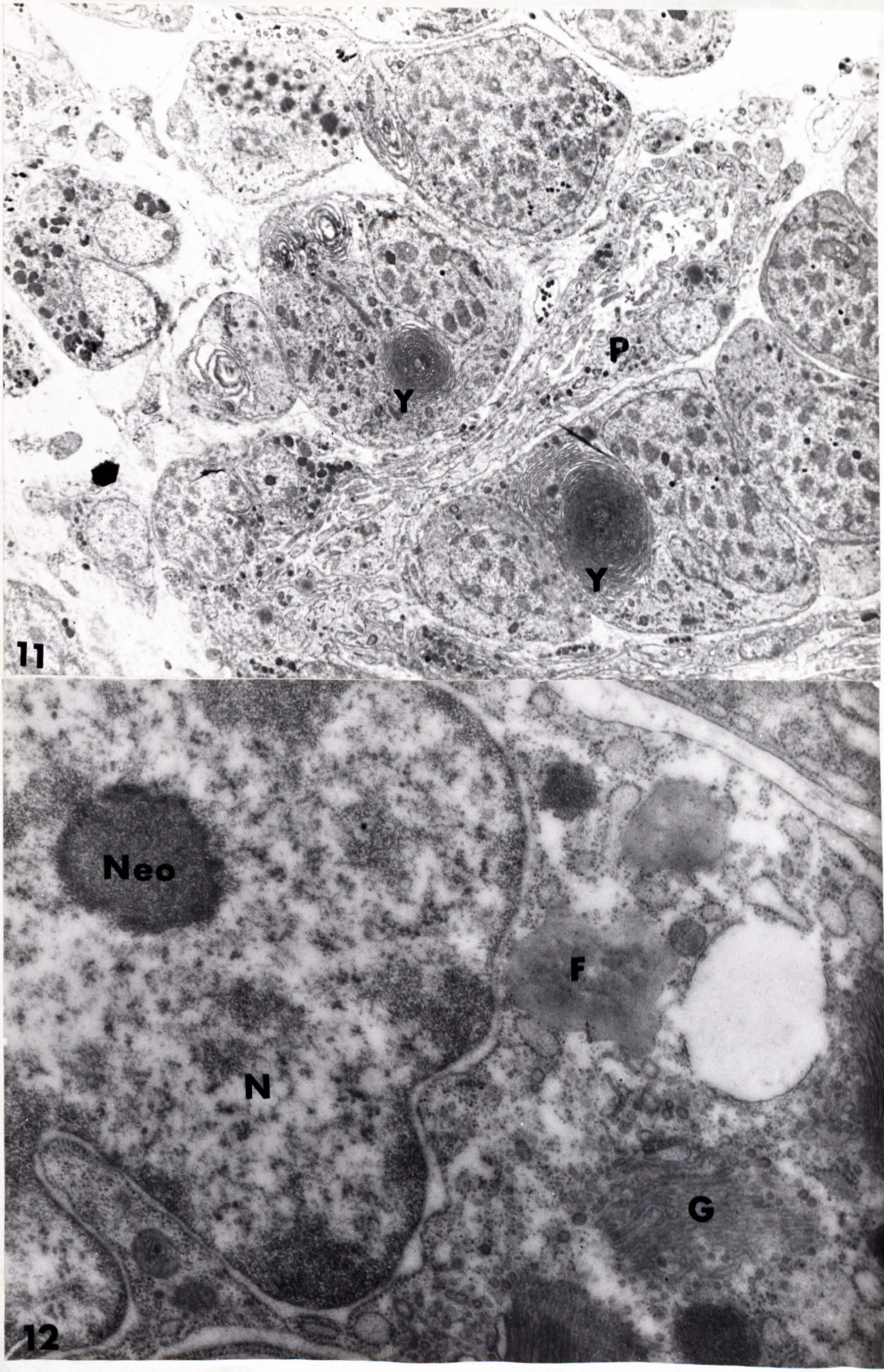

Y
P
Y
11
Neo
F
N
G
12

Fig 13 Dividing Golgi from fig 11. X 150,000.

Fig 14 Details of concentric rings of endoplasmic reticulum (Y). X 40,000.

F = fat droplets.

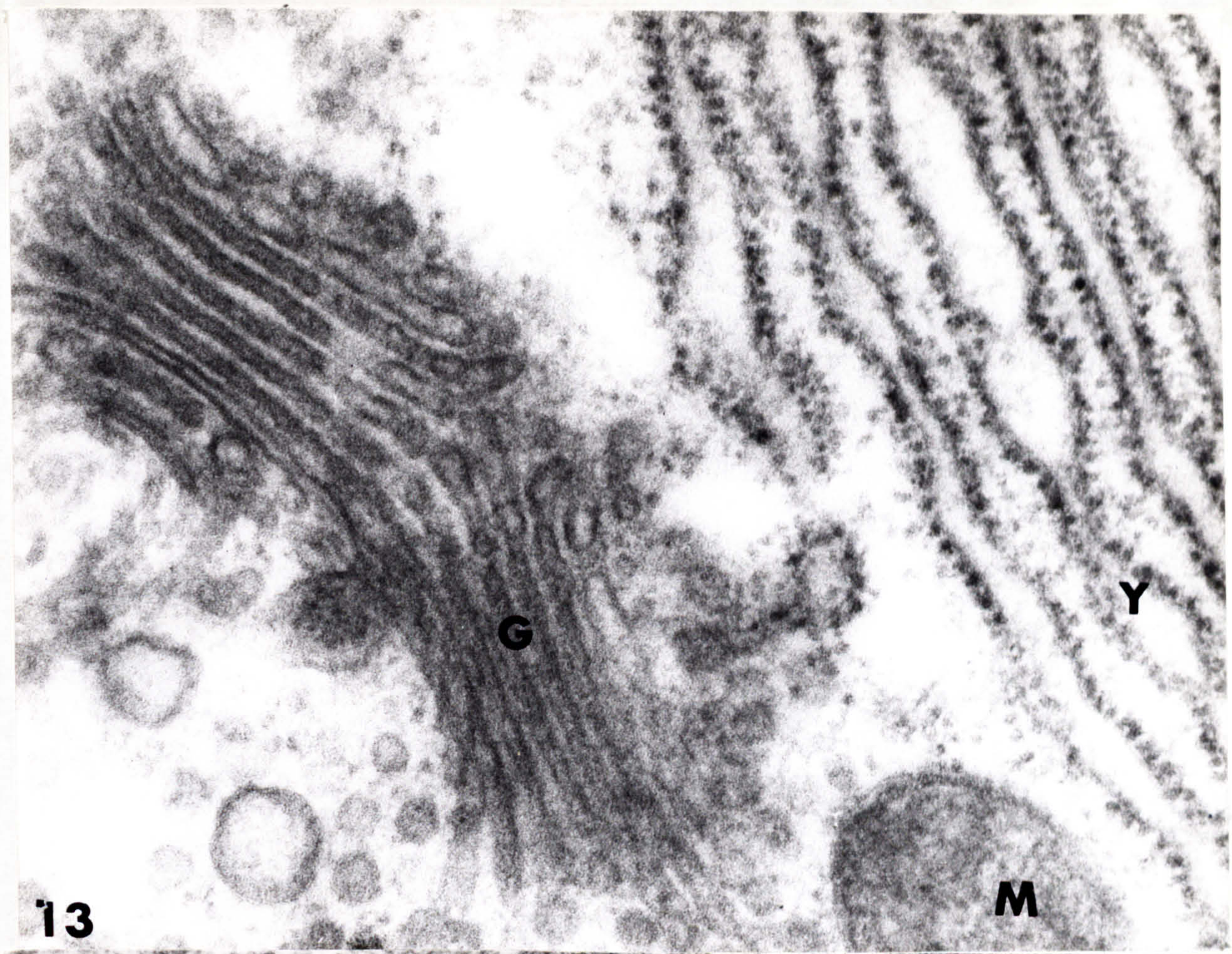
G = Golgi body.

M = Mitochondrion.

N = Nucleus.

W = Cell wall.

Y = concentric rings of endoplasmic reticulum.



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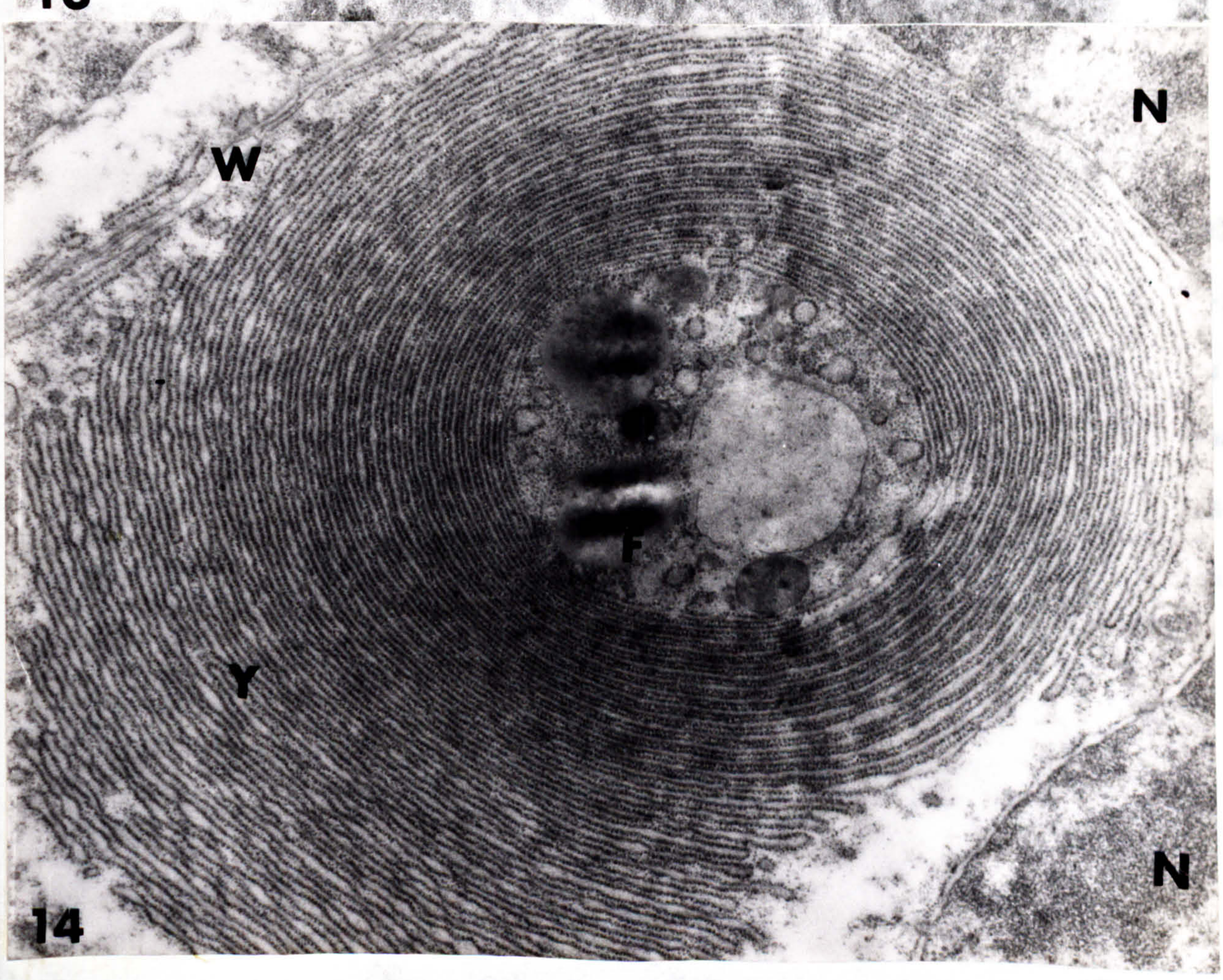

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masses of swollen rough endoplasmic reticulum. This swollen rough endoplasmic reticulum is only present in small amounts in cultured secretory cells of approximately the same stage in development. If the cells have begun secretion prior to being explanted, then secretion continues in culture. No cases of the production of secretion when it was not present in the control have occurred.

In the oviducal gland the changes are less dramatic. The epithelium becomes slightly thinner and only one cell thick. The secretory cells produce necks if they are not already present. The golgi bodies appear to become larger and thus possibly more active but the remainder of the cytoplasmic organelles remain the same.

It therefore appears that the gonad and the brain-tentacle association is capable of producing some factor which stimulates the common duct to differentiate further. This factor appears to influence the male secretory cells more than those of the female. This appears to be true even in cases where associated explants were taken from animals which possessed a female oviducal gland.

CONCLUSIONS.

From these results, it would appear that when they are cultured together there are interactions between some slug reproductive organs. The isolated gonad does not survive very well, the male cell line in particular disappearing after about two weeks. However the association of the homologous brain, homologous brain/tentacle complex and of the brain/tentacle complex from another animal in the "male" stage of reproductive development, improves the survival of the gonad and especially the spermatogenic stages. Cell divisions may also be frequently seen in such spermatogenic stages. The association of the gonad with the brain/tentacle complex of an animal in the "female" stage of reproductive development does not appear to cause such an improvement. Thus it would appear that some factor is produced by these associations of organs from animals which exhibit "male" characteristics which favours the male cell line. Furthermore this factor would appear to be produced by the brain rather than by the tentacles.

The overall survival of the gonad is also improved by the addition of 10% of tissue extracts to the culture medium. This addition favoured the female cell line while also giving some benefit to the connective tissue cells. No apparent benefit was noticed in the case of the male cell line. This improvement in the female cell line was found with all cases of

added extracts irrespective of the type of tissue. Thus there would appear to be some non-specific nutritive substance present in these extracts which is lacking in the original culture medium.

The survival of the common duct, and other parts of the reproductive system tried, was excellent when cultured in isolation. In such cases no differentiation or secretion was noticed. These organs would appear to behave as "Neutral" organs (Streiff 1966, 7) when cultured alone.

If explants of the common duct are associated with explants of gonad, irrespective of the stage of either organ, no difference is noticed when compared to the isolated common duct explants or the controls. Thus the gonad alone does not affect the common duct. The same is also true of the common duct and brain/tentacle association.

In the case of the gonad, brain/tentacle and common duct association an effect may be seen. Large onion-shaped bodies of rough endoplasmic reticulum appear in the secretory cells of the prostate. This phenomenon is probably some attempt at secretion by these cells. Occasionally the cells do in fact produce secretory granules. Since this effect is only produced by the association of the gonad and the brain/tentacle complex with the common duct, it would appear that some factor is produced

by this association which causes further differentiation or secretion of the prostate. Whether the active substance is produced by the gonad explants or by the brain/tentacle complex is unknown.

Although preliminary, these results give indications of the presence of interactions between certain explants. Such interactions require further study. However, the culture technique must be improved so that gonad and brain explants may be cultured in isolation. This probably requires some non-specific and/or specific substances which may be found in extracts of certain organs or even whole embryos. With such an improvement in the culture technique, further study of these interactions would yield much information on the hormones of Agriolimax reticulatus.

ABELOOS, M., 1943, Éffets de la castration chez un mollusque Limax maximus. C.r.hebd.Séanc.Sci.Paris, 216: 90-92.

BAILEY, T.G., 1969, A new anaesthetic technique for slugs. Experientia, 25: 1225.

BERRY, A.C. & CHAN, L.C., 1968, Reproductive condition and tentacle extirpation in Malayan Achatina fulica (Pulmonata). Aust.J.Zool., 16: 849-855.

CHOQUET, M., 1965, Recherches en culture organotypique, sur la spermatogenèse chez Patella vulgata L. (Mollusque gastéropode). Rôle des ganglions cérébroïdes et des tentacles. C.r.hebd.Séanc.Sci.Paris. 261: 4521-4524.

FILHOL, J., 1938, Recherches sur la nature des "epidosomes et les phenomènes cytologiques de la sécrétion chez Gastropodes pulmonés. Arch.Anat.Mic., 34: 155-439.

GOMOT, L. & GUYARD, A., 1964, Évolution en culture in vitro de la glande hermaphrodite de jeunes escargots de l'espèce Helix aspersa Mull. C.r.hebd.Séanc.Sci.Paris. 258:2902-5.

GUYARD, A., 1967, Féminisation de la glande hermaphrodite juvénile d'Helix aspersa Mull associée in vitro au ganglion cérébroïde d'escargot adulte ou de Paludine femelle, C.r.hebd.Séanc.Sci.Paris., 265: 147-149.

LAVIOLETTE, P., 1950, Rôle de la gonade dans le morphogenèse du tractus génital, chez quelques Mollusques Limacidae et Arionidae, C.r.hebd.Séanc.Sci.Paris., 231: 1567-1569.

———————— 1950, Sur un retard de la maturité génitale observé chez Arion rufus, Bull.Soc.Linn.Lyon., 19: 52-56.

———————— 1951, Régénérats germinaux multiples chez Arion rufus, C.r.hebd.Séanc.Sci.Paris., 233: 1139-1141.

———————— 1953, Régénération germinale après castration chez un mollusque, gastropode, pulmoné Arion rufus, XIV Internat.Cong.Zool., 233-235.

———————— 1954, Rôle de la gonade dans le déterminisme hormonal de la maturité glandulaire du tractus génital chez quelques gastropodes Arionidae et Limacidae, Bull. biol.Fr.Belg., 88: 311-332.

———————————— 1954, Étude cytologique et expérimentale de la régénération germinale après castration chez Arion rufus, Annls Biol., 30: 481-492.

———————————— 1956, Rôle de la gonade dans la maturation glandulaire du tractus génital chez quelques gastéropodes. Annls.Sci.nat.,18: 171-173.

PELLUET, D. & LANE, N.J., 1961, The relation between neurosecretion and cell differentiation in the ovotestis of slugs. Can.J.Zool., 39: 789-805.

SPEEG, K.V. & CAMPBELL, J.W., 1968, Formation and volatilization of ammonia gas by terrestrial snails. Am.J.Physiol., 214: 1392-1402.

STREIFF, W., 1966, Autodifférenciation ovarienne chez un mollusque prosobranche hermaphrodite protandre Calyptraea sinensis (L) . C.r.hebd.Séance.Acad.Sci. Paris., 263: 539-542.

———————————— 1966, Étude endocrinologique du déterminisme du cycle sexuel chez un mollusque hermaphrodite protandre Calyptraea sinensis L. I Mise en évidence par culture in vitro de facteurs hormonaux conditionnant l'évolution du tractus génital mâle. Annls.Endocr., 27: 385-400.

———————————— 1967, Étude endocrinologique du déterminisme du cycle sexuel chez un mollusque hermaphrodite protandre Calyptraea sinensis L. II Mise en évidence par culture in vitro de facteurs hormonaux conditionnant l'évolution du tractus femelle. Annls. Endocr., 28: 461-472.

———————————— 1967, Étude endocrinologique du déterminisme du cycle sexuel chez un mollusque hermaphrodite protandre Calyptraea sinensis L. III Mise en évidence par culture in vitro de facteurs hormonaux conditionnant l'évolution de la gonade. Annls.Endocr., 28: 641-656.

STREIFF, W., & BRETON, J., 1970, Étude endocrinologique des facteurs régissant la morphologenèse et la régression du pénis chez un Mollusque Prosobranche gonochlorique Littorina littorea, C.r.hebd.Séanc.Sci.Paris., 270: 547-549.

———————————— 1970, Étude comparée en culture in vitro des facteurs responsables de la morphogenèse et de la régression du tractus génital mâle externe chez deux Mollusques Gastérpodes Prosobranches: Crepidula fornicata (espèce protandre) et Littorina littorea (espèce gonochlorique)., C.r.hebd.Séanc.Sci.Paris., 270: 632-634.

WOLFF, Et. & HAFFEN, K., 1952, Sur une méthode de culture d'organes embryonaires in vitro., J.exp.Zool., 119: 381-404.

GENERAL DISCUSSION.

The aim of the studies reported here was to investigate the possible hormonal control of reproduction in *Agriolimax reticulatus*. Establishment of the various new techniques proved lengthy, and the information obtained about the hormones of this animal, rather preliminary.

An effective anaesthetic was essential and the one developed proved very reliable in this study and is now used routinely in this laboratory.

The freezing point depression values obtained for the haemolymph of this animal were found to be similar to those published in related species. The laboratory reared animals seem to have a more constant freezing point depression value.

The pH 8.86 obtained for a pooled sample of the haemolymph agreed with most published work on the subject. However, culture media buffered to this pH value were found to be unsatisfactory for organ culture. Trial and error showed that cultures survived at a pH of about 7.7 and subsequent *in vivo* pH measurements, together with published work by Speeg & Campbell in 1968, showed that the normal haemolymph of this animal had a pH of 7.72.

Disc electrophoresis on a polyacrylamide gel proved

the most sensitive technique for the separation of the haemolymph proteins. A total of 11 proteins were found, of which two appeared to be related to the sexual state of the animal. These proteins were estimated to be present in very small amounts. Separation of the individual fractions was not attempted since the organ culture technique was proving very difficult to establish.

The organ culture technique was established after the main difficulties of pH stabilisation and sterility had been overcome. It was found necessary to obtain absolute sterility since antibiotics proved ineffective at preventing infection. A combination of Ultra Violet radiation and sterile washes eventually overcame this problem. The pH stabilisation was difficult because of the relatively high pH at which the medium was to be maintained, and the high concentration of sodium bicarbonate present in the medium. The solution to this problem was found when Tricine was used as a buffer. The results obtained when organs of A. reticulatus were cultured using this technique were encouraging and organs such as the common duct survived almost unchanged. Further development of this technique to incorporate extracts of slug organs or non-specific fractions of the haemolymph might improve the hitherto poor survival of the brain and isolated hermaphrodite gland.

The structure and development of the hermaphrodite gland had been described in detail using material examined in the electron microscope. A study of the cell dynamics within the acinus and hermaphrodite ductules using radioactive markers such as tritiated thymidine, would probably lead to a greater understanding of this organ.

The structure and development of the prostate have been readily followed while those of the oviducal gland proved more difficult to understand due to distortion and fixation difficulties, especially in the later stages of development. A great deal is still to be discovered about the nature of the various secretions in these two glands. A histochemical study, followed by the use of radioactive isotopes would yield much information about these secretions.

Various organs were cultured in isolation and in association with one another using the organ culture technique developed. The preliminary results obtained seem to point to reproductive hormones similar to those discovered in other groups of molluscs. However a vast amount of work still needs to be carried out repeating the experiments described and following new lines of research. It is hoped that such research could lead to an understanding of the hormonal control of reproduction. Armed with this knowledge, combined with techniques of protein

fractionation, it should be possible to isolate active constituents from the haemolymph and from crude tissue extracts. The activity of such active constituents may easily be established by incorporating them into culture media. This might then be followed by purification, identification and possibly synthesis of the hormones. Such information may then be of use in the future control of these important agricultural pests.