Some Cytological Features of Epididymal Cells in the Rat

By B. L. REID

(*From the Department of Histology and Embryology, University of Sydney*)

With three plates (figs. 1–3)

SUMMARY

Living material was photographed by phase-contrast miscroscopy. Fixed material was stained for mitochondria or post-osmicated.

The findings show a close conformity between the pictures in living and fixed cells, except that the so-called Golgi apparatus, whilst very apparent in some zones by both techniques, in other zones is well represented after osmication but not in the living cell. Reasons for this are proposed.

Studies by earlier workers on the mitochondrial pattern are confirmed. There is an increased density and decreased length of rods down the length of the epididymal duct. The relationship of the chondriome to the osmiophil material varies in the different zones; in some, close association with permeation is found, while in others the separation is complete.

General observations upon living epididymal cells are recorded. These include the effects of different suspending media and the changes undergone by the cells after their isolation. A close study has been made of the free (stereociliated) border of the cells.

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INTRODUCTION

IT became apparent in previous studies of the epididymis (Reid and Cleland, 1957) that there were marked differences in the appearance of the cells in the different zones of the epididymal duct of the rat. In the present communication these histological differences have been given a cytological interpretation. The more conspicuous of these, Golgi apparatus and chondriome, have been closely studied in both living and fixed material, and a communication devoted solely to these structures is considered both a necessary extension to the histological studies and an essential prerequisite to an investigation of the detailed physiology of the organ.

Most of the early cytological work on the rodent epididymis was done on the mouse, occasionally on the guinea-pig, but rarely on the rat. Nassonov (1924) credits Negri with the first description of the Golgi apparatus in gland

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cells (organ not specified) by the silver impregnation method. Aigner (1900), who studied the rat epididymis, described the vacuolated area (probably analogous with the Golgi area) of zone I C or II, the fine granules of the isthmus region, as well as the juxtanuclear vacuoles of zone 4 A and B. Regaud (1901) described the granules present throughout the rat epididymis and the variations in nuclear shape. Fuchs (1902) certainly recognized the Golgi structure in the mouse epididymis but always refers to it as *Fadenknäuel*, his figures showing it in contact with the cell surface by a bundle of filaments and so discharging its secretion. Holmgren's trophospongium is undoubtedly the same area.

Benoit (1926) described the cytology of the rat epididymis. He observed the abundance of the mitochondria in body and tail and their invasion of the Golgi region in these segments. By contrast, in the mouse, he noted the relatively poorly developed mitochondria of the epithelium of this initial segment and their filamentous shape, and implies that here they do not invade the Golgi region. Benoit also homologized the trophospongium of Holmgren with the *Fadenknäuel* of Fuchs and gave an accurate description of the disposition of this material in the zones which he distinguished, viz. elongated in the *segment initiale*, rounded in the end of the head region, flattened in the body, and compactly globular in the tail. In the same paper he described the scant mitochondria and Golgi apparatus of the basal cells.

Nassonov (1924), Ludford (1925), and Parat (1928) have all contributed to the Golgi and mitochondrial picture in the mouse and more recently Laurent (1932) and Mietkiewski (1935) have studied these structures in the guinea-pig. Using more recent techniques Dalton and Felix (1953, 1954) have directed attention to the Golgi apparatus of the mouse as shown by phase-contrast and electron microscopy. Christie (1955) noted the mitochondrial pattern and described a 'supranuclear' body of osmiophil material in the mouse epididymal cell.

Throughout this paper the author has used the term 'Golgi apparatus' as applying to that osmiophil organelle more generally described as 'apparatus' or 'area', in the full knowledge that the justification for so grouping what is probably a heterogenous complex with doubtful inter-tissue homologies has only the sanction of usage and convenience.

Similarly the word 'chondriome' has been used generically to cover those cytoplasmic inclusions, including rods, rodlets, and granules, which do not allow further subdivision into mitochondrion, secretion granule, &c., from their appearances in living cells.

Materials and Methods

Fresh and fixed material has been studied. In the fixed material, small pieces of tissue dissected from the appropriate anatomical region were fixed either in Mann's fluid, which was followed by Ludford's (1926) modification of the Mann–Kopsch technique, and in Helly's fluid, followed by postchroming (48 h at 37° C in 2·5% $K_2Cr_2O_7$) and staining by the Bensley–Cowdry

(Cowdry, 1918) or Kull (1914) procedures. Blocks were embedded in paraffin and sectioned at 3 μ.

For fresh preparations similar small pieces were usually suspended in homologous serum charged with carbon dioxide and kept at 35° C, but in one experiment isotonic sucrose was used. They were then minced with scissors and teased with fine needles upon siliconed slides and promptly examined by phase-contrast microscopy. For an average preparation these manipulations were completed in 15 min after death.

RESULTS

Description of the micro-anatomy of the epididymis in these observations will conform to that proposed by Reid and Cleland (1957). A short summary of the principal histological features will precede each section.

Efferent ducts

The efferent duct is divisible histologically into two zones: (i) an initial zone characterized by a fairly tall epithelium with basal nuclei. The nuclei are smooth in contour with fine chromatin material. The cytoplasm is characterized by a finely vacuolated region occupying $\frac{1}{3}$ to $\frac{3}{4}$ of the supranuclear area, occasional large granules just beneath the surface, and a free border beset with brush or apocrine cupula terminalis; (ii) a terminal zone with absence of supranuclear vacuolation and with large numbers of small granules.

Both zones contain ciliated cells of varying height and shape with rounded nucleus and characteristic row of basal granules at the insertion of the cilia.

Initial zone. Fresh preparations of the initial zone confirm the vacuolated state of the cytoplasm. It is to be noted, however, that the living cell does not appear as translucent as it does in fixed preparations stained with eosin. Mitochondria are not clearly seen, but the cytoplasm contains granules scattered throughout the cell. The nature of these is uncertain; they have no obvious counterpart in stained material. The general appearance is of a featureless cell.

These preparations are not so informative as to the structure of the free border of the cell. Phase-contrast pictures merely show an accumulation of homogeneous structureless material whose border, both with adjacent cells and with lumen, is ill defined. This is, presumably, some secretion product, or it may be a brush border as seen at the limit of resolution in serum. Sections of material fixed in Helly sometimes show an ultrafine fringe at the free border about 5 μ tall.

Single-cell fresh preparations suggest a higher frequency of binucleate cells than was apparent in fixed tissue. In one sample of over 200 cells, 10% of the cells were binucleate.

Variation in the shape of ciliated cells as seen in histological preparations becomes very evident in single-cell preparations. Even from the same zone, initial or terminal, they vary from cuboidal through wine-glass (the shape usually figured) to elongate cylindrical. A tenuous extension at the cell base

in the shape of a 'foot' is also common. The basal granules are clear-cut. Mitochondria can be discerned throughout the cell and there are numerous granules especially about the apical pole of the nucleus in the form of a crescentic mass. Chance preparations show ciliary movement with an approximate frequency of 5 to 10 per sec.

In fixed preparations mitochondria were filamentous and evenly distributed throughout the cell, skirting the spaces which we have called vacuoles. Fig. 1, B shows this distribution.

Sections from Mann–Kopsch preparations show a rather special distribution of the Golgi apparatus. A collection of three or four osmiophil threads in randomly directed arrangement lie in the apical third of the cytoplasm only a short distance from the cell border. They surmount a featureless zone of cytoplasm immediately above the nucleus. The latter zone is devoid of cytoplasm apart from a few sparsely scattered granules (fig. 1, C). On the other hand, the ciliated cells are usually heavily blackened. The black material consists of a supranuclear 'stringy' or fibrillar mass of osmiophil substance and infranuclear lines of rodlets reminiscent of the arrangement of the mitochondria in these cells. In non-ciliated cells the osmium has no special affinity for mitochondria.

Terminal zone. In fresh preparations, the terminal zone of the efferent ducts is characterized by variations in cell size and shape ranging from spherical-cuboidal to elongate-columnar. A corresponding variation occurs in the ciliated cells. The general appearance of the cells is not greatly different from those in the initial zone. The vacuolation is not so marked, although the cells are beset with numerous granules irregularly placed about the cytoplasm, again bearing no relation to the fuchsinophil granules so conspicuous in this area in stained preparations. Narrow perinuclear spaces evident in some of these cells in fixed preparations are conspicuous in the fresh material. Fresh material does not help the understanding of the cell border. The cell here terminates merely in a structureless irregular coagulum.

In fixed preparations mitochondria are seen to be scattered throughout the cytoplasm, as in the initial zone. Pallisades of filaments occur beneath the free border of the cell and shorter plumper rods are found between the nucleus and the basement membrane.

The Golgi zone is not prominent, occurring at some distance above the nucleus although to a less pronounced degree than in the initial zone. It consists of a few interconnected strands of osmiophil material (fig. 1, D). Impregnation was never good. In no instance was there a great development of osmiophil material associated with chains of vacuoles, as seen elsewhere in the epididymal duct.

The obvious difference between living and fixed cells was the general opacity of the living cells of the initial zone, which do not appear as highly vacuolated as the fixed cells. The granules of the cells of the terminal zone, which are so conspicuous in sections stained with fuchsin, are not seen in living cells.

However, as regards other cytological details and cell shape there is a remarkable correspondence between the living and fixed cells.

Epididymal duct

Zone I A and B is characterized by very tall principal cells with apical cells interposed. There is a separate, finely vacuolated, lightly staining region immediately above the nucleus, occupying the second quarter of the cytoplasm. Nuclei are smoothly contoured. From the apical surface project long, fragile stereocilia.

Fresh preparations of dissections from zone I A and B were all characterized by optical homogeneity and low refractive index of nucleus and cytoplasm, caused largely by the absence of cell inclusions. These cells were characteristically flexible, so that when seen flowing along in a stream they would temporarily assume contorted shapes, only to revert to their tall columnar shape when at rest. No such variation in shape was seen in cells from elsewhere in these ducts.

Favourable resultion of stereocilia showed them as a bundle of about 10 slender, immobile hairs, projecting in the long axis of the cell from the central portion of the free surface for about $\frac{1}{3}$ to $\frac{1}{2}$ of the length of the cell. Mitochondria were often seen as long filaments lying haphazardly down the length of the cell but were most numerous in the inner half (with respect to the lumen). Apical cells were often found and mitochondria were conspicuous in them. The presence of the Golgi area was inconstant and this could not be related to any other observation, e.g. the state of surrounding cells, the state of mitochondria, or the presence or absence of nucleoli. When present it took a roughly spherical shape. It was separated from the nucleus by a thin strand of cytoplasm and composed mostly of rows of vacuoles peripherally enclosing an area of the same appearance as the remainder of the cytoplasm.

The nucleus in these cells has a refractive index nearly the same as that of the cytoplasm. When apparent, the nuclei lie at some distance from the basement membrane. In fresh preparations little can be seen of the nuclear membrane or nucleoli. Binucleate cells were seen just as is the case in the efferent duct cells.

Postchromed preparations for mitochondria confirmed the picture of the randomly orientated, long filaments seen in living cells. They lie mainly parallel to the length of the cell and are especially concentrated toward the apical half of the cell. The concentration referred to is relative only, since the mitochondria of zones I A, B, and C are nowhere as dense as in more caudal zones. The Golgi area is free of mitochondria.

The fraction impregnated by osmium methods conforms to the vacuolated area in the living cells and consists of a well-defined area on the luminal side of the nucleus about twice the length of the nucleus and separated from it by a small strip of cytoplasm. Favourably impregnated areas of tissue show the osmiophil material to be generally distributed over the supranuclear area; the periphery of this area is vacuolated. The occasional apical cells which were

impregnated showed osmiophil material extending from the supranuclear position along one side of the nucleus into the pointed base of the cell. This extent does not correspond with the vacuolated area in the living cell.

With regard to specificity, the whole question of osmium impregnation of the epididymis is unsatisfactory. Certain zones always seem to impregnate well (e.g. zone I C and II), others well here and there only (zones IV, V, VI), whilst others are most capricious, with only scattered cells in samples of several dozen tubules binding the osmium (coni vasculosi, zone I A and B). No reason can be given for these observations. Similar observations by Nassonov (1924) and Parat (1928) on the irregularity of impregnation are amply confirmed. Even in adjacent cells of otherwise satisfactory preparations one may see an impregnation, beautiful in every detail according to prevailing concepts of this organelle, on the one hand, or merely a small amorphous group of osmiophil granules, on the other.

The conspicuous supranuclear vacuolated area in histological preparations is identified with the Golgi area of osmium preparations but not always with a vacuolated area in living cells.

Living cells confirm the length and tenuousness of the stereocilia and of the long filamentous mitochondria in zone I A and B.

Zone I C is a shorter epithelium than I A and is characterized by an especially prominent, supranuclear, clear vacuolated zone, which, toward the end of the zone, becomes less prominent although increasing in volume. The nuclei show regular alignment a few microns from the basement membrane.

The majority of cells in zone I C have terminal processes almost as long as the cell itself and usually projecting at an angle of approximately 45° from the cell surface. Often entrapped in these filaments is an ovoid, homogeneous secretion-globule. The precise relationship of the globule to the filaments could not be resolved. The most favourable preparations suggested that the globule was leaving the cell along a column made by the filaments. The problem may be clarified by electron microscopy. No filamentous continuity between stereocilia and Golgi area as figured by Fuchs was ever seen despite careful search.

The Golgi area is a very conspicuous feature of the living cell in this zone. The area, as suggested by the fixed histological preparations described above, is relatively large, occupying the middle three-fourths of the cell. The area is usually predominantly vacuolated, the vacuoles occurring in chains around the periphery. Occasionally the vacuolation is much reduced so that the area cannot be delimited from the cytoplasm elsewhere. No other correlated cytological conditions seem consistently related to the presence or absence of a Golgi zone.

Space not filled by the nucleus, Golgi apparatus, and mitochondria is occupied by rounded granules. They are similar in form and distribution to granules found in all the other living cells examined.

The nucleus is well defined and rounded, separated from the base of the

cell by only a small quantity of cytoplasm containing the granules referred to immediately above.

The basal cytoplasm, sometimes rounded as in zone I A and B, is squared in contour or drawn to a point at one angle of the square as if underlying the rounded base of an adjacent cell.

Sections of zone I C stained for mitochondria show filamentous rods filling the apical zone of cytoplasm and ranging down on each side of the Golgi area to skirt it and the nucleus, so terminating in the basal portion of the cell as a collection of shorter rods and granules. Most sections suggest that the bulk of these filaments form a cylindrical investment to the whole of the cell content, so that when the apical portion of cytoplasm is crowded one may suspect a tangential (more peripheral) plane of section. Transverse cuts exemplify this contention.

Sections of material impregnated with osmium show a very prominent mass of osmiophil material immediately above the nucleus, occupying up to 50% of the cell volume. Rims of osmiophil material surround chains of vacuoles distributed approximately linearly in the direction of the lumen. There may be three to five of such rows occupying the width of the cell. There is no doubt that this (or its equivalent in other rodents) is the epididymal zone used in most studies of the Golgi apparatus previously published (Fuchs, 1902; Ludford, 1925; Nassonov, 1924; Dalton and Felix, 1954).

There is a close similarity in the appearance of living and fixed cells in this zone. The living cells exhibit a better-preserved terminal structure, and the length of the stereocilia is much more apparent. Otherwise cytological details of cell size and shape, Golgi area, chondriome, and nuclear size and position are similar.

Zone II. The distinguishing feature is the presence of vacuoles, often of considerable size, in the apical $\frac{1}{4}$ to $\frac{1}{3}$ of the cytoplasm. The nuclei are regularly arranged basally. There is no very prominent clear cytoplasmic area as in preceding zones of the epididymal duct. Within this zone there is variation to the extent that the more posterior portion has a shorter epithelium with fewer or even no apical vacuoles.

Cells of fresh preparations from zone II, like their fixed and stained counterparts, are identified by the conspicuous vacuolation of the apical cytoplasm. As has been remarked, there is considerable variation down the length of zone II in this vacuolation. This is confirmed in the wet preparations. Dissections from the junctional area with zone I C show a shorter, plumper cell with few apical vacuoles. Samples from the junctional area with zone III similarly show a shorter, plumper cell, whilst in the bulk of the zone are typical tall columnar, highly vacuolated cells. The vacuoles, which all show similar degrees of translucency, are distributed as follows. One constant set is immediately supranuclear, arranged in a long U-shaped chain open apically, the centre of the U occupied by more opaque material, also beset with smaller vacuoles. This is the classical Golgi area, so conspicuous in this and adjacent zones I C and III; it may or may not be connected directly by a vacuolar

chain with the apical vacuoles which occupy the luminal third of the cell. These vacuoles, two to seven in number and becoming smaller as their number increases, may be found either fenestrating the attachment of a conical terminal secretory mass to the cell, within the apical third of the cytoplasm, or forming a chain down one side of the cell to connect with the Golgi area group.

Mitochondria are prominent in preparations less than one-half-hour old, but after this they either lose their refractility or become granular, so losing their identity amongst the other cytoplasmic components. They are long and filamentous and scattered throughout the cell, sparing the Golgi region and the luminal end of the cytoplasm immediately beneath the border. They may, however, infiltrate between the apical vacuoles.

The stereocilia are shown by these preparations to be three-quarters of the length of the cell, protruding directly out in the direction of the long axis of the cell. Their bases are surrounded with an amorphous mass of low refractility; this is probably secretion, which in most cells is present as a low flat cap over the free surface but occasionally prolonged into a conical or bifid villiform protuberance, the base of which is often vacuolated.

The refractility of the nucleus is low. There is a well-marked nuclear membrane and the nucleus rests against the basal pole with a collection of highly refractile, dark granules between it and the pole of the cell. These granules appear to be continuous with a row of granules of similar refractility lying beside the nucleus and Golgi area, and others scattered in the apical cytoplasm.

Altmann preparations show a distribution of mitochondria similar to that in zone I C, in that rows of smaller rods than those in zone I C are linearly distributed within a cylinder around the periphery of the cell, invading the centre of the cell only at each extremity. Their number appears to be in reciprocal relation with the apical vacuolar content of the cell. The distribution is best confirmed in transverse sections of the cell (fig. 2, c).

Tissues impregnated with osmium confirm the wet preparation picture of the Golgi area. Three or four long chains of vacuoles surrounded by an osmiophil rim occupy the central half of the cell length in a site immediately above the nucleus. The nuclear ends of the chains are more or less defined at one level from cell to cell but the apical end tapers off into the cytoplasm in straggling fashion and the ends of some columns, at least, become continuous with the apical vacuoles. These vacuoles, in turn, are devoid of osmium impregnation (fig. 2, c).

The vacuoles of the Golgi region and those situated apically are seen equally clearly in the living cell, but in the fixed cell only the apical vacuoles are prominent. Otherwise the distribution of the mitochondria, the shape and position of the nucleus, and the appearance of stereocilia is similar in living and fixed cells.

Zone III. The epithelium is much shorter than in the preceding zones and the cells have a supranuclear clear area which occupies most of the cytoplasm

in this region. The shape of the nucleus is irregular, varying from ovoid to more or less rectangular. The margin is often notched or folded. Stereocilia are not prominent in this zone.

Unlike the cells of other zones, zone III cells prepared from fresh bits of tissue show cytological detail very clearly. Two cell types appear.

The commoner type is a short, rectangular cell usually without stereocilia (occasionally short, vaguely defined hairs can be seen, about one-quarter of the length of the cell). Homogeneous, slightly refractile material, presumably secretion products, is present in a few cells, where they usually form a low cap or flattened cone.

The bulk of the supranuclear portion of the cell is occupied by the Golgi area, but it is not always as sharply defined from the general cytoplasm as in previous zones because of its content of mitochondria and granules, and because it is not so strikingly vacuolated. The general conformity of its vacuoles to a spheroid, however, suffices to delimit it quickly in wet preparations.

The nucleus is usually conspicuous and its membrane is clearly notched and often folded. Occasionally a very prominent refractile nucleolus is present.

Characteristically the outline of the basal border of the cell is square rather than round, and contains an area beset with large numbers of small, highly refractile granules. Whilst such grouping and refractility of basal granules is common in wet preparations throughout the duct, it appears particularly strikingly in zone III.

The less common cell type is about 10% longer and is narrower, usually with a more rounded nucleus and a greater content of cytoplasm above the Golgi area (fig. 2, F). It is found mixed with the commoner cell in most samples of zone III and on reference to fixed sections, groups of the taller cells are scattered side-by-side with shorter cells.

Preparations stained for mitochondria reveal large numbers of small rods and granules crowded into the thin rim of cytoplasm surrounding the Golgi area and even invading this area. These surround the nucleus and are collected again at the cell base as a clump of granules. The identity of this collection with the refractile granules of the wet preparations has not been established, but seems likely. Nassonov's (1924) and Benoit's (1926) observations of the increasing density of the chondriome in this *unter* portion of the organ is verified in the case of zone III.

The negative image of the supranuclear clear zone referred to above and of the corresponding area in wet and Altmann preparations is brought into strong relief in osmium preparations as a system of tortuous rods. Vacuolation of the rods is present but not as commonly nor as conspicuously as in the previous zones. In this respect the zone simulates the efferent ducts more closely. The Golgi apparatus occupies some 50% of the cell volume, extending from immediately above the nucleus to just beneath the free surface. This relationship holds for both shorter and taller cell types.

The prominence of the Golgi area as a negative image in standard histological preparations and as a positive image in osmium preparations is in marked

contrast to the picture in the living cell, where the Golgi area is not picked out from the remainder of the cytoplasm. However, other features are similar in fresh and fixed preparations, e.g. the lack of prominence of stereocilia, the short mitochondrial rods interspersed with Golgi area, and the basal, irregularly shaped nucleus, whose margin is folded.

Zone IV. This zone is characterized by the presence of prominent juxta-nuclear vacuoles, many of which indent the nucleus. The vacuoles may be either supranuclear or perinuclear in extent. The outline of the nucleus is very irregular, owing to marked notching and folding of the nuclear membrane.

Conspicuously vacuolated cells, most of whose cytoplasm is given over to a meshwork of vacuoles, occur very commonly in zone IV. These cells have been designated 'clear cells'.

Wet preparations of zone IV show the juxtanuclear vacuoles to contain an irregular, highly refractile mass occupying about 85% of the volume of the vacuole; the remainder of the content is a clear rim or moat about the mass. An indentation of the nuclear membrane is opposed to the vacuoles, and these preparations show this apposition to be very precise. Chains of vacuoles, often of smaller size than those immediately in association with the nucleus, lead up toward the cell surface and each contains its own irregular refractile mass.

The luminal surface of the cell is lost in a narrow, indistinct, amorphous zone, probably secretion. Stereocilia are not prominent.

The Golgi area is not prominent and is permeated by mitochondria, other granules, and the rows of vacuoles referred to above, rather similar to those of zone III. Vacuolation in the Golgi area itself is not obvious.

In fresh preparations, mitochondria fill the cell throughout its extent except the nucleus. As slender short rods they are often seen disseminated in the Golgi area and concentrated at the cell base.

Indentation of the nucleus reaches extremes in this and the following zones. Notching of irregular depth covers the whole of the surface. The nucleolus is usually prominent.

From its relatively high position in the cell, the nucleus has beneath it a quantity of cytoplasm containing the usual refractile granules and mitochondria.

No recognizable clear cells have been recorded in wet preparations, but within many clumps not broken up by teasing there were certain cells, which, although not recognizable when isolated, were presumably cells of this type.

Zone IV B is characterized by the position of the juxtanuclear vacuoles, which are both infra- and perinuclear as well as supranuclear. Otherwise the content and relationship to nuclear notching is similar.

The Golgi area and chondriome are similar to those of zone IV A, and the high incidence of binucleation referred to by Reid and Cleland is confirmed. The nucleolus is usually as prominent as in zone IV A.

The mitochondrial arrangement in stained preparations is characteristic. Very numerous short rods crowd all the space in the cell left by the nucleus

and vacuoles, in marked contrast to more anterior zones I and II. Running down the side of the nucleus in three or four lines they aggregate in greater numbers in the basal cytoplasm (fig. 2, I). There are large numbers of basal mitochondria in these cells. As in the more anterior zones, the more basal mitochondria are shorter rods and granules.

The striking feature of osmium preparations of this zone is the marked osmiophilia of the juxtanuclear vacuoles and of the chains of vacuoles as they proceed toward the cell surface. The Golgi area itself extends the full width of the cell and is not markedly rounded as in more anterior zones, the overall effect being the production of a uniform band around the lumen of the tube. In common with zones V and VI, vacuolation of the network of osmiophil rods comprising the area is not conspicuous; these rods seem solid throughout all grades of osmium impregnation.

Clear cells are common in this zone and are represented as interlacing threads or small rods surrounding large numbers of vacuoles, giving an overall sponge-like appearance. The threads are too tenuous to assess a real colour value in Altmann-type preparations but they resemble mitochondria. The nucleus is often in the apical half of the cell and may be pycnotic. Osmium preparations show substantially the same arrangement of interconnected rods, each osmiophil, and although the rods are too slender for accurate resolution there appear to be three or four of them surrounding a nearly spherical space. The impression is, again, that the rods are mitochondria.

The so-called 'vacuoles' of ordinary histological preparations are seen to be almost filled in life with a highly refractile mass. For the remainder of cell structures, e.g. free surface, mitochondria, Golgi apparatus, and nucleus, there is close conformity between the appearances of fresh and fixed cells. Thus the Golgi area is not prominent in either kind of preparation.

Zone V. In this zone the juxtanuclear vacuoles decrease in number until they are absent. The nuclei have irregular outlines and are roughly rectangular. The nuclear chromatin, as in the above zones, is distributed in finely granular form. The infranuclear cytoplasm may be large (zone V A) or very small (zone V B) in amount.

Clear cells are especially abundant. Binucleate cells are also common.

Living cell preparations from zone V are favourable subjects for phase-contrast microscopy for the reasons given above. Suspension in serum shows a hazy coagulum at the cell surface which is about 10% of the cell height. In isotonic sucrose solutions a mass of fine hairs is seen projecting almost vertically from the cell surface. The Golgi zone is vaguely defined in most cells. It is strewn with mitochondria as small rods, and with granules. Occasionally some cells show a well-defined, rounded, vacuolated area occupying 90% of the supranuclear cytoplasm. The nucleus is relatively large, notched, and indented; it contains well-defined nucleoli. This difference in refractive index between nucleolus and nucleoplasm is a feature of zones V and VI. Binucleation is common.

The subnuclear cytoplasm is variable in amount and is characterized by a

content of large numbers of rodlets. No increased translucency or vacuolation of this cytoplasm was seen, such as characterizes zone V A in fixed material.

Juxtanuclear vacuoles of similar form and content to those of zone IV are found in random cells. They are quite distinctive, though not as numerous as in zone IV.

Preparations of postchromed material confirm the density and form of the chondriome as seen in wet preparations, i.e. large numbers of rodlets scattered diffusely throughout the cell, unimpeded by any other organelle.

The Golgi picture is similar to that of zone IV in that there is a tangle of osmiophil rods occupying most of the supranuclear cytoplasm abutting closely on the Golgi area of the neighbouring cell; there is no obvious vacuolation.

There is a general conformity between the appearance of fresh and fixed cells as regards the free surface, cell shape, and nuclear shape. However, the vacuolation of the Golgi area occasionally seen in living cells is not seen in osmicated preparations. Further, the qualitative difference between the cytoplasm above and below the nucleus in zone V A with respect to lightness of staining and appearance of fine vacuoles is not apparent in the living cell. The good definition of nucleoli in living cells of zone V is not a feature of fixed cells.

Zone VI is characterized in histological preparations by the presence of coarse chromatin in the nucleus; it is adherent to the nuclear membrane. Binucleation is common and the cytoplasm is homogeneous in haematoxylin / eosin preparations, but contains large granules in sections stained with acid fuchsin. Clear cells are common.

The characteristic feature of the cell picture in wet preparations of zone VI is a variation in shape, which ranges from ovoid-spherical to tall-columnar. In fixed preparations, on the contrary, the epithelium is low and of nearly uniform height. The long axes of each cell must be at varying angles to one another, that of the shortest cells being perpendicular to the basement membrane, that of the longest being very nearly flat or tangential. Careful observation of cell boundaries in fixed material confirms this. Vacuolation is a common feature in the supranuclear region. The cytoplasm is strewn with granules which are larger than mitochondria, have a greater refractility, and appear to be concentrated in the tapering base of these cells. The unusual distribution of chromatin, so typical of fixed preparations, is not a feature of living cells.

The form and distribution of mitochondria in fixed preparations is similar to that in zone V. The granules referred to in the living cells are not obviously identical with fuchsinophil granules common in these cells in Altmann preparations.

The Golgi area, as seen in osmicated preparations, is similar to that in zone V.

The true constitution of the epithelium of zone VI is not revealed by mere inspection of a histological preparation. The variation of cell shape in the wet preparations reveals the true constitution. The nucleus of the living cell fails

to show the chromatin distribution so characteristic of sections stained with haematoxylin.

General observations on living cells

Apart from the formal description of the structure of living cells, a number of general points emerged during the study of numerous preparations.

With attention to minor detail of slide preparation, fresh serum, and temperature control, cells showed no observable change in cytological detail beyond the following observation. Up to half-an-hour after mounting (i.e. $\frac{3}{4}$ to 1 h after death of the animal), cells from most zones are difficult to perceive except as a collection of spherical granules interlaced with mitochondria. This is particularly true of zone IV A and B. The cells resemble a conglomerate of spheres of varying size, with no cell or nuclear outline visible. After this time, quite insidiously, the cell outline materializes, the nuclear membrane becomes distinct, and fine hairs, the stereocilia, are seen. No illustrative photographs are available, since the changes are below the resolution limits of the photomicrographic apparatus used. Comparison of exposures before and after this change fail to bring out its subtlety. After 48 h (and sometimes as early as 12 h in zone II), clear vesicles appear from random points on the cell surface, reaching up to 50 μ in diameter after a few hours, evidently by coalescence, and distorting the cell in bizarre fashion. Short of this gross disruption there is no evidence from close scrutiny that the cell detail is altered in any way from that in well-fixed material.

Apart from the ciliary movement found in efferent duct cells there is no appreciable movement in other cell components. Thus stereociliary hairs, vacuoles in the Golgi area and those near by in the apical cytoplasm (zone II), the secretion cupolas at the cell surface, and the nuclear membrane were unchanged over prolonged periods of observation. Nothing equivalent to the movement of pancreatic zymogen granules (Hirsch) or to currents, as in plant cells, which might account for the transport of particles of pigment (Mason and Shaver, 1954), was to be found.

Preparation of suitable disaggregated cell suspensions varies with the zone. Both efferent ducts and epididymal duct down to the end of zone II are difficult to fractionate in this respect. In contrast, more caudal zones produce a rapid yield of free cells. In any case, merely allowing the tissue to remain at 35° with or without added homologous serum, provided steps are taken to avoid dehydration, will produce free cells after an interval of about 2 h. There is no resolvable difference in cells prepared thus compared with those sampled immediately after death, and left under the microscope for an equal time. In other words, cells left for some time before teasing have apparently undergone the small phase-change referred to above, whether they were in the organ or on the slide. The enzyme hyaluronidase was used in an attempt to facilitate disaggregation, without appreciable effect.

The value of varying the refractive index of the suspending agent was borne out when resolution of fine structure like stereocilia was required. When

the stereocilia were very short or tenuous, more watery media (isotonic sucrose) gave much better visibility.

Discussion

Integration of these findings with those of earlier workers has been made difficult by the inability to localize the zones to which they referred by reference to the gross anatomy of the ducts. However, the non-motility of the stereocilia is amply confirmed and the much figured Golgi area of zone I C and II of the studies of Aigner (1900), Benoit (1926), Nassonov (1924), Ludford (1925), and Dalton and Felix (1954) is placed in its correct sequence down the length of the tube. The distribution of mitochondria supports the findings of Nassonov, Ludford, and Benoit as regards increasing density and decreasing size toward the tail, and absence from the Golgi area in cranial zones. These authors have generally stressed the basal concentration of mitochondria, which is amply confirmed here. Besides mitochondria, wet preparations show that granules, although occurring ubiquitously in the cytoplasm of all cells studied, are also concentrated basally. There was no relationship between these most obvious basally situated granules and any cell component of fixed preparations stained for mitochondria, except in zone VI. Benoit raised the question of association with ergastoplasm in this connexion; alternatively it may be associated with some cell activity in relation to the basement membrane, e.g. secretion.

The problem of the exact nature of the free cell surface in the epididymis is still in doubt. Homologous serum has disadvantages, as has been pointed out, but even with more watery fluids it is difficult to resolve any organized structure in the end of the efferent duct cell, although postchroming methods do reveal a brush border on some cells. This accords with Benoit's observations. He draws attention to the similarity of such terminal processes to those in other secretory cells, e.g. the gut and placenta. The classical stereocilia of zones I and II appear in these studies to be no more connected with cell activity than that they occasionally enmesh a secretion globule. This agrees with the description of Fuchs on this point, although observation failed to reveal any intracytoplasmic connexion with the Golgi zone as figured by this author. His preparations, however, were of the mouse, where they may be more obvious, and he used iron haematoxylin staining. Likewise Laurent (1932) traced the course of the hair roots to the supranuclear region in the epididymis of the guinea-pig, but gives no illustrations. This has not been found in the rat. The main feature brought out by the examination of living cells in this study is the extraordinary length of these hairs, and the ease with which they are damaged mechanically. Where the cell is disaggregated with a minimum of teasing, e.g. zone III, long stereocilia are regularly seen. The hairs appear to be rigid throughout their length, which would weigh against the suggestion that they might be a mechanism for increasing the secreting surface, after the style of an overgrown brush border.

The position of apocrine secretory knobs is confirmed in efferent ducts and

in zone I, occasionally in zone II. Benoit thought they did not exist in the living cell. Whilst this would be very difficult to investigate, having regard to the opacity of the most tenuous efferent ducts, it does not seem likely that gross and rapid changes in shape follow the death of the animal and the subsequent isolation of the cell.

The findings here presented bear at some points on the enigma of the Golgi complex. Where it is represented in what has been called classical form (zones I and II), the structure of the complex in living cells accords closely with the detailed account given by Baker (1945, 1957). A substantial proportion of cell area near the nucleus is given over to chains of vacuoles in spheroidal mass surrounding a central, less vacuolated area. The osmicated material accords point-for-point with this interpretation and the age-old contention of artifacts in such impregnation is no longer significant. However, in zones III, IV, V, and VI, where there is a more or less well-defined vacuolated area in living cells (although not as clear cut as in the classical zones), the corresponding osmicated areas are not vacuolated, but more in accord with the usual description of a network of osmiophil rods or plates (the 'classical metazoan' structure of Benoit). Nassonov (1924) suggests that the degree of impregnation determines the appearance of vacuoles. Whilst this may be true of zones I and II (his plates seem to correspond with zone II), even the lightest binding of osmium in more caudal zones fails to reveal chains of vacuoles. Benoit also observed the absence of vacuoles in the Golgi of the tail region, describing it as a network of black trusses with irregular contours. No such counterpart could be found in the living cells and one must conclude that in these specific zones the Golgi complex *is* indeed altered by osmium impregnation. In summary, the concordance of findings in living and osmicated material is dependent on the zone under consideration. This, of course, does not elucidate how the osmium alters the vacuolated area in the zones concerned. It is of interest here to correlate this with the other clear-cut distinction between the two groups of zones in question; that is to say, the intimate association of the chondriome with the Golgi area in the caudal groups. This is one major factor in the lack of precise definition of the Golgi picture. Dalton and Felix (1953) refer to and picture the Golgi network, but obviously only one optical section can be photographed and their illustrations conform more nearly to the concept of a system of rods associated with vacuoles in a spheroidal mass than to a network. Such condensed positive images were rarely seen in the cells that formed the subject of this study.

The continuity of the vacuoles of the Golgi complex with those of the apical cytoplasm in zone II was seen many times in fresh and fixed material and the original observations of Fuchs in this respect were amply confirmed. The association is more vivid in the fresh cells, where the vacuoles of both parts show equally clearly, than in routine stained sections, where the vacuoles of the Golgi area take up more of the counterstain. Elsewhere in the epididymis the association of the Golgi area with cell function is not obvious from these studies. Parat and Nassonov remarked on the variability of the effects of

osmication. One should avoid drawing conclusions too confidently from the study of osmicated preparations. A recent paper by Baker (1957), pointing out the variety of chemical combinations into which osmium tetroxide can enter and the subsequent physical union that it can make with non-specific substances, is interesting as a background to the possible causes of the variety of effects mentioned by Parat and Nassonov.

The picture presented here of the mitochondria is substantially in agreement with that obtained in earlier studies. Their transient appearance as rods in living cells may be simply due to the violence of handling during teasing. The interesting point is that the time of disappearance varies in random fashion from ½ to 4 h and a traumatic cause is more likely than some deficiency in the medium. The mitochondria were the most labile of the cell components studied.

The association of mitochondria with the Golgi area in caudal zones may be significant from the viewpoint of the proponents of the direct transition theory. On the other hand, they may represent a granular secretion product of the Golgi apparatus in those zones, as initially proposed by Fuchs and supported by Nassonov.

The basal concentration of mitochondria may be of significance in connexion with a suggestion of Benoit that in the epididymis of the horse, nuclear material in the form of granules remains in the basal cytoplasm, awaiting transfer across the basement membrane to near-by wandering tissue cells and so to capillaries.

The lack of appreciation of the sequence of zones has led, in the past, to a good deal of confusion as regards the significance of the juxtanuclear vacuoles of zone IV. They do not seem to have been separated from the Golgi apparatus either morphologically or physiologically by Benoit or Ludford. The latter author uses the term 'complex granules' for these vacuoles after Nassonov, who, in turn, described a large granule surrounded by a fluid space in cells of the seminal vesicle of the mouse. There is no doubt from Nassonov's description that he refers to a similar structure as the contents of the juxtanuclear vacuoles of zone IV: 'Jeder Granula stellt ein stark glänzendes gelbliches Körnchen vor, welches von einem hellen Rande von Wahrscheinlich flüssigerer Konsistenz umgeben ist.' Moreover, from his plates the vacuoles do appear to be intimately associated with the Golgi apparatus. However, in the present study, an entire sequence of stages from intimate contact with the nuclear membrane to the typical row of vacuoles along one side of the cell in both vital and fixed material show that these extraordinary envacuolated granules are apparently more concerned with the nucleus than with the Golgi apparatus. The other proposition, put forward first by Benoit in 1921 for the bull, confirmed by Ludford for the mouse, but subsequently refuted by Benoit in 1926, was that these vacuoles may enclose nucleolar fragments extruded by the nucleus. The close apposition between such vacuoles and an indentation of the nuclear membrane would easily account for such a false impression from all but the most careful observation in living

cells. This is Benoit's explanation of his earlier misapprehension and is borne out in these studies. Christie (1955) recognized them as closely related to the nucleus. He called them juxtanuclear bodies and showed histochemically that they consisted of phospholipid and cerebroside, the phospholipid being distributed peripherally as a thin rim; this may well correspond to the fluid space of vital preparations. Baker (1957) draws attention to the presence of similarly constituted 'globules' in the sympathetic ganglion of the rabbit. In presaging the ubiquitousness of these globules in other cells Baker suggests the name *cerephos* globules.

The status of the 'clear' cells of zones III and IV is not clarified by these studies. The findings are in agreement with Nassonov as regards the degeneracy of the Golgi area, although there is no evidence of his concept of its explosive rupture and dissemination throughout the cell. Nuclei are not always pycnotic and the cell often appears to be formed by the confluence of three or four cells. The failure to recognize these cells in living preparations is interesting and may mean that they are unusually fragile and do not survive teasing.

Polymorphism of nuclei in zones III and IV, first studied by Regaud, is fully confirmed. Most elegant pictures of nuclear pattern are seen in living cells. However, there is no evidence from this study that the deep fissures, which almost transect the nucleus, proceed to nuclear division, as first postulated by Regaud and supported by Benoit. Although their frequencies vary, there are binucleate cells in all parts of the epididymis, unrelated to nuclear folding. The problem must await quantitative studies, for short of actually seeing the two portions move apart, the contact between them is extremely intimate and the plane of transection would not allow the two separate parts to be seen until they were well separated. By that time, of course, a normal mitosis would have effected the same result.

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FIG. 1 (plate). A, efferent duct of the terminal zone. 3-μ paraffin section of material fixed in Helly, postchromed 2 days at 37°; stained by toluidin blue, aurantia. The mitochondria in the apical half are filaments, clustered together subapically, whilst basally they are shorter rods. Conspicuous granules surmount the nucleus in cone-like masses.

B, efferent duct of the initial zone. 3-μ paraffin section of material fixed in Helly and postchromed 2 days at 37°; stained by toluidin blue, aurantia. The apical mitochondria are filamentous, the basal are shorter rods.

C, efferent duct of the initial zone. 3-μ paraffin section of material fixed in Mann's fluid; Ludford's modification of Mann's method of osmication. Two well-defined ciliated and several principal cells are seen. The osmiophil material in the former is distributed as a supranuclear cap. This is continued into the cell base as a mass of filaments and granules. In the principal cells, the osmiophil material surmounts an unusually clear area of cytoplasm at some distance from the nucleus.

D, efferent duct of terminal zone. 3-μ paraffin section of material fixed in Mann's fluid; Ludford's modification of Mann's method of osmication. The osmiophil strands, representing the Golgi area, are situated much closer to the nucleus.

E, zone I A. 3-μ paraffin section of material fixed in Mann's fluid; Ludford's modification of Mann's method of osmication. The Golgi area is immediately supranuclear and osmiophobe vacuoles may be seen in it. The area is quite small compared to the length of the cell. The nucleus occurring between the middle and right-hand cell borders is that of a 'halo' cell.

FIG. 2 (plate). A, zone II. 3-μ paraffin section fixed in Helly, postchromed 2 days at 37°; stained by toluidin blue, aurantia. The mitochondria are short rods, peripherally distributed. The apical vacuoles characterize this zone histologically.

B, zone II. 3-μ paraffin section of material fixed in Mann's fluid; Ludford's modification of Mann's method of osmication. The Golgi area is extensive and composed of 3 or 4 rows of osmiophobe vacuoles surrounded by osmiophil rims. The sharp contrast between osmiophilia of the Golgi and the apical cytoplasmic vacuoles is evident.

C, zone II. 3-μ paraffin transverse section of material fixed in Helly, postchromed 2 days at 37°; stained by toluidin blue, aurantia. The peripheral distribution of the chondriome is shown. The central vacuolated area is almost devoid of mitochondria.

D, zone IV, living cell from teased preparation suspended in homologous serum and photographed under phase-contrast microscopy. The nucleus is just below the middle of the cell and is surrounded by four cerephos granules in varying planes of focus. The granules are typically related to notches in the nucleus. The clear moat surrounding these granules is best seen in the lower right-hand granule.

E, zone III. Living cell from teased material suspended in homologous serum at 37° and photographed under phase-contrast microscopy. The apical border is devoid of secretion. The Golgi area is ill-defined because of its content of refractile granules. Numerous granules can also be seen at the cell base. The nucleus contains a prominent nucleolus.

F, zone III. Living cell from teased preparation suspended in homologous serum at 37° and photographed under phase-contrast microscopy to show the alternate cell shape in this zone, which is not so manifest in fixed preparations. The nucleus is rounded and is just outside the plane of focus. It is surrounded by granules.

G, zone III. 3-μ paraffin section fixed in Mann's fluid; Ludford's modification of Mann's method of osmication. The photomicrograph shows the relatively large size of the Golgi area, corresponding to the 'clear' area of haematoxylin / eosin preparations and the absence of vacuoles within the osmiophil rods.

H, zone III. 3-μ paraffin section fixed in Helly, postchromed 2 days at 37°; stained by the Bensley–Cowdry modification of Altmann's aniline fuchsin. The mitochondria are short plump rods crowded about the periphery of the cell, but also invading the Golgi area.

I, zone IV. 3-μ paraffin section fixed in Helly, postchromed 2 days at 37°; stained by the Bensley–Cowdry modification of Altmann's aniline fuchsin. The mitochondria are short rods scattered throughout the cytoplasm, about the periphery of the nucleus, and in the subnuclear cytoplasm. The prominent fuchsinophil nucleolus is often seen in zone IV. The nuclear membrane is notched.

FIG. 3 (plate). A, zone IV. 3-μ paraffin section of material fixed in Mann's fluid; Ludford's modification of Mann's method of osmication. The Golgi material forms a uniform band around the tubule and is non-vacuolated. The cerephos granules are markedly osmiophil and their apposition to the nucleus is again apparent.

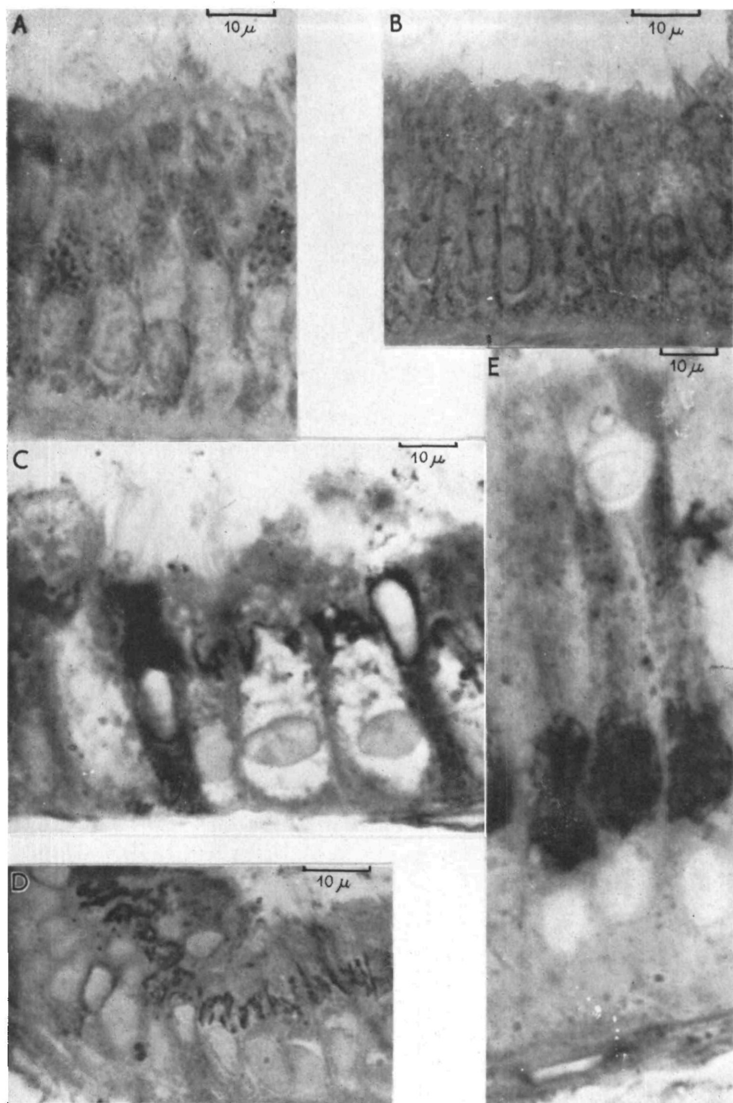

FIG. 1
B. L. REID

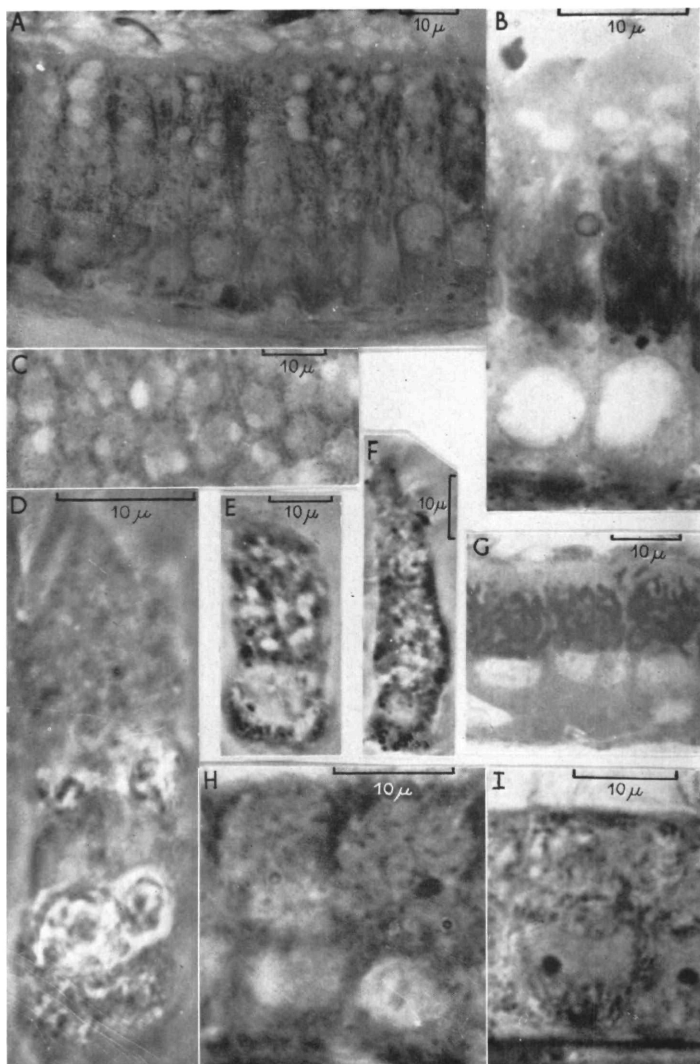

FIG. 2

B. L. REID

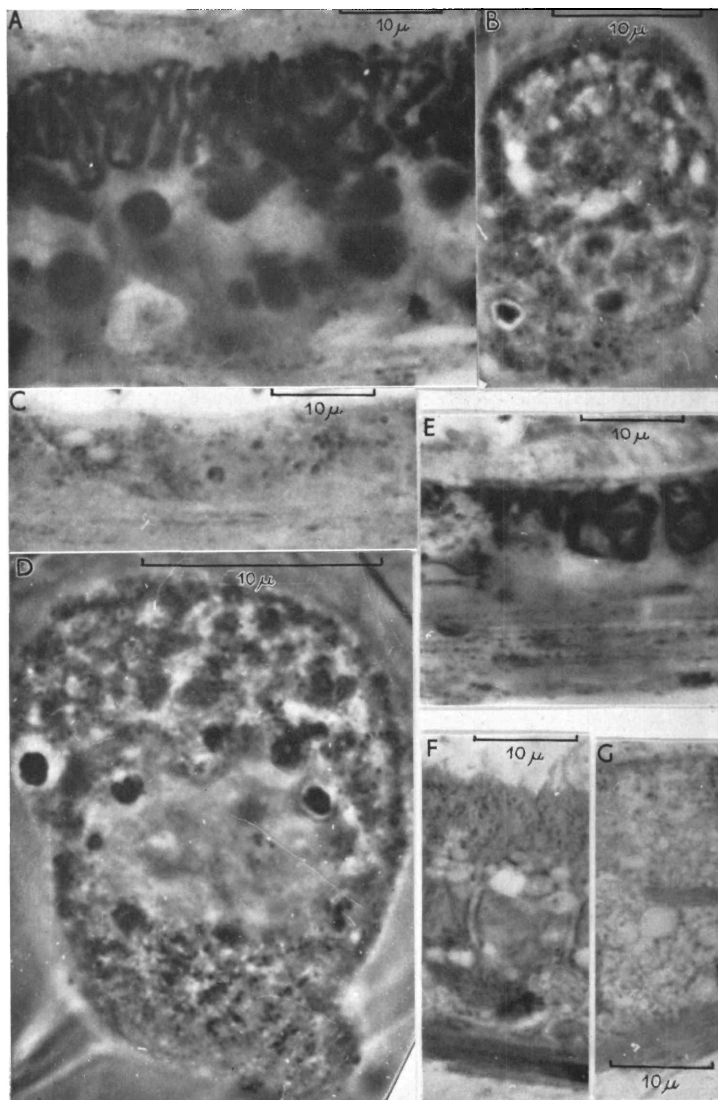

FIG. 3

B. L. REID

B, zone V. Living cell from teased preparation suspended in homologous serum and photographed under phase-contrast microscopy. The Golgi area is vaguely demarcated by a ring of vacuoles occupying most of the supranuclear cytoplasm. The nucleus is ovoid and is elongated in the transverse axis of the cell. Small granules are concentrated at the cell base. One cerephos granule is present at the left-hand side of the basal cytoplasm.

C, zone VI. 3-μ paraffin section of material fixed in Helly, postchromed 2 days at 37°; stained by toluidin blue, aurantia. The epithelium is very short and the lower half of the picture is taken up with the muscle-coat, which is thick in this zone, as the vas deferens is approached. About 5 epithelial cells are shown. The mitochondria are inconspicuous short rods scattered throughout the cells and the muscle-coat. The larger granules, more apparent toward the centre of the picture, are apparently separate from mitochondria and may represent some sort of secretion product.

D, zone V. Living cell from teased preparation suspended in homologous serum. At a higher magnification than fig. 3, B, to show small granules present throughout the cytoplasm, including the Golgi area. The latter is not defined. The nucleus is ovoid and has three cerephos granules associated, the upper right-hand one of which is seen in characteristic close apposition with the nuclear membrane.

E, zone VI. 3-μ paraffin section of material fixed in Mann's fluid; Ludford's modification of Mann's method of osmication. The Golgi material is in the form of black trusses which are not vacuolated. The interruption to the continuity of the Golgi material as a band around the tubule, in contrast to fig. 3, A, is apparently the result of the heterogeneous constitution of the epithelium in this zone.

F, zone IV. 3-μ paraffin section of material fixed in Helly, postchromed 2 days at 37°; stained with toluidin blue, aurantia. The widespread distribution of the small rod-like mitochondria is shown. The perinuclear vacuoles are the sites of the cerephos granules.

G, zone IV. Same technique as F. Clear cell containing large numbers of small spaces surrounded by small rods which stain like mitochondria.