The Histochemistry of the Male Germ-cells of the Nematode *Porrocaecum angusticolle*, a Parasite in the Vulture

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With two plates (figs. 1 and 2)

SUMMARY

The cytoplasm of the male germ-cells of *Porrocaecum angusticolle* contains (1) phospholipid granules, (2) 'refringent bodies' consisting of ribonucleoproteins at first, but some masked lipids as well in the late spermatid, (3) mitochondria of the usual lipoprotein nature. The refringent bodies arise in the spermatocytes in close association with the phospholipid granules ('Golgi bodies') by the aggregation of cytoplasmic basiphil material. In the spermatids the refringent bodies gradually fuse to form a single cone-like structure which occupies the narrow posterior region of the spermatozoon.

This cone-like structure, judging from its origin in close association with the 'Golgi bodies' (acroblasts), is the homologue of the acrosome of a normal flagellate sperm; but it is completely devoid of polysaccharides and seems unable to function as an acrosome, since it occupies a position posterior to the nucleus in the spermatozoon. On the contrary, some polysaccharides have been demonstrated by the authors in the broad anterior region of the spermatozoon, which may be functioning as an acrosome.

INTRODUCTION

NEMATODE spermatogenesis has been studied by a number of cytologists. Some of the previous work on the subject has been reviewed by Bowen (1925), Sturdivant (1934), and Nath and Singh (1956).

The nematode spermatozoon is characterized by the presence of a remarkable structure—a large, conical, refractile body, variously called the 'refringent body', the 'ascaridine granule', the 'acrosome', &c. The homology of this rather unique structure with the acrosome of a typical flagellate sperm was first suggested by Bowen (1925). This author considered the refringent body of the nematode sperm as a differentiated product of the 'Golgi material', like the acrosome of the typical spermatozoon. This homology has been confirmed by some of the later workers (Sturdivant, 1934; Collier, 1936; Nath & Singh, 1956; Singh, 1957; Hovasse, 1958).

It has been found during the past few years that the acrosome of the flagellate sperm of widely different groups of animals is characterized by the presence of a mucopolysaccharide, demonstrable by the periodic acid / Schiff reaction (Wislocki, 1949; Leblond, 1950; Schrader & Leuchtenberger, 1951; Clermont & Leblond, 1955; Moriber, 1956; Clayton & others, 1958; &c.).

There have been various speculations about the chemical composition of the refringent bodies of the nematode spermatozoon. Since these bodies

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appear highly refractile in the living condition, they were usually supposed to consist of a fatty substance.

Romieu (1911), however, considered the refringent bodies as consisting of 'nucleo-albumen'. This observation has recently found support in the histochemical studies of Pasteels (1948), Panijel and Pasteels (1951), Delavault (1952), and Favard (1958), who stated that these bodies consisted of ribonucleoproteins. Similarly, Makarov (1958) has denied the presence of any polysaccharides in these structures.

It may, therefore, be concluded from this brief review that chemically the refringent body of the nematode spermatozoon bears no resemblance to the acrosome of a flagellate spermatozoon.

The origin of the refringent body has been also disputed (see Sturdivant, 1934; Nath & Singh, 1956; Hovasse, 1958). Very recently Favard (1958) has put forward evidence from electron microscopy that the ascaridine granules (refringent bodies) of the spermatocytes of *Parascaris equorum* arise from the 'grains de Palade' of the ergastoplasm. The lipid inclusions represent simply the sites of this synthesis; they disappear during the formation of the ascaridine granules.

It was considered worth while to undertake a histochemical study of spermatogenesis in a nematode to ascertain the chemical nature, origin, and probable homology of the refringent body.

Material and Technique

Porrocaecum angusticolle is a parasite in the intestine of the vulture. The parasites were removed from freshly killed vultures and dissected in paraffin-lined dishes containing physiological saline (Baker, 1944). The testis, which is a single, coiled, thin thread about 5 to 8 cm in length, was transferred immediately to various fixatives. The uteri of the females were fixed for study of the ripe spermatozoon.

The various histochemical techniques employed have been listed in the appendix to this paper. Some of the paraffin sections of material fixed in various ways were stained with Heidenhain's iron-haematoxylin. The acid fuchsine technique of Cain (1948), the 'Hermann PO' technique of Baker (1957), and the Kolatchev technique were also employed.

Living cells were studied by phase-contrast microscopy, and with supervital dyes like neutral red and Janus green B.

Results

The cytoplasm of the male germ-cells of *P. angusticolle* shows the following inclusions:

1. Lipid bodies (granules and duplex spheres), consisting of phospholipids.
2. 'Refringent bodies', rich in ribonucleoproteins and developing some masked lipids also, in later stages.
3. Mitochondria, consisting of lipoproteins.

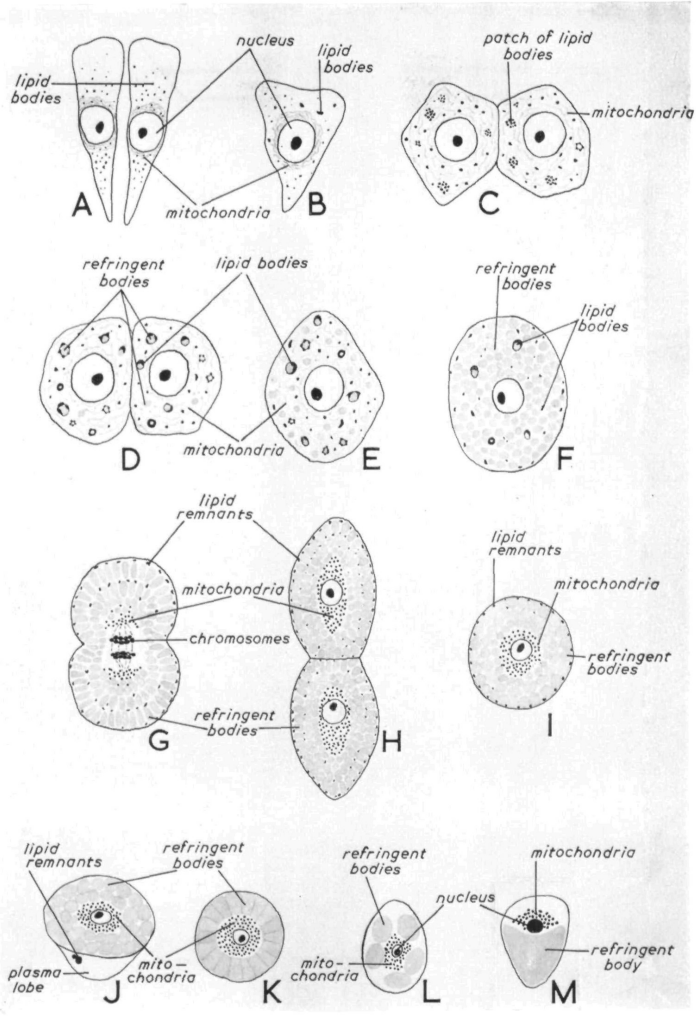

FIG. 1

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The morphology, histochemistry, and ultimate fate of these inclusions in spermatogenesis has been studied. The results of the various histochemical tests employed have been given in the appendix to this paper; only the relevant conclusions drawn from such tests are given here. Figs. 1 and 2 present process of spermatogenesis diagrammatically.

Spermatocytes

The earliest spermatocyte appears as an elongated, conical cell with a centrally placed spherical nucleus containing a single prominent nucleolus (fig. 1, A). The cytoplasm and the nucleolus are intensely basiphil. The various extraction-controls show that the cytoplasmic basiphilia is attributable to RNA. Besides this, some minute mitochondria can be seen, forming an aggregation round the nucleus, both in the fixed and living cells. A few minute phospholipid granules are dispersed in the cytoplasm. However, the marginal cytoplasm is free from any such inclusions.

The spermatocyte now enters the growth phase and gradually assumes a polyhedral shape in sections (fig. 1, B, C). The mitochondria, which now appear as distinct fibrils, become uniformly dispersed (C). They can easily be demonstrated in living cells under the phase-contrast microscope, or with Janus green B, and in the processed material by various techniques (acid haematein (AH), iron-haematoxylin applied to Lewitsky-fixed gelatin sections, acid fuchsine (Cain, 1948), &c.). They are phospholipid-protein in nature. They do not show any distinct basiphilia. The nucleus remains centrally placed and grows slightly. It is very uncommon to find the stages of regular prophase; most of the cells encountered are in the resting or growth phase (C).

The phospholipid granules proliferate and grow slightly. Occasionally some of these granules appear as short straight or curved rods (B); this is probably due to the fusion of individual granules. Very soon these granules arrange themselves in small groups; and 4 or 5 such groups can be seen in each spermatocyte (C). However, separate phospholipid granules are also abundant. Cytoplasmic basiphilia continues to be intense.

The formation of refringent bodies is ushered in by the appearance of small spherical spaces in the centre of the phospholipid aggregations (C); the lipid granules become applied to the surface of these vacuole-like spaces, thus giving the appearance of the typical 'mulberry spheroids' of Thomas (1948). A strongly basiphil material quickly fills this vacuole-like internum of the mulberry spheroids. This material represents the refringent body or ascaridine

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FIG. 1 (plate). A–M, diagrammatic representations of the process of spermatogenesis in *Porrocaecum angusticolle*. The diagrams are based on the study of the living as well as processed material.

A, earliest primary spermatocyte.

B–F, subsequent stages in the development of the primary spermatocyte.

G, first maturation division.

H, second maturation division.

I–L, stages of spermateleosis.

M, ripe spermatozoon.

granule (D). The refringent body grows into a separate sphere about 1 μ in diameter; it gets rid of its phospholipid 'satellite' granules, and comes to lie free in the cytoplasm. Very commonly the phospholipid material appears in the form of an irregular thickening or a crescent, or even a ring around the developing refringent body (D, E). These appearances are due to the confluence of the individual lipid granules. The formation of new refringent bodies continues till the whole of cytoplasm of the spermatocyte becomes filled up with them (F). The lipid remnants in the form of small granules or short straight or curved rods lie interspersed among these refringent bodies.

It is very difficult ordinarily to locate any mitochondria in this stage with any of the techniques, owing to an overcrowding of the refringent bodies in the cytoplasm (F).

The single nucleolus and the nucleoplasm do not show any basiphilia while the refringent bodies are being formed (fig. 2, A). The nucleolus is eosinophil.

While the formation of the refringent bodies proceeds in the cytoplasm, the latter gradually loses its basiphilia. During prometaphase I and the subsequent stages the cytoplasm of the cell is almost completely negative to the various basic dyes.

During the prometaphase and the subsequent stages of meiosis I and II, which appear in quick succession without any resting stage, the refringent bodies appear slightly elongated and oval; they are arranged in the form of three or four concentric rings in the cytoplasm (fig. 1, G).

The mitochondria now assume a spherical appearance; they occupy the two poles of the spindle during meiosis (G). They become more sudanophil and more AH-positive, but remain unchanged in their general histochemical nature.

Spermatids

The spermatid receives more or less equal quantities of all the three cytoplasmic inclusions. The refringent bodies continue to be arranged in three concentric rings. Some clear cytoplasm is left around the nucleus; this is occupied by the granular mitochondria. All the remaining phospholipid granules, on the contrary, move to the extreme periphery of the cell (fig. 1, H, I). The nucleus is small and spherical and continues to show a single prominent nucleolus. Now there occurs the well-known process of plasma-lobe-formation, in which the excess of cytoplasm along with all the lipid remnants is sloughed off. The cytoplasm of the spermatid and of the plasma-lobe gives a distinct PAS-positive reaction, owing to the presence of polysaccharide.

Before moving into plasma-lobe, the phospholipid granules fuse to form an irregular mass (J). Simultaneously, the refringent bodies begin to fuse with each other to form a tight ring of slightly elongated, cuboidal structures in the maturing spermatid (K). With a further process of condensation of the ribonucleoproteins of the refringent bodies, 5 to 6 large spheres are formed (L);

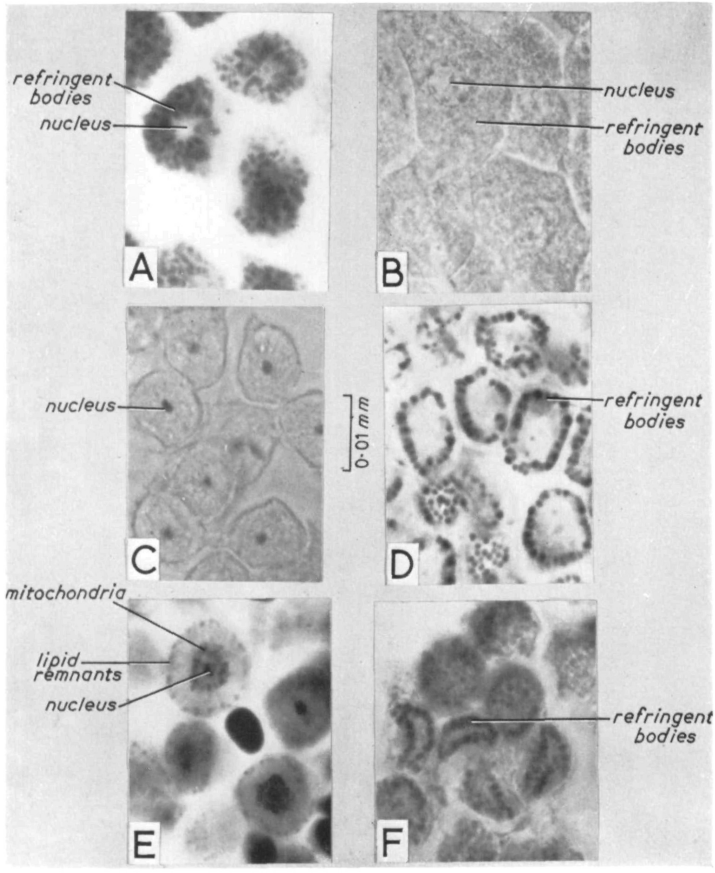

FIG. 2

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ultimately all of them fuse to form a single conical refringent body in the ripe spermatozoon (M). The mitochondria continue to be situated round the nucleus (fig. 2, E). They become larger and fewer, apparently by fusion of the smaller granules. The nuclear material also becomes condensed into a small, spherical, Feulgen-positive mass (figs. 1, M; 2, C).

The circumnuclear region of the cytoplasm of the maturing spermatids and the spermatozoon gives a slight PAS-positive reaction without any metachromasy. However, it is difficult to say whether the polysaccharide is present in the mitochondria of this region or in the ground cytoplasm, or in both.

The histochemical changes in the refringent bodies during the process of maturation are interesting. It has already been pointed out that these bodies are strongly basiphil, owing to the presence of large amounts of ribonucleoproteins (fig. 2, A, B). However, as soon as the refringent bodies arrange themselves in a single ring in the maturing spermatid, they suddenly lose all their basiphilia in routine preparations. These stages are by far the most abundant in the testicular material of *Porrocaecum*. However, in the ripe spermatozoon (which is found only in the uterus of the female), the single refringent body again develops slight basiphilia.

It has been mentioned earlier that there are no histochemically demonstrable lipids in the refringent bodies till the early spermatid stage. The various unmasking techniques given in the appendix also do not impart any sudanophil coloration to these structures. However, the burnt Sudan black B technique of Berenbaum (1958) colours quite intensely the refringent bodies of the maturing spermatid, particularly at the stage when they lose their basiphilia (fig. 2, F). The chromosomes of all the stages are also strongly coloured. This would show that the refringent bodies during the later stages of maturation develop some quantities of lipids, probably in the form of a lipoprotein complex.

It has been further observed that the sudden loss of basiphilia by the refringent bodies at a particular stage of the spermatid is not due to the absence of ribonucleoproteins as such, but rather to their masking by a lipoprotein complex, as shown by the following experiment. When paraffin sections of material fixed in Zenker (3 h) or in Carnoy are treated with 4% trichloroacetic acid (TCA) at 90° C for 15 min. as a control for RNA, the refringent bodies of all other stages except the maturing spermatids lose their

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FIG. 2 (plate). Photomicrographs of the male germ-cells of *Porrocaecum angusticolle* from various histochemical preparations.

A, spermatocyte from a methyl green / pyronin G preparation, showing pyroninophil refringent bodies.

B, same as in A but after digestion in 4% TCA for 15 min.

C, spermatids after Feulgen nucleal reaction.

D, spermatids from methyl green / pyronin G after extraction in 4% trichloroacetic acid for 1 h.

E, spermatids from Lewitsky-saline gelatin sections stained with 0·5% iron haematoxylin.

F, spermatids from Berenbaum's burnt Sudan black B preparation, showing sudanophil refringent bodies.

basiphilia (fig. 2, B). The refringent bodies of the maturing spermatids, on the contrary, develop slight basiphilia after this treatment, although they are not basiphil in ordinary preparations. When the slides are kept in TCA for longer periods, the basiphilia of these refringent bodies increases progressively; they become strongly basiphil after a treatment for 1 h (fig. 2, D). A further increase in the time of digestion in TCA causes a rapid loss of basiphilia from these bodies, till they become completely non-basiphil.

It is, therefore, logical to conclude that the loss of basiphilia of the refringent bodies during the maturation stages of the spermatid is due to some kind of masking of RNA, perhaps by the formation of a complex containing proteins, lipids, and RNA.

It is now generally accepted that digestion by TCA breaks the RNA/lipoprotein bond and thus causes the extraction of RNA from the cell. But in this particular material TCA first causes an unmasking effect on the refringent bodies, perhaps by breaking the complex mentioned above, and later causes the extraction of RNA.

A similar effect of digestion by TCA has also been noticed in the refringent body of the ripe spermatozoon found in the uterus.

The ripe spermatozoon of *Porrocaecum* is of typical ascarid type, being cone-like. The nucleus along with the mitochondria occupies the anterior blunt end, while the conical refringent body fits into the posterior pointed half of the spermatozoon.

It has been further confirmed that during fertilization the spermatozoon enters the egg with its blunt end forward. When the whole spermatozoon has entered the ooplasm, the refringent body becomes rounded. Gradually its material disappears in the cytoplasm of the egg.

Discussion

The ripe spermatozoon of *P. angusticolle* is of typical ascarid type (Nath and Singh, 1956). The most prominent structure in this spermatozoon is the large 'refringent body', also called the 'acrosome' (Bowen, 1925; Sturdivant, 1934; Collier, 1936; Nath & Singh, 1956; Singh, 1957; Hovasse, 1958) or the 'ascaridine granule' or 'body' (Pasteels, 1948; Panijel & Pasteels, 1951; Favard, 1958). This refringent body occupies about two-thirds of the cone-like spermatozoon, lying behind the nucleus towards the narrow posterior end of the cone.

The chemical nature of the refringent body has been variously described in the earlier work (Bowen, 1925; Sturdivant, 1934; &c.). Quite recently Pasteels (1948), Panijel and Pasteels (1951), Delavault (1952), Favard (1958), and Makarov (1958) have produced evidence to show that it contains ribonucleoproteins.

Our observations on *Porrocaecum* spermatozoon fully confirm the presence of ribonucleoproteins in the refringent body. In addition, we have also found some small quantity of masked lipids in the refringent body of the maturing spermatid. The lipids seem to be present more or less in the

masked state described recently by many workers (Berenbaum, 1958; Clayton, 1958; Chayen & others, 1959).

Pasteels (1948) observed that the refringent bodies (his 'ascaridine granules') lose their basiphilia suddenly in the maturing spermatid and do not regain it till fertilization. Our observations also reveal a similar behaviour of the refringent bodies in normal preparations. However, treatment of the sections in 4% TCA at 90° C for 1 h completely restores the capacity of these bodies to stain with pyronine G. A further prolongation of extraction, even for a few minutes, again removes the basiphilia. This indicates that the RNA in these bodies is present at this stage in some complex form with lipoproteins; i.e. it is 'masked' and not absent, as Pasteels believed (1948).

The single conical refringent body of the ripe spermatazoon is formed by the gradual fusion and condensation of the material of the smaller spherical bodies which arise in the primary spermatocyte. In fact, the major cytoplasmic activity of the male germ-cells in this species seems to consist of the formation of these structures. Besides this the spermatid shows very little activity while transforming into the mature spermatozoon, except that small quantities of the excessive cytoplasm along with lipid remnants are cast off as the plasma-lobe.

Our observations fully support the conclusion of previous authors that the mitochondria of the spermatocyte, which are in the form of filaments and granules, do not play any direct role in the formation of the refringent bodies (Sturdivant, 1934; Collier, 1936; Nath & Singh, 1956; Hovasse, 1958; Favard, 1958).

Our observations lead us to believe that the refringent bodies arise by condensation of the cytoplasmic basiphil substances (ribonucleoproteins) into spheres. In the early spermatocyte the cytoplasm, containing sparsely distributed mitochondria and phospholipid granules, is intensely basiphil, the basiphilia being attributable to the presence of RNA (which is labile to salivary ribonuclease).

As the process of spermatogenesis advances and the spermatocyte enters the growth period, small vacuoles or spaces appear within the localized aggregations of phospholipid granules; the latter become attached to the surface of the former. These vacuoles or spaces become quickly filled up with the basiphil material (ribonucleoproteins). Gradually the ribonucleoprotein sphere grows; ultimately it gets rid of its phospholipid satellites and forms a refringent body. When the primary spermatocyte approaches the prometaphase stage, its cytoplasm has become choked with these refringent bodies, obliterating the mitochondria from view. The phospholipids show an obvious decrease during these stages. However, it may be that the phospholipid granules do not directly contribute any substance in the formation of the refringent bodies; perhaps they help in the segregation of the dispersed basiphil material by forming lipid membranes. The role of lipids in the formation of such segregation membranes around the secretion products in the various animal cells has been stressed by many recent workers, particularly Hirsch (1955).

A point of great interest is that the nucleus of the spermatocyte throughout

the process of refringent body-formation contains a single inactive (though prominent) nucleolus. The rest of the nucleoplasm is almost devoid of any pyroninophil substance. The origin of the cytoplasmic RNA is usually attributed to the nucleus (Brachet, 1957; Chantrenne, 1958; Zalokar, 1959). The lack of activity of the nucleolus particularly and of the nucleus as a whole might be interpreted as meaning that no new RNA is synthesized in the nucleus for the formation of the refringent bodies.

Favard (1958), working with the electron microscope, believes that the ascaridine granules in the spermatocytes of *Parascaris equorum* are formed by the aggregation of the 'grains de Palade' of the ergastoplasm or of the endoplasmic reticulum. Such aggregations to begin with are associated with lipid droplets, but become free from them ultimately, forming ascaridine granules. Favard (1958) considers these lipids as neutral glyceride droplets.

Bowen (1925), after studying the work of Hirschler (1913) and other investigators of the nematode spermatozoon, was the first to suggest tentatively the homology of the refringent body with the acrosome of other animals. He based this idea on the fact that the refringent body arises by the confluence of similar spheres which appear in the spermatocyte in association with the 'Golgi bodies', like the acrosome of the flagellate sperm. It must be noted, however, that Bowen fully realized the shortcomings of such a homology and stressed that the term 'acrosome' was inadequate to explain the chemical properties and the physiological role of the structure thus named in the various spermatozoa.

Many workers on the spermatozoon of *Ascaris* have accepted this supposed homology (Sturdivant, 1934; Collier, 1936; Nath & Singh, 1956; Hovasse, 1958).

In our view Bowen (1925) and almost all other earlier workers correctly considered the 'Golgi bodies' of nematode spermatocytes and the 'Golgi dictyosomes' of male germ-cells of other animals as homologous.

The separate phospholipid bodies of the spermatocytes described by us in *Porrocaecum angusticolle* are no doubt the structures that have been described under the name of 'Golgi bodies' or 'Golgi dictyosomes' by the earlier workers on nematodes (Tretjakoff, 1905; Scheben, 1905; Hirschler, 1913; Sturdivant, 1934; Collier, 1936; Nath & Singh, 1956; Hovasse, 1958). Recent work with the help of electron microscope has shown that the 'dictyosomes' of the male germ-cells of a large variety of animals have a very characteristic structure of double lamellae associated with some small vesicles, corresponding respectively to the chromophil and chromophobe regions seen with the light microscope (Burgos & Fawcett, 1955; Clermont, 1956; Palade, 1955; Clayton & others, 1958; Gatenby & others, 1958). Favard (1958), however, has found that the lipid granules of the spermatocytes of *Ascaris* do not have any such characteristic ultrastructure.

A good deal of work has been done recently on the chemical nature of the acrosome of a variety of flagellate spermatozoa, and it has been found that the acrosome invariably contains some PAS-positive substance, perhaps neutral mucopolysaccharides (Wislocki, 1949; Leblond, 1950; Leuchten-

berger & Schrader, 1950; Schrader & Leuchtenberger, 1951; Clermont & Leblond, 1955; Moriber, 1956; Clayton & others, 1958). The acrosome does not contain even the slightest trace of any basiphil substance at any stage (Clermont, 1956). In fact, the ripe flagellate spermatozoon does not have any appreciable quantity of basiphil substances in the cytoplasm. Most of the cytoplasmic basiphilia or ergastoplasm is discarded during the process of maturation and by sloughing off of the cytoplasm (Daoust & Clermont, 1955).

The refringent body of the *Porrocaecum* spermatozoon, on the contrary, does not contain any PAS-positive substances whatsoever. On the contrary, it is solely composed of the cytoplasmic ribonucleoproteins—a substance which is completely discarded in the normal flagellate sperm.

Again, apart from the fact that the refringent body lies behind the nucleus in the ascarid spermatozoon, it gives no indication during fertilization that it initiates the egg-reaction like the acrosome of a typical flagellate sperm. It is generally believed that the acrosome contains some 'active substances' which help in the dissolution of the membranes around the unfertilized eggs. These substances are believed to be present in the form of mucopolysaccharides and proteins (Rothschild, 1958). Even when the acrosome of an atypical flagellate sperm like that of *Lepisma* is present behind the nucleus (Bowen, 1925; Nath & Bhatia, 1953), it continues to have at least some PAS-positive substances (unpublished observations).

The cytoplasm and the mitochondrial granules at the anterior blunt end of the sperm of *Porrocaecum* are slightly PAS-positive. Perhaps they contain the enzymes normally carried by the acrosome of the flagellate sperm.

It is quite clear that the refringent body of the ascarid sperm is, at least chemically and physiologically, different from the acrosome of the flagellate sperm.

It is worth while to mention that some of the very early workers on the nematode spermatozoon anticipated the ribonucleoprotein nature of the refringent body. Scheben (1905) considered this body as composed of the 'achromatic' material of the nucleus. Romieu (1911) considered this structure as the 'nucleo-albumen'.

We do not know the function of the refringent body in fertilization. The senior author (Nath) believes that, in view of its origin in close association with the so-called Golgi bodies, the refringent body of the ascarid sperm is homologous with the acrosome of the typical flagellate spermatozoon, but it has undergone clearly a change in function. This has involved changes in its chemical composition, morphology, and position in the ripe sperm.

We have already stated that there is overwhelming evidence in favour of the view that the acrosome of the normal flagellate sperm is the seat of PAS-positive substances (Rothschild, 1958) which initiate the fertilization reaction on the part of the egg. If this is true, the broad anterior end of the cone-like sperm of *Porrocaecum* in which we have demonstrated some PAS-positive substances may be functioning as the acrosome. This view is strongly supported by the complete absence of PAS-positive substances from the refringent body.

APPENDIX

Table showing the histochemistry of the cytoplasmic inclusions during the spermatogenesis of Porrocaecum angusticolle

Technique	*Fixation*	*Embedding medium*	*Reference*	*Spermatocyte*			*Spermatid*		
				Lipid bodies	*Refringent bodies*	*Mito-chondria*	*Lipid bodies*	*Refringent bodies*	*Mito-chondria*
SBB in 70% ethanol; at 60° C; in acetone, hot; after phenol; after 90% ethanol, and dioxane	FCa, FCa+Pc, L, Z, C	P and G	Baker, 1949; Pearse, 1954; Berenbaum, 1958; Clayton, 1958	++	O	+	++	O	++
Burnt SBB	Z, C	P	Berenbaum, 1958	+++	O	+	+++	++	++
AH	FCa+Pc	P and G	Baker, 1946	++	O	+	++	O	++
AH after PE	WB+PE	P	Baker, 1946	O	O	O	O	O	O
NB	FCa, FCa+Pc	G	Cain, 1948	Bl	O	O	Bl	O	O
MG/PG	Z 3 h	P	Jordan & Baker, 1955	O	++	O	O	O	O
MG/PG after saliva	Z 3 h	P	Bradbury, 1956	O	O	O	O	O	O
MG/PG after 4% TCA 15 min.	Z 3 h	P	Pearse, 1954	O	O	O	O	+	O
MG/PG after 4% TCA 1 h	Z 3 h	P	..	O	O	O	O	++	O
MG/PG after 4% TCA 70 min.	Z 3 h	P	..	O	O	O	O	O	O
Eosin/MB	Z 3 h	P	Gatenby & Beams, 1950	O	++Bl	O	O	++Bl	O
Eosin/MB after saliva	Z 3 h	P	Bradbury, 1956	O	O	O	O	O	O
Eosin/GW	Z, B	P	Pearse, 1954	O	++Bl	O	O	++Bl	O
Feulgen	C, B, H	P	Feulgen & Rossenbeck, 1924	O	O	O	O	O	O
Hg-BPB	C, B, Z, FCa	P and G	Mazia & others, 1953	O	O	+	O	O	+
Hg-BPB after PE	WB+PE	G	..	O	O	+	O	O	+

Millon's reaction	Z, C	P	Bensley & Gersh, 1933; Pearse, 1954	O	O (?)	+	O	O (?)	+
CTZ	Z, C, B	P	Danielli, 1947; Pearse, 1954	O	++	+	O	++	+
CTZ after DNFB	,,	P	,,	O	O	O	O	O	O
CTZ after PFA	,,	P	,,	O	+	O	O	+	O
CTZ after benzoylation	,,	P	,,	O	O	O	O	O	O
Sakaguchi	B, C, FCa	P	Baker, 1947	O	O	O	O	O	O
Mercuric sulphate test for phenols	FS	P and celloidin	Baker, 1956	O	O (?)	O	O	O (?)	O
PAS	FCa, B, C	P and G	Hotchkiss, 1948; Pearse, 1954	O	O	O	O	O	+ (?)
Best's carmine	Z, C, B	P	Pearse, 1954	O	O	O	O	O	O
HPO	Hermann $+OsO_4$	P	Baker, 1957	++	O	+	++	O	+

KEY: AH = acid haematein; B = Bouin; Bl = blue; C = Carnoy; CTZ = coupled tetrazonium; DNFB = dinitrofluorobenzene; Eosin/MB = eosin/methylene blue; Eosin/GW = eosin/Gram/Weigert; FCa = formaldehyde-calcium; FS = formalde-hyde saline; G = gelatin; H = Helly; Hg-BPB = mercuric bromophenol blue; HPO = Hermann + post-osmication; L = Lewitsky-saline; MG/PG = methyl green/pyronin G; NB = Nile blue; P = paraffin; PAS = periodic acid/Schiff; PE = pyridine extraction; PFA = performic acid; PC = postchroming; SBB = Sudan black B; TCA = trichloroacetic acid; WB = weak Bouin; Z = Zenker. O = negative; +, ++, +++ = positive; (?) = doubtful.

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