THE MIDPIECE OF THE MOUSE SPERMATOZOO: ITS FORM AND DEVELOPMENT AS SEEN BY SURFACE REPLICATION

D. M. WOOLLEY

Institute of Animal Genetics, A.R.C. Unit of Animal Genetics, University of Edinburgh, Scotland

SUMMARY

A method is described for making carbon replicas of the surface of fixed spermatozoa and spermatids. In a study of the undulations of the surface of the midpiece, it has been shown that in the mouse spermatozoon the mitochondria are arranged predominantly in two parallel helices—without exception sinistral—totalling about 87 windings around the flagellum. The variability of this number has been calculated. The origin of this arrangement in spermateleosis has been examined in replicas of spermatids, revealing a rather complicated morphogenesis. It seems that, following the migration of the terminal annulus, elongated mitochondria form a single dextral helix around the flagellum; they then divide, making an orderly arrangement of spherical units; finally, each unit elongates, pushes between the contiguous units, and, by end-to-end apposition, contributes to the formation of the regular system of two sinistral helices. A detailed account of this transformation is presented. It is suggested that, for tissues of sufficient hardness, the replica technique can usefully complement the cutting of thin sections.

INTRODUCTION

The mitochondria of the mammalian spermatozoon are arranged around the proximal part of the flagellum in the region called the middle-piece or midpiece. The long-standing view, now widely accepted, is that their configuration is helical. Attempts to provide a more detailed description have concerned the number of constituent mitochondria and the extent to which they retain their individuality, the number of gyres or windings of the spiral around the flagellum, the number of parallel helices involved, and the direction of their spiralization. From the progress already made in answering these questions it has become clear that the mature structure has an impressive regularity, and is evidently the result of a strictly determined developmental process. The complex geometry of its morphogenesis, however, cannot easily be worked out from the study of sectioned material. In this paper a method of surface replication is described which has the advantage of providing an overall view of the arrangement of the mitochondria for the entire midpiece of a spermatozoon. The technique was developed in connexion with a genetical study of the variation in the length of the spermatozoan midpiece (to be published), for which a means was required of verifying that apparent differences in the length of the midpiece were equivalent to differences in the mitochondrial content. It was found that the number of gyres in the helical arrangement could be counted from a replica of the undulations in its surface, making possible an analysis of the variation in this characteristic. The
method has also proved suitable for studying the development of the midpiece, as
will be shown. In this context, replication usefully provides information at a level
between light microscopy and the electron microscopy of thin sections, and so can
facilitate the interpretation of the latter. For the particular purpose of demonstrating
the direction of a helix, replication is the method of choice.

Surface replication has been widely used in electron microscopy but seldom applied
to biological material since the introduction of the technique of cutting thin sections.
A review of the methods and materials available is given by Bradley (1965). The
method used in this work, single-stage carbon replication, has the advantages of high
resolution and stable preparations, but the procedure involves severe desiccation of the
specimen. Probably the first application of the replica technique to the study of
spermatozoa was made by Kessler (1954), who made impressions of the cell surface in
a varnish (Zaponlack) composed of pyroxylin and amyl acetate. More recently,
Kojima (1966) has made collodion replicas of the bull spermatozoon. Carbon replicas,
with their higher resolution, have been made of frozen-etched bull spermatozoa by
Koehler (1966) in a study which emphasized the structure of the spermatozoan head,
but did, in one of the electron micrographs, show the mitochondria clearly visible
beneath the cell membrane of the midpiece.

MATERIALS AND METHODS

The essential features of single-stage carbon replication are as follows. The specimen is dried
on to a smooth, clean surface and a layer of carbon applied over it by evaporation. The carbon
film and the specimen are then removed from the surface, and the specimen dissolved away,
leaving its impression in the carbon; the replica is afterwards given greater contrast by shadowing.

The mice used in this work were adult males of the non-inbred 'Q' strain. Mature sperma-
toza were obtained from freshly killed animals by flushing the vasa deferentia with a phosphate-
buffered saline composed of equal volumes of 0.15 M NaCl and Sorensen's phosphate buffer,
\( \text{pH } 7.2 \) (made from 0.15 M stock solutions); immature cells were obtained by teasing out and
cutting up the seminiferous tubules in phosphate-buffered saline. In both cases a suspension of
cells resulted. Neutralized formaldehyde solution was then added to the suspension to a final
concentration of 2% formaldehyde. The suspension was immediately centrifuged at 2000 \( \times \) g for
20 min and the supernatant withdrawn as completely as possible. The pellet was then partially
resuspended in distilled water and the suspension was spread on to a polished microscope slide
and allowed to dry. In this way the cells were dried down in a clean, salt-free state. Their
density and distribution could be examined under the microscope before proceeding. Baylor,
Nalbandov & Clark (1943) first employed formaldehyde fixation to avoid the disruptive effects
of distilled water on spermatozoa prepared for whole-mount electron microscopy.

The preparation was then placed in an evaporator and left to dry for 3 h at a pressure of
1.33 \( \times \) \( 10^{-2} \) N m\(^{-2}\) (10\(^{-4}\) mm Hg). After this, a layer of carbon approximately 25 nm thick was
evaporated on to the surface of the slide. The resulting carbon film was cut into small squares
and floated off the slide on to distilled water. The formaldehyde-fixed cells, especially their
nuclei, were very resistant to digestion. First the film was transferred in a wire loop to the surface
of a trypsin solution at 37 °C and left for 1 h. Then the digestion of the spermatozoa was com-
pleted by the drastic measure of putting the film on a 4 N solution of sodium hydroxide at
60 °C for 2 h. After this the film was washed on several surfaces of distilled water and picked
up on uncoated 200-mesh copper grids. The grids were shadowed with a 40% palladium-gold
alloy at an angle of about 25°, and examined in a Philips EM 75 electron microscope. Most of
the electron micrographs were made at an actual magnification of 2000-5000, and printed as
positives: the white areas of the print, therefore, are those parts of the replica over which no
metal has been deposited. Some of the dark areas on the prints are due to incomplete digestion.
Replicas of the spermatozoan midpiece

of tissue. The two major potential sources of artifact are from the process of fixation, and, more important, the drying of the specimen from the liquid phase. No attempt has been made in this work to obviate this distortion by the use of the Anderson critical point technique.

OBSERVATIONS

The mature spermatozoon

The mature cell will be described first since a knowledge of its structure will subsequently act as an established end-point in spermateleosis when cells from the testis are described. The following description applies to spermatozoa from the vas deferens.

The mitochondria are visible in parts of most midpieces. They begin distal to the neck region and extend posteriorly to the terminal annulus, which abuts against the fibrous sheath of the main-piece of the flagellum. The length of the individual mitochondria could not be distinguished in the replicas of mature spermatozoa. The spiral structure, however, was clearly discernible. Often, the windings of the spiral (or gyres) could be counted for the entire length of the midpiece. For the purpose of this description, the mean number of gyres for 'Q' males can be calculated indirectly from another study (unpublished) which involved the estimation of a regression coefficient of gyre number on midpiece length (in \( \mu m \)): a study of 136 spermatozoa from 9 mice, 7 of which were from lines artificially selected for extremes of midpiece length. Knowing, from measurements made using the light microscope, the midpiece length of unselected 'Q' mice to be about 226 \( \mu m \), and using the calculated regression coefficient 3.84, the mean number of gyres in the midpiece of unselected animals is 86.8. The standard deviation within males was estimated as 2.0 gyres, and between males, 0.87 gyres.

To determine the number of helices in the configuration, measurements have been made of the angle formed by the long axis of the mitochondria with the transverse axis of the midpiece. It can be shown that for a region of midpiece in which both the width of the midpiece \( (w) \) and the width of the mitochondria \( (m) \) are constant, this angle \( (\theta) \) is proportional to the number of parallel helices involved \( (n) \).

\[ n = 2 \sin \theta \left( \frac{w}{m} \right). \]

A typical analysis is shown in Fig. 1, where the angle of each mitochondrion has been plotted and related to the calculated number of helices. Almost the entire length of one midpiece, the specimen shown in serial electron micrographs in Figs. 3-5, is included. The calculation of the number of helices in the proximal region of this specimen has allowed for the relative narrowness of this part of the midpiece. (The proximal region was commonly found to be slightly narrower.) Measurements made on other spermatozoa have been consistent with this analysis in indicating that there are two helices. This deduction can, in fact, be made with some confidence by visual assessment alone, and the general conclusion, from an examination of many specimens, is that the mitochondria form, very regularly, two helices. Occasionally what seems like a short region of triple helix, resulting from the interposition of an extra mitochondrion, is seen. It seems a rare irregularity, however, and it has not been studied systematically because of the difficulty of distinguishing it from chance distortion and damage.
The direction of the mitochondrial helices has been recorded for a great many spermatozoa and has been found without exception to be left-handed or sinistral, i.e. in the opposite direction from that of the ordinary carpenter's screw. This apparently obvious fact was established only after a careful consideration of the technical processes which can affect it. The reasoning is, briefly, as follows. The carbon replica, viewed on the impression surface, shows the helices with their direction reversed; and the replicas were always put in the microscope with impression surface uppermost. Therefore, since the electron microscope does not produce an inverted image, the direction of the helices in the screen-image was also reversed. The photograph of this image was printed conventionally through the back of the plate, giving a print with the helix direction the same as in the original specimen.

Finally, the terminal annulus or so-called ring centriole is visible at the distal end of the mitochondrial helices, except when it is obscured by the overlying cytoplasmic droplet. It appears more turgid than the mitochondria, and lies not parallel to them but perpendicular to the long axis of the flagellum. In Fig. 18 the terminal annulus is seen in high relief in a replica of a spermatid from which some mitochondria have been displaced.

The spermatid

The different stages in the development of the midpiece are shown in Figs. 6–12, where they are arranged in the supposed developmental sequence. They will be described in this order, allowing that it is naturally an interpretation of what has been observed. Only the final stage in the development was already established (from replicas of mature spermatozoa). Unfortunately, no correlation could be detected.
Replicas of the spermatozoan midpiece

between the later succession of changes in the midpiece and any other contemporaneous, well-established development (for example, in the head of the spermatid) which might have provided a chronological reference to the changing configuration of the mitochondria.

The earliest stage illustrated (Fig. 6) precedes the morphological demarcation of the midpiece region. In what corresponds to the acrosome phase (Oakberg, 1956) the flagellum is seen originating from behind the elongating nucleus. The acrosome is visible. The flagellum is obviously not yet enclosed by the protein sheath in the region of the future main-piece. The length of the flagellum which is embedded in the spermatid cytoplasm cannot be judged in these preparations.

<table>
<thead>
<tr>
<th>Mouse</th>
<th>No. spermatids</th>
<th>Estimated no. gyres in helix</th>
<th>Mean no. gyres</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>41, 40, 42, 37</td>
<td>40°</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>39, 39, 36, 37</td>
<td>37.75</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>41, 39, 37, 38, 39, 40, 37, 41</td>
<td>39°</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>37</td>
<td>37°</td>
</tr>
</tbody>
</table>

Grand mean 38.8

In the next stage of development (Fig. 7) the nucleus has a more mature shape. The protein sheath is now present on the future main-piece, thus defining by its absence the length of the midpiece. The terminal annulus can be seen encircling the flagellum in the neck region, and no mitochondria are associated with the flagellum.

In the stage of spermateleosis shown in Fig. 8, the terminal annulus is now in its final position at the distal end of the midpiece, and the flagellum of the midpiece is surrounded by large elongated mitochondria which form a single, dextral or right-handed helix. No examples of this single helix were seen to have a sinistral direction. Again, it was not possible to estimate the length of the individual mitochondria in these spermatids, but the number of gyres in the helix could be counted in favourable specimens. The results obtained for 17 spermatids of reasonable clarity are given in Table 1. The between-male differences were tested and found to be non-significant. Sometimes, spermatids are seen in which the terminal annulus has not completed its migration, and in these the helix has consequently fewer gyres. No structure has been seen which might determine the direction of this mitochondrial helix. It has been noted, for example, that the outer coarse fibres of the flagellum run parallel to the long axis, confirming the general view that the growth of the flagellum is not, even slightly, helical (Figs. 17, 18).

In some spermatids the mitochondria of this single helix appear to be dividing into spherical units. A stage is seen in which the dextral helix is evident, but is made up of these approximately spherical mitochondria (Fig. 9). It has not been possible to determine whether this process begins consistently in any particular region of the midpiece. Figure 10 shows a stage in spermateleosis in which a single dextral helix is no longer apparent in the midpiece. The flagellum is entirely surrounded by spherical mito-
chondria. They are aligned in columns, which are not quite co-linear with the long axis of the flagellum but always have a slight dextral twist (Figs. 10, 14). The number of columns has been estimated at 4 after making clay models of the several possibilities and comparing their appearance with the surface replicas. By manipulating such clay models, it has also been noticed that if the 4 columns of spheres have a dextral twist, it means that each 360° gyre of the original dextral helix has given rise to slightly fewer than 4 spheres, the exact number depending upon the degree of twist. This varies somewhat (perhaps owing to secondary twisting of the entire flagellum as the specimen dries down), and only an approximate estimate has been made: it seems that 6 gyres of the dextral helix become 23 spherical units, i.e. about 3.8 spheres per gyre.

Fig. 2. A–F. The changing configuration of the mitochondria during spermateleosis in the mouse. Fusion between mitochondria is not implied in A and F.

A prediction from this model is that an exactly transverse section would cut either 4 or 5 spheres with about equal frequency. The total number of spherical mitochondria in the midpiece can now be estimated (38.8 $\times$ 3.8) at 149. A consequence of this arrangement is that each sphere, making contact with 6 others, often has an hexagonal appearance (Fig. 14).

Later in development, presumably, the mitochondrial units appear more or less elongated along an axis through two opposite angles of this hexagon (Fig. 2). They become roughly diamond-shaped, forming an interlocking pattern on the surface (Fig. 15). Eventually they become crescentic and each one extends until it occupies one half of one gyre. This produces a surface pattern in which the points of contact between the extremities of any two elongating mitochondria appear in alternate gyres of the configuration. Two helices result from this transformation when the elongating mitochondria meet. This complicated situation is shown in Fig. 11, particularly in the more distal region of this midpiece, and is illustrated schematically in Fig. 2 which has been drawn from clay models. The expected number of gyres in the final two sinistral helices, added together, is now 149 $\times$ 0.5 or about 74. It has been possible to count the number of gyres in only 2 specimens at this stage. In one of these the number was 72, and in the other, about 70. This figure is obviously less than the number of gyres in the midpiece of the mature spermatozoon (87). It is supposed, then, that in the last stages of maturation, in the testis, a further elongation of the mito-
Replicas of the spermatozoan midpiece

chondria occurs so as to lengthen each of the two helices by about 6 gyres. If this is correct, one can calculate that each mitochondrion of the mature midpiece occupies 87/149 or 0.58 of one gyre.

Spermatozoa with a mature midpiece structure are frequently seen in preparations from the testis, and the number of mitochondrial gyres has been found to be between 80 and 90 in all the cells examined. The only difference seen in the midpiece between these cells and those from the vas deferens is that the cytoplasmic droplet has a post-nuclear position in testicular spermatozoa.

To summarize this interpretation of the changing surface pattern of the midpiece: the mitochondria are at first elongated, and surround the flagellum as a single dextral helix. They then divide to form 4 rows of spherical units. Each unit under compression acquires a hexagonal outline and elongates, becoming first diamond-shaped, then sausage-shaped, until it extends half-way around the cylindrical surface of the flagellum. The spherical units of one of the 4 rows thus become segments of the same mature, sinistral, helix, alternating with the units from the diametrically opposite row of spheres. From the 4 rows, two helices are produced. Further elongation then increases the number of gyres to the number found in the mature spermatozoon.

DISCUSSION

By the use of surface replication it has been possible to make a quantitative morphological study of the mitochondrial complement in the mouse spermatozoon. It has been shown that, in replicas, the gyres of the helices can be enumerated, the number of helices deduced, and their direction observed. The number of mitochondrial gyres can, of course, be discovered from longitudinal sections which pass through the entire structure, but such sections would be expected only rarely in this species. Shadowed, whole-mount preparations can potentially yield this information, too, if the problem of low penetrability to the electron beam can be overcome. André (1962) has reviewed the findings of the earlier electron microscopists on this question and tabulates the approximate numbers which have been obtained for several species. The variability of this feature within species and within individuals has not been examined except in a short series of counts made by André on rat spermatozoa.

The number of parallel helices in the midpiece can also be determined in several ways. In oblique sections, the difference between the number of transected mitochondria on the two edges of the section is equal to the number of helices (Challice, 1953; André, 1962). Using this approach, Challice concluded that the number of helices in the mouse spermatozoan midpiece is two, and André found that in the rat there are predominantly two helices, with occasional intercalations forming regions of triple helix at an irregular frequency. The number of helices may also be derived from the angle made by the mitochondria with the long axis of the flagellum, as can be measured in subtangential sections: Kojima (1966), studying the bull spermatozoon, deduced that there were two helices, whereas André's results with this method conflicted somewhat with his conclusions drawn from the oblique sections. The angle may also be measured in whole-mount shadowed preparations of sufficient penetra-
bility. Randall & Friedlaender (1950) used this method on ram spermatozoa and demonstrated a double helix of mitochondria. The present findings from replicas reinforce the general opinion that the mammalian midpiece has two helices of mitochondria.

The direction of the spirals cannot be found from sections unless they are serial. The information can in theory be gained from good shadowed preparations if carefully interpreted. But in simple whole-mount preparations the spirals are seen in transparency and their direction is not apparent. The question can best be answered by an examination of surface replicas. Kojima (1966) has used collodion replicas to this end but, unfortunately, did not augment his electron micrographs with an unambiguous description. To describe a spiral as clockwise or anti-clockwise is not meaningful without formally specifying the position of the observer in relation to the structure. A further caution is that several authors (e.g. Fawcett, 1958; André, 1962) in drawing 3-dimensional reconstructions from sectioned material have necessarily shown the spiral with one of the two possible directions, but the choice has been arbitrary and not meant to indicate the actual direction.

The most detailed description of the development of the midpiece applies to the rat (Yasuzumi, 1956; André, 1962). In this species, according to André, elongated mitochondria divide into 3 or 4 spherical units which migrate to the cell membrane: the phenomenon of 'margination'. Later, these spheres move towards the flagellum and in transverse section 4–6 mitochondria are seen. Then these units elongate and diminish in volume, at the same time undergoing internal changes in structure. They ultimately meet to form a spiral, yet, though closely apposed, retain their individuality. Each mitochondrion occupies just less than three-quarters of one turn. Fawcett & Ito (1965) have examined the length of the mitochondria in the spermatozoa of certain bats and for one species (Myotis) have described an exceptional arrangement in which each mitochondrion comes to occupy exactly half of one gyre of the spiral.

In the mouse it is probable that margination of the spermatid mitochondria does not occur. Gardner (1966) described a tendency towards their peripheral location in the early spermatid, but stated that later their distribution in the cytoplasm is more even. Gresson (1942) also held that the mitochondria are evenly distributed. However, Gresson & Zlotnik (1945) did not remark on the phenomenon of margination in their study of the rat either. The division of the mitochondria apparent in surface replicas conforms with André's account, though in the mouse it would appear to occur after their migration to the flagellum. Bell (1953) demonstrated the spherical mitochondria of the mouse spermatid with iron-haematoxylin, and deduced, by focusing through thick sections, that they are arranged in a spiral. This has been confirmed in this study. A much earlier description of these spherical mitochondria was given by Retzius (1909), who also illustrated (what we now know to be an impression of) the spiral arrangement in the mature midpiece. According to Retzius' review of the literature, the notion of a spiral midpiece had been current then for more than 20 years. In most recent studies of spermateleosis in the mouse (e.g. Gardner, 1966) it is stated that the mitochondria become organized in a spiral fashion around the flagellum, but a more detailed analysis of the transformation has not been possible. From the present work, the low-magni-
Replicas of the spermatozoan midpiece

Replicas of the spermatozoon midpiece have permitted the construction of a geometrical scheme for the developing midpiece. Certain predictions have been made from the replicas, which can now be tested in sectioned material; and, conversely, findings from sectioned material—for example, that the fibrous sheath of the main-piece is laid down in the late cap phase (Nicander, 1962)—have been confirmed by surface replication.

At this stage the causal morphogenetic processes can be discussed only speculatively. Clearly, the mitochondria are attracted to the flagellum, and, since this occurs after the migration of the terminal annulus, the hypothesis can be made that this attractiveness is conferred by the annulus or triggered by its passage (Lommen, 1967). Regarding the derivation of the annulus, there has been a provisional report (Courot & Loir, 1968) suggesting that it develops from the chromatoid body of the early spermatid. The importance of the membrane reported to enclose a periflagellar canal in the early midpiece (Yasuzumi, 1956; Sotelo & Trujillo-Cenoz, 1958) remains to be elucidated. It is clear, though, that the deposition of the fibrous sheath of the main-piece, rather than the extent of the migration of the terminal annulus, determines the length of the midpiece. The variation in this length between males has been attributed largely to additive gene action (Woolley & Beatty, 1967), while the greater, within-male variance is as yet unexplained. Although some of the latter can safely be described as 'developmental' variance, the possibility of genetic variance due to post-meiotic gene action cannot be discounted. It is known, however, that at least one gene in the mouse which has an effect on the course of spermateleosis, hpy (recessive), is not expressed in heterozygotes (Bryan, 1968). There are more mitochondria in the spermatid cytoplasm than are incorporated in the midpiece (Bishop & Walton, 1960) review the considerable evidence on this point), and those left in the residual cytoplasm apparently undergo the same internal structural changes as those in the future midpiece (Dietert, 1966), suggesting that the proximity of the flagellum does not necessarily induce the transformation of the mitochondria.

This work was supported by a grant to Dr R. A. Beatty from the Agricultural Research Council made specifically for this research. The author would like to thank Dr R. A. Beatty for his interest and enthusiasm, and for his criticism of the manuscript. He wishes to express his gratitude, also, to Mr E. D. Roberts for his expertise in drawing Fig. 2, and to Mr F. Johnson for his careful photographic work.

REFERENCES


(Received 9 August 1969)

---

Figs. 3-5. Serial electron micrographs of a replica of one spermatozoan midpiece. The distal extremity has been omitted. x 27 000.
Replicas of the spermatozoan midpiece
Figs. 6–12. Replicas of spermatids (Figs. 6–11) and an immature spermatozoon (Fig. 12) arranged in the presumed developmental sequence. The terminal annulus (in the post-nuclear region) and the beginning of the fibrous sheath of the main-piece are arrowed in Fig. 7. Fig. 6, $\times 3000$; Figs. 7–12, $\times 4000$. 
Replicas of the spermatozoan midpiece
Figs. 13–18. Replicas of spermatids.

Fig. 13. Mitochondria arranged in a single dextral helix. × 9000.

Fig. 14. Spherical mitochondria, each with a roughly hexagonal surface. × 7000.

Fig. 15. Diamond-shaped mitochondria producing an interdigitating surface pattern. × 9000.

Fig. 16. Elongated mitochondria meeting to form a double sinistral helix (enlarged from Fig. 11). × 9000.

Fig. 17. Damaged spermatid. Spherical mitochondria adhering to the coarse fibres of the flagellum. × 7000.

Fig. 18. Damaged spermatid. Terminal annulus displayed. × 30000.
Replicas of the spermatozoan midpiece