A DISRUPTION AND FRACTIONATION OF BOVINE EPIDIDYMAL SPERMATOZOA

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Summary. The colloid mill was found capable of disrupting bovine epididymal spermatozoa in such a way that even the very resistant sperm middle piece was opened. The homogenate thus produced was fractionated by both differential centrifugation and isopycnic density gradient centrifugation. The latter method was found very useful in producing homogenous fractions of cell components including in order of increasing density: mitochondrial fragments, acrosomes, membranes, tail principal pieces, middle pieces, middle piece exteriors, contractile fibres and denuded heads. The mitochondrial particles consumed oxygen while the cell membranes had hexokinase-like activity.

INTRODUCTION

The structure of bovine spermatozoa varies in sensitivity to mechanical damage. The very fragile external membranes are broken merely by gentle shaking with 85 μ diameter glass beads (Morton & Lardy, 1967b). The head–tail juncctures require low intensity sonic oscillation for separation (Zittle & Odell, 1941) while the principal piece of the tail is fragmented only by higher intensities of sonic oscillations (Mohri, Mohri & Ernster, 1965). The remaining heads, as well as the middle pieces of the tail which house contractile elements surrounded by mitochondria, represent the upper extreme in resistance to mechanical damage. (For an excellent study of the anatomy of bull spermatozoa see Saacke & Almquist (1964a, b).

After testing Potter Elvehjem-type homogenizers, a blender, a sonicator and a ball mill without success, the colloid mill was found to disrupt the tail middle piece to produce a sperm cell homogenate in which the total contents of the spermatozoa with the exception of the nucleus were freed into the medium.

It was clearly shown by De Duve & Berthet (1953) that a contamination of the more rapidly sedimenting fractions by those migrating more slowly is inherent in the fractionation of a heterogenous suspension by differential centrifugation. Thus, in the isolation of rat liver mitochondria by differential centrifugation (Schneider, 1948), there is a large contamination of lysosomes, a significant contamination of microsomes and a small amount of other slowly sedimenting particles in the final mitochondrial preparation.

In the fractionation of spermatozoa disrupted in the colloid mill, this problem

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is accentuated because the components of the complex flagellum are segmented into pieces of widely varying lengths. It would appear from the results presented here that differential centrifugation which separates the components of mixtures primarily on the basis of particle size is, in terms of sperm components resolved, inferior to isopycnic density gradient centrifugation (De Duve, Berthet & Beaufay, 1959) which separates the components on the basis of density.

**EXPERIMENTAL METHODS**

Bovine epididymal spermatozoa, obtained through the courtesy of Oscar Mayer Inc., Madison, Wisconsin, were extruded from the epididymis and washed as described by Morton & Lardy (1967a). The washed cells (about 10⁷/ml) were suspended in 0.25 m sucrose with 50% v/v glass beads, 75 or 85 μ diameter (Superbrite, Minnesota Mining and Manufacturing Co.) and homogenized at 0° C in a colloid mill (Gifford-Wood Minimill) for 2 min at the maximum power setting and at a rotor–stator clearance just greater than that causing audible slowing of the instrument at low speed when beads were present. Light microscope observations at 1944 diameters after homogenization revealed that most cells had been decapitated. A high percentage of the resulting tail segments were severed at least once. Some middle pieces were split lengthwise causing them to appear much wider in the centre than the ends while other middle pieces were not only split but severed as well. The severed principal piece sections appeared in various states of disintegration, the final extreme being thin filaments which were assumed to be part of the contractile elements themselves.

The beads, along with some heads and most whole cells, were removed from the homogenate by centrifugation at 200 g for 10 min at 2° C in a Lourdes 9RA head. Only about 20% of the homogenate appeared above the sedimented beads due to the large interstitial volume between the beads. If a greater yield was desired, the homogenate containing beads, was diluted 6- to 20-fold with 0.25 m sucrose followed by centrifugation similar to that above. Then the supernatant fractions were either directly fractionated in the same rotor by differential centrifugation, or they were concentrated by 5 min centrifugation at 30,000 g and resuspended in a minimal volume of 0.25 m sucrose for use in density gradient centrifugation. In the latter case, the sample was layered upon linear sucrose gradients and centrifuged at 5° C in a Spinco SW25.1 swinging bucket rotor at 25,000 rev/min for 1 to 4 hr. The resultant visible bands were isolated by use of a pipette or by drop counting after insertion of a mountless number 25 hypodermic needle into the tube bottom.

Oxygen consumption was measured polarographically with a Gilson Medical Electronics oxygraph. The 2 ml, pH 7.4, incubation medium contained: 10 m-mole KH₂PO₄, 10 m-mole MgSO₄, 160 m-mole sucrose, 10 m-mole succinate or β-hydroxybutyrate, and 0.5 m-mole ADP (when added).

**RESULTS**

Table 1 describes the results of experiments using differential centrifugation as a means of fractionation of a colloid mill-produced sperm homogenate. None of the fractions appeared homogenous under the light microscope.
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Table 1
THE EFFECT OF INCREASING CENTRIFUGAL FORCE UPON THE SEDIMENTATION OF A SPERM HOMOGENATE

<table>
<thead>
<tr>
<th>Gravities (10 min)</th>
<th>Microscopic observations at 1944 diameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>510</td>
<td>All whole cells, majority of heads</td>
</tr>
<tr>
<td>1,200</td>
<td>Majority of midpieces (most damaged), a few heads and small particles</td>
</tr>
<tr>
<td>2,000</td>
<td>Remaining midpieces, many contractile fibre-like strands, a few small particles</td>
</tr>
<tr>
<td>3,200</td>
<td>Many contractile fibre-like strands, many small particles</td>
</tr>
<tr>
<td>4,600</td>
<td>Very small debris and hair-like objects, many small particles</td>
</tr>
<tr>
<td>6,300</td>
<td>Very few strands, many very small particles</td>
</tr>
<tr>
<td>8,200</td>
<td>Many extremely small particles</td>
</tr>
<tr>
<td>10,300</td>
<td>Many particles so small as to be almost invisible</td>
</tr>
<tr>
<td>28,000</td>
<td>Many particles so small as to be almost invisible</td>
</tr>
<tr>
<td>2,500 to 10,000</td>
<td>No midpieces or large midpiece fragments, many smaller objects described above (&gt; 3,200 g)</td>
</tr>
<tr>
<td>5,300 to 10,000</td>
<td>No midpieces or large contractile fibre-like strands, many smaller objects described above (&gt; 6,300 g)</td>
</tr>
</tbody>
</table>

Text-fig. 1 summarizes the results obtained by isopycnic density gradient centrifugation of the colloid mill-produced homogenate. The broad white band occurring at about 20% sucrose contained mitochondrial fragments. Electron microscopic observation of negatively stained samples showed this band to contain particles which looked like cristae in that they had corrugations upon
their surface similar to appendages found upon the surface of cristae in other mitochondria (Parsons, 1963). Occasionally there was a low amount of oxygen consumption associated with these particles which was not altered by the addition of ADP or 2,4-dinitrophenol. No net phosphate uptake could be detected by use of the assay of Morton & Lardy (1967a).

The broad white band occurring at about 29% sucrose did not appear in gradients layered with colloid mill-disrupted samples but was observed only when the spermatozoa were treated by gentle shaking with glass beads as described by Morton & Lardy (1967b). In a separate experiment of this type 10-min centrifugation at 1200 g removed all beads and cells leaving only acrosome-like solids in the supernatant fraction. Acrosomes were earlier demonstrated to be removed from spermatozoa by this treatment (Morton & Lardy, 1967b). These most delicate structures are evidently too fragile to withstand, undamaged, the rigours of the colloid mill. It is at present uncertain to what density the disintegrated acrosomal membranes would migrate.

The heavy yellow band near 40% sucrose was unusual because it contained what appeared under the light microscope to be very large sections of cellular membrane. Some were rectangular and about the size that one would predict would have come from the middle piece, i.e. they were about the same length as the middle piece and roughly three times as wide. Others were of a shape more like that of the head. There were many smaller pieces of membrane as well. The band which was concentrated to 0.5 ml by dilution and centrifugation was then assayed for hexokinase by the method of Darrow & Colowick (1962). An activity of 1 µmole glucose phosphorylated/min/ml was observed for the membranes of about 10^9 cells. This was estimated to be a four-fold excess over that required for the anaerobic formation of lactate at the rates recorded in the literature for this cell (Lardy & Phillips, 1943).

Since this assay for hexokinase depends upon pH change, it is possible that other enzymes such as membrane ATPase could contribute to the activity. However, the membrane ATPase inhibitor, ouabain, at 6 x 10^{-6} M caused no decrease in activity nor did 0.1 M KF, an inhibitor of contractile fibre and mitochondrial ATPase.

The next band appeared at 53% sucrose and contained sections of tail principal pieces.

The heavy white band at around 55% sucrose contained middle pieces and middle piece segments.

The light band immediately following at about 57% sucrose contained damaged middle piece sections. As the mitochondrial fragments appear to be among the least dense particles of the homogenate, it seems reasonable that these denser middle piece fragments would be partially devoid of mitochondria. The anatomical forms in this band, as in the case of those in some of the other bands, have never been reported. If the entire length of the middle piece was present, at 1944 diameters, it looked like a collapsed skeleton of a Japanese lantern because the many strands which were attached at each end bent out at the centre. If, however, the middle piece had been severed at the centre, what remained appeared as an end plate with strand-like appendages extending from one surface and whose ends curled away from the central axis at the tips. These strands
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of the middle piece structure did not appear to be as fine as the threads which occasionally protruded from the stump of broken tails or middle pieces and which probably were contractile fibres. It is suggested that the strands observed are associated with the protective structure of the middle piece which makes it more resistant than the rest of the tail to sonic and other physical attack.

The pale yellow band occurring near 60% sucrose concentration appeared to be composed of contractile fibre segments. If this were true, one would expect this band to have ATPase activity which would be inhibited by fluoride. As yet this has not been tested.

At about 64% sucrose cells were found which were severed only in the principal piece of the tail (except for the probable removal of exterior membranes). As would be predicted, these cell segments were more buoyant than those in the upper portion of the following band which contained heads attached to long middle piece segments. Lower in that band the attached middle piece fragments were shorter.

Near the bottom of the tube in about 70% sucrose was a ring-like pellet of denuded sperm heads.

The relative concentrations of the various bands depended upon the severity of the colloid mill disruption conditions.

DISCUSSION

The density data presented here complement and extend those of other workers. Benedict & Davis (1966), using rabbits, found epididymal spermatozoa to migrate to about 1·12 g/ml (30%) and ejaculated spermatozoa to migrate to two positions, about 1·18 (41%) and 1·28 (61%) g/ml, in sucrose gradients. In the case of bull and human as well as rabbit spermatozoa, the ejaculated cells of the upper band could be converted to those of the lower by treatment of the spermatozoa in a manner evidently deleterious to membrane integrity (Benedict, 1965). Lindahl & Thunquist (1965), using bulls, obtained similar results with Ficoll gradients with the exception that they did not observe the less dense category of ejaculated spermatozoa. In addition, they decapitated both ejaculated and epididymal spermatozoa, using very mild sonic oscillation, and found the tails of both cell types to migrate to about 1·06 g/ml. The epididymal heads appeared to have a density of about 1·13 g/ml while the heads from ejaculated cells appeared at 1·25 to 1·35 g/ml.

It has been demonstrated (Morton & Lardy, 1967b, c, d; Bishop & Hoffmann-Berling, 1959) that physical and chemical damage of certain bull-sperm membranes causes a loss of motility that can be regained only by the addition of necessary components. Therefore, the genetic defect in cattle which causes spontaneous sperm decapitation with the cell body retaining full motility (Mann, 1964) argues for the separateness of the membrane compartments of cell head and tail. Saacke & Almqquist (1964a, b) give anatomical evidence which supports this concept. The lower density of intact spermatozoa and of sonically decapitated cells, compared with that recorded here for other structural components, could be due to the retention of water within one or more cellular compartments. Thus, it is only after the membranes of the spermatozoa have been damaged that the true density of the structural components of the cell
becomes apparent. Furthermore, since the removal of the acrosome and other head components by the colloid mill was found here to produce a cell nucleus apparently as dense as that of the ejaculated cell, it is probable that the conversion of the epididymal sperm head to the much more dense head of the ejaculated cell during maturation does not involve the modifications of DNA packing but is associated with hydration changes in the extranuclear portions of the head.

That undamaged spermatozoa are not significantly dehydrated by the osmotic pressure of sucrose of equal density is indicated by the similar density observed for those cells when dextran, a high molecular weight polymer, was used to form the gradient (Lindahl & Thunquist, 1965). However, intact mitochondria remaining in fractions containing middle piece may be somewhat dehydrated by high concentrations of sucrose. This could cause a slight increase in the apparent density of those fractions but probably not in other fractions containing no intact membranes. Beatty (1964) has reported attempts to control tonicity and viscosity in density gradient media.

Whether or not it is possible to isolate undamaged sperm mitochondria is uncertain. It is not known how many individual mitochondria are contained in the mitochondrial helix of bull spermatozoa which makes about seventy-five turns around the contractile fibres (Rahlmann, 1961). If there are much fewer than seventy-five mitochondria/cell it may be impossible to remove a mitochondrion from around the tail without breaking it. In the bat (Fawcett, 1962) it appears that each mitochondrion only covers a portion of a turn and thus might not be damaged if disengaged from the tail. If the latter were true in the bull, and no whole mitochondria were freed by the disruption technique of this report, one might suspect that the mitochondria may adhere very tightly to themselves, to the inside of the middle piece cover, or to the outside of the axial bundle, thus preventing their release. That some form of attachment exists was suggested by electron micrographs of middle piece fragments. In these small segments which consisted of only two or three turns of helix, the mitochondria were roughly torn on either side and yet retained their middle piece configuration. Possibly treatment with a proteolytic enzyme or mild sonic oscillation would loosen the adhesions and permit the mitochondria to be removed.

Finally, it is suggested that the disruption and fractionation methods presented here have potential for the preparation of specific cell fractions for enzymatic, immunological or compositional studies.

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