INTRODUCTION

It has been known since the nineteenth century that the microscopical structure of living cells may be modified by fixation and by post-mortem or sub-mortem changes (Hardy, 1899), but inadequate attention seems to have been paid to factors which may injure spermatozoa before fixation and microscopical examination. One such factor is the effect of sudden cooling.

If semen is exposed to a sudden fall in temperature there is an irreversible decline in the activity of the spermatozoa (Chang & Walton, 1940), and this reduction in activity is associated with an increase in the number of dead, i.e. eosinophil cells (Lasley, Easley & McKenzie, 1942). This phenomenon of 'temperature shock' is encountered in bull semen cooled abruptly by pipetting semen at 30° C. into an aqueous solution of nigrosin and eosin at 20° C. (Hancock, 1951). The depressed rate of respiration and fructolysis of temperature-shocked samples can be correlated with the increase in the proportion of eosinophil cells (Bishop & Hancock, 1951).

When bull spermatozoa are stained to differentiate living from dead spermatozoa, the acquisition of staining affinity by the dead spermatozoa is associated with obvious structural changes. These changes are likely to be encountered in fixed specimens unless special precautions are taken to avoid lethal damage from 'temperature shock' before fixation.

The introduction of the electron microscope has given a new impetus to the study of the morphology of spermatozoa. The spermatozoa of the bull have been studied and described in great detail (Bretsneider & van Iterson, 1947; Bretschneider, 1949, 1950) and Randall & Friedlaender (1950) have used the method to investigate the structure of ram spermatozoa. The published results of these studies contain, however, no observations on the need for special precautions in the treatment of spermatozoa before fixation, and it is possible that some of the structural features which have been described may be absent from the intact living cell and appear only as artifacts after its death.

In this paper the morphological features of living and dead bull spermatozoa are described. The differences which have been observed relate to the structure of the head. It is therefore convenient at this stage to record the principal structural features which have been described in the head of the bull spermatozoon. These are illustrated diagrammatically in Text-fig. 1a.
The galea capitis is shown as a loose cap investing the anterior half of the head and overlying a second more compact cap, the acrosome, which is closely applied to the head. The posterior boundary of the galea capitis marks the anterior border of the equatorial segment which is bounded posteriorly by the posterior margin of the acrosome. This reconstruction of the head of a bull spermatozoon is based on a number of sources discussed throughout this paper. It should be compared with the head shown in Text-fig. 1b. Here, the head is covered by a single structure called the acrosome. This is the 'cap' referred to throughout this paper. Text-fig. 1b is based on the experiments described below, which deal with the variations in the morphological appearance of the acrosome; the appearance of the acrosome in Text-fig. 1b is characteristic of the living spermatozoon.

Text-fig. 1.  

a. Conventional diagram of head of bull spermatozoon.  
b. Head of living spermatozoon (see text).

MATERIALS AND METHODS

Semen was obtained from four bulls at the Animal Research Station, Cambridge, and twelve animals at the Cambridge Cattle Breeding Centre. It was collected in an artificial vagina and held in a water-bath at 30°C. before any subsequent treatment. To preserve the vitality of samples selected for study of the characteristics of the living cell, ejaculates were maintained at 30°C. during examination and fixation. These operations were usually completed within 30 min. of collection. When examination was delayed the samples were placed in a 100 ml. beaker containing water at 30°C. and transferred to a refrigerator at 4°C. so that cooling to this temperature occupied not less than 1 hr., and temperature shock was avoided.

The characteristics of the dead cell were studied in spermatozoa killed by cooling rapidly to 0 or -10°C., and in samples of semen cooled, with precautions...
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against temperature shock, to 4°C. and stored at this temperature for 2-3 weeks. Additional observations were made on the dead spermatozoa encountered in varying proportions in freshly ejaculated semen.

The proportions of live and dead cells were determined, for all samples studied, at the time of collection and at the time of examination or fixation. It was thus possible to relate the cytological characteristics of test populations to their viability as determined by the differential staining technique. The following techniques were used:

* Nigrosin-eosin stain. This was prepared by dissolving 5 g. water-soluble eosin Y (Gurr) in 300 ml. of a 10% solution of nigrosin (Gurr) in distilled water.

The stain was used to differentiate living and dead spermatozoa and for the preliminary study of their cytological differences (Hancock, 1951). The number of living cells was determined in suspensions prepared by diluting one drop of fresh semen at 30°C in eight drops of the stain at this temperature. After 5 min. one drop of the suspension was smeared on to each of two slides on a warm stage, allowed to dry in air and mounted in DPX. Counts of a total of two hundred spermatozoa per slide were made under a 4 mm. objective and ×8 oculars, using a blue filter and Beck 'Tenslite' illumination.

The cytological characteristics of cells killed by temperature shock were studied in suspensions made by diluting one drop of semen at 30°C in eight drops of stain at 0°C.

* Giemsa stain. Smears of whole semen were dried in air on a warm stage, fixed in neutral formal-saline (5% formaldehyde) for 30 min. and then stained for 1½ hr.

in the following solution:

- Giemsa (Gurr) 3 ml.
- Sorensen phosphate buffer, pH 7·0 2 ml.
- Glass-distilled water 35 ml.

* Heidenhain's iron haematoxylin. Smears of whole semen could not be stained satisfactorily by this technique. They were therefore prepared from semen diluted in M/15-phosphate buffer (pH 7·4). These were dried in air, fixed in osmium tetroxide vapour for 10 min. at 37°C., washed overnight in running tap water, mordanted for 24 hr. in 2½% ferric alum solution, stained for a similar period in haematoxylin, differentiated in fresh alum solution and mounted in DPX.

Control suspensions were made by pipetting one drop of semen at 30°C. into eight drops of buffer solution at this temperature.

Suspensions of 'temperature-shocked' material were prepared by dilution with buffer at 0°C. This treatment was sufficient to kill all spermatozoa in the suspension.

* Microscopical examination of unfixed material. Living semen was cooled slowly and handled at room temperature, after storage, if necessary, at 4°C. For study of the dead cell, spermatozoa were killed by pipetting 0·5 ml. semen at 30°C., drop by drop, into a 6 in. Pyrex test-tube pre-cooled to 0°C. in an ice-filled vacuum flask.
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Samples of the whole semen were mounted under a cover-glass on a thick film of 1% agar in mammalian Ringer. Details of the technique will be published by Mr J. Smiles, National Institute of Medical Research, Mill Hill, London.

Direct observations were made with Beck phase contrast microscope equipment. Living and dead spermatozoa were photographed by direct and phase-contrast ultraviolet light. I am indebted to Mr Smiles for the photomicrographs which are reproduced in this paper.

Electron micrography. The spermatozoa were washed by slow centrifugation in phosphate buffer (pH 7.4), fixed in formal-saline for 10 min. and washed twice in distilled water. The grids were shadowed with gold-palladium (40–60 %) at an angle of 20° to the horizontal.

Control material was cooled slowly to room temperature and handled subsequently at room temperature. 'Shocked' samples were obtained by pipetting 0.5 ml. whole semen at 30 °C, drop by drop, into a 6 in. Pyrex test-tube pre-cooled in a freezing mixture of ice and salt to —10 °C. and exposed to this temperature for 15 min. This treatment ensured death of all cells in any sample of whole semen. It was employed because the cytological characteristics of cells from semen ‘temperature-shocked’ in this manner could be definitely related to the structure of the dead cell. Because of the much larger sample sizes available for study by light microscopy, the significance of any morphological heterogeneity within samples containing both live and dead cells could readily be determined. This might not have been possible in samples of the size studied with the electron microscope. The electron micrography was carried out by Dr J. R. G. Bradfield, Cavendish Laboratory and Department of Zoology, University of Cambridge.

RESULTS

The appearance of the spermatozoa in films prepared by the nigrosin-eosin technique from freshly ejaculated active bull semen is shown in Pl. 17a.

The outline of the spermatozoon is clearly defined, and its bright refractile head is in sharp contrast to the dark background. The anterior end shows a bright distinct boundary zone, which becomes indistinct in the region of the equatorial axis. This appearance is closely comparable with that in the photomicrographs of unfixed living material (Pl. 18a, c) and in those of material fixed in the ‘living' condition and stained with iron haematoxylin (Pl. 17c). The distinct boundary to the anterior pole of the sperm head marks the limits of the cap which, in Giemsa-stained preparations, is seen to invest this region of the head (Pl. 17e). The electron micrograph of fixed material illustrates this general appearance in greater detail and confirms the impression that the structure is closely applied to the sperm head, the outline of which is regular and uninterrupted (Pl. 19a). The posterior boundary of the cap is straight and bisects the head equatorially.

The appearance of dead spermatozoa in fixed and unfixed material is characteristicly different from that of the living cell and of cells in which the living structure has been fixed.

In the nigrosin-eosin preparations, the ‘dead’ spermatozoa are stained with
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Eosin and no longer contrast sharply with the background stain (Pl. 17b); the outline of the sperm head is indefinite and, in fact, the 'dead' spermatozoa in such preparations are readily distinguished from the 'living' ones without reference to their affinity for eosin. The cap is now inconspicuous; it shows an irregular outline and loosely envelopes the anterior part of the sperm head.

This loosening of the attachments of the cap is more evident in the photomicrographs of dead unfixed spermatozoa. There is evidence of shrinkage along the posterior border of the cap which results in the appearance of an equatorial segment (Pl. 18b, d). The general pattern conforms with that in the photomicrographs of stained dead material (Pl. 17d). The profound nature of the structural change in the cap is clearly illustrated in the electron micrograph (Pl. 19b). These changes are also found in the dead spermatozoa in freshly ejaculated semen and in samples which have been stored for several days, but are more pronounced in the latter. The wide separation of the cap and sperm head which is frequently observed in such material appears to represent a later post-mortem change preceding complete detachment of the cap. These features are illustrated in Pl. 17c.

Table 1. The relationship between the numbers of capless and dead spermatozoa

<table>
<thead>
<tr>
<th>Bull</th>
<th>No. of ejaculates studied</th>
<th>Average % dead cells</th>
<th>Average % capless cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>22.9</td>
<td>2.20</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>17.1</td>
<td>1.12</td>
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<tr>
<td>3</td>
<td>3</td>
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<tr>
<td>4</td>
<td>2</td>
<td>13.0</td>
<td>2.88</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>22.0</td>
<td>2.62</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>15.8</td>
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</tr>
<tr>
<td>7</td>
<td>2</td>
<td>10.0</td>
<td>2.50</td>
</tr>
<tr>
<td>8</td>
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</tr>
<tr>
<td>9</td>
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<tr>
<td>12</td>
<td>2</td>
<td>45.0</td>
<td>33.13</td>
</tr>
</tbody>
</table>

Table 1 summarizes some observations on the incidence of capless spermatozoa in freshly ejaculated semen. The proportion of capless cells was determined by counting 200 spermatozoa in each of two Giemsa-stained smears of whole semen.

The table shows that the proportion of capless cells is related to the proportion of dead cells. Analysis of the total data for individual ejaculates shows that this relationship is significant at the 0.1% level and provides clear evidence that denudation of the sperm head is associated with death of the cell.

DISCUSSION

Bull spermatozoa exhibit markedly altered morphological characteristics after death; any conclusions about the normal morphology of spermatozoa may, therefore, be open to serious criticism unless steps are taken to determine and control the viability of the material studied. In this way structural variations due to morbid
changes in the cell may be recognized and their significance appreciated. The sus-
ceptibility of spermatozoa to lethal damage inflicted by sudden cooling suggests
that particular care is required in the handling of semen prior to cytological study.

Of immediate interest are the changes which have been shown to occur in the
sperm cap. In the living spermatozoon the cap is closely applied to the head, while
in the dead spermatozoon it appears as a looser structure which may become
detached as a fragile envelope. The changes in the structure of the cap which are
seen after death are also responsible for the appearance of the equatorial segment
which is not visible in the living spermatozoon. A survey of the published results of
previous studies shows that there is still some doubt as to the identity and nature of
the cap. An attempt will be made here to show that some of this confusion is
traceable to the failure of previous observers to recognize the existence of post-
mortem changes in its structure.

Blom (1945) confirmed the existence of a sperm cap in the bull and stallion and
identified it as the galea capitis or kopfhappe previously described by Ballowitz
(1891 a, b) and Retzius (1909). His review of earlier work suggests that he regards it
as homologous with the structure described in other species as the perforatorium
(Waldeyer, 1906), and acrosome (Bowen, 1924), to mention only two of the several
names which exist. The present observations have shown that the sperm cap of the
bull possesses a uniformly characteristic appearance in the living cell and confirm
Blom’s suggestion that detachment of the cap may be evidence of the death of the
spermatozoon.

An examination of the literature which has followed Blom’s paper suggests that
while there is widespread acceptance of the existence of the galea capitis there is
reluctance to identify it with the acrosome.

Rao & Hart (1948) noted the variations in the morphological appearance of the
sperm cap which have been described above. They failed, however, to appreciate
their significance. They suggest that in certain forms the acrosome can be seen
within the cap. Their photomicrograph in support of this suggestion illustrates the
features seen in PI. 17 a. The evidence for the existence of a second structure is
unconvincing and does not justify the implication that the protoplasmic cap (galea
capitis) is not the acrosome.

Bretschneider & van Iterson (1947) and Bretschneider (1949) have illustrated the
galea capitis in situ as a fragile, loose envelope, and it is obviously identical with the
cap dealt with in this paper. In all their illustrations, however, the characteristic
features of the cap of the living cell have been lost. Bretschneider (1950) has
recently brought forward evidence suggesting that the galea capitis originates as a
double structure which sometimes persists as such and is encountered as a specific
sperm defect associated with sterility in the bull (Teunissen, 1946; Hancock, 1949).
He emphasizes, however, that he has been unable to demonstrate more than one
cap-like structure in the mature spermatozoa of the normal bull, and his discussion
of the results of comparative studies with the electron microscope of the sperma-
tozoa of the guinea-pig, bee, squid and bull leave no doubt that he regards the galea
capitis of the bull as the homologue of the acrosome of other species.
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Randall & Friedlaender (1950), in a study of the ram spermatozoa with the electron microscope, claim to have demonstrated two cap-like structures. These are identified as the galea capitis and acrosome. The galea capitis is said to lie over the acrosome which only becomes visible after removal of the former; their separate illustrations of these two structures show that the acrosome corresponds closely in appearance with the cap of the bull spermatozoon in Pl. 19a; there is a similar correspondence between their illustration of the galea capitis and that of the cap of the bull spermatozoon in Pl. 19b.

Bishop (1951) has since claimed to have demonstrated in bull spermatozoa structures comparable with those reported for the ram by Randall & Friedlaender, although his illustrations furnish little evidence in support of this claim.

It is probably significant that in none of the papers cited above is there any evidence of an attempt to determine and preserve the viability of the spermatozoa in the semen studied. There can be little doubt that failure to distinguish between the structure of the cell before and after death has produced an erroneous picture of the normal appearance of the sperm cap. Equally there can be little doubt that the alleged existence of a galea capitis and an acrosome in the spermatozoon of the ram and bull is due to the failure to distinguish between the characteristic appearances of the cap before the death of the cell, and after. The structure which exhibits these characteristic changes is identical with the galea capitis described by Blom. In the absence of convincing evidence of other comparable structures it may be concluded that the terms acrosome and galea capitis have been used to describe the same structure.

It seems worth while to examine this conclusion in the light of other evidence provided by studies of spermatogenesis. Gatenby & Woodger (1921), in their study of spermatogenesis in the guinea-pig, attempted to resolve some of the confusion surrounding the identity of the cytoplasmic investment of the spermatozoon. It is noteworthy that the term galea capitis is absent from the list of terms tabulated by them in an attempt to systematize the nomenclature relating to this structure. It would seem probable that the term galea capitis has only been applied to the mature sperm. According to the above workers the kopfkappe is the equivalent of the structure described by Papanicolaou & Stockard (1918) as the spermiocalyptrotheca of the guinea-pig sperm. Gatenby & Woodger apparently did not identify any similar structure in their own studies of this species, but suggest as a simple alternative 'the covering membrane of the acrosome'. This structure was said by Papanicolaou & Stockard to invest the whole sperm head and cannot therefore be the homologue of the kopfkappe of Retzius. Gresson & Zlotnik (1948) have described the origin of the acrosome of the bull spermatozoon in terms which correspond with those used by Gatenby and his collaborators (Gatenby & Woodger, 1921; Gatenby & Beams, 1935) to describe its origin in other species, although the acrosome in the guinea-pig is exceptional in that it appears as a double structure. There is no doubt that the acrosome of the bull, described by Gresson & Zlotnik, is the cap dealt with in this paper and the structure which Blom described as the galea capitis. Thus the terms acrosome and galea capitis as applied to the bull
spermatozoon are synonymous and it becomes necessary to reject one. The universal use of the term acrosome by cytologists in this country and the U.S.A. is sufficient justification for its retention.

SUMMARY

The microscopical features of living and dead bull spermatozoa are described. The findings are discussed in relation to previous observations on the structure of the head of the bull spermatozoon. The cytoplasmic cap of the living cell possesses a characteristic structure which is identical with that which has been ascribed to the acrosome. The altered structure of the cap of the dead spermatozoon is identical with that previously described as characteristic of a second cap-like structure, the galea capitis. It is concluded that the terms galea capitis and acrosome have been used to describe the same structure. It is suggested that the cytoplasmic cap of the bull spermatozoon should be called the acrosome.

I wish to thank Lord Rothschild, Department of Zoology, University of Cambridge, for his unremitting encouragement and criticism.

I am also indebted to Dr W. Jacobson, Strangeways Institute, Cambridge, for his generous guidance, to Dr A. Walton, Animal Research Station, Cambridge, for his help and interest, and to my colleague, Mr R. C. Campbell, for his statistical analysis of the data in Table 1.

REFERENCES


HANCOCK—THE MORPHOLOGY OF BULL SPERMATOZOA
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EXPLANATION OF PLATES

**PLATE 17**


**PLATE 18**


**PLATE 19**


In Pls. 17 and 19, 'living' spermatozoa means spermatozoa in which the structure of the living cell has been preserved; 'dead' spermatozoa means spermatozoa in which the structure of the living cell has been destroyed.