WATER CONTENT, SPECIFIC GRAVITY AND CONCENTRATIONS OF ELECTROLYTES IN BULL SPERMATOZOA

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Summary. The water content of epididymal bull spermatozoa in media of osmolalities corresponding to those of seminal plasma and to the fluids from the cauda epididymidis was determined by a gravimetric-volumetric method as 0.586±0.0052 and 0.512±0.0086 ml per ml of cells, respectively. It was related to the ionic concentrations in mmol/litre of spermatozoa determined by the conversion of the mean values of the major cations expressed as mequiv./litre, mg/100 g, or mg/100 ml, established by previous authors. The osmolalities of the intracellular fluids were also calculated. They were compared with the osmolalities of the extracellular fluids, the comparison making it possible to perform an introductory analysis of the osmotic-equilibrium states of bull spermatozoa.

The calculated osmolality of the cytosol of epididymal spermatozoa was found to be about 40 mosmol less than that of the epididymal fluids. This discrepancy from the state of osmotic equilibrium is not serious when considering the errors, which are probably inherent in the values of both the ion and water-content determinations. The osmolality of the cytosol of ejaculated spermatozoa was calculated as 1.7 times greater than that established for seminal plasma. If the calculated value is reasonably correct, this should imply that, for the existence of identical concentrations of osmotically active particles on both sides of the cell membrane of spermatozoa suspended in seminal plasma, a fraction of the intracellular ions, amounting to about 38%, must be assumed to be 'bound'.

Determinations of the water-content of the spermatozoa furnished data from which it was possible to calculate the specific gravity of the cells. This was compared with those actually established by gradient centrifugation in various media by different authors.

INTRODUCTION

Drevius & Eriksson (1966) studied the swelling in hypotonic media of bull, rabbit and human spermatozoa. In order to interpret such changes quantitatively, the concentrations of water and of ions in spermatozoa under isotonic
conditions must be known. In the present study, the fractional water content of epididymal bull spermatozoa has been determined and has been related to published values for ionic concentrations. On the basis of this comparison, osmotic equilibria can be given a preliminary analysis. The data so obtained permit indirect determinations of the specific gravities (sp. gr.) of the spermatozoa.

MATERIAL AND METHODS

Bull spermatozoa were pooled from the cauda epididymidis of gonads from slaughterhouse material. The hydrostatic pressure inside the epididymis was raised by injection into the vas deferens of 0.2 to 0.4 ml of the appropriate suspending medium (see below). The ducts of the epididymides were then gently bisected and the spermatozoa from the organs available in a given experiment (6 to 16) were collected and thoroughly mixed. In the experiments, these dense stock suspensions were diluted from four to eight times.

Specific gravities of suspending media and suspensions

These were established by pyknometry at 20.5 to 21.0°C (volume of pyknometers, 10 ml). The media used were NaCl solutions of 0.154 M and 0.191 M, M denoting molality. The first-mentioned solution has the same freezing-point depression, Δ, as seminal plasma and as Mann’s Ringer solution (Mann, 1964), i.e. −0.53°C (Rothschild & Barnes, 1954), whereas Δ for 0.191 M-NaCl is −0.66°C, a value equalling that of the fluids from the cauda epididymidis (Salisbury & Cragle, 1956). The molalities of these saline solutions were calculated from the formula Δ = 1.86 × 2 × φ × M, by ‘trial-and-error’ selection of the concentrations of NaCl which, together with the corresponding osmotic coefficients φ, yielded values of Δ of 0.53 and 0.66, respectively. The values of φ used, 0.928 and 0.925, were obtained by interpolation of the φ values given for various concentrations of NaCl by Robinson & Stokes (1949).

The fractional water content of the spermatozoa (ml of water/ml of cells)

This was determined with the aid of values for (1) the sp. gr. of suspensions and suspending media, (2) the dry- to wet-weight ratios of suspensions and suspending media, established by weighing portions of suspensions and media before and after evaporation to constant weight (105°C for 12 hr) and (3) the relative volume of the cells in the suspensions, calculated by dividing spermatocrit values, corrected for trapped extracellular medium, by the volume of the centrifuged suspension aliquots (see below). It would have been desirable to obtain values for (1) to (3) from the same suspension. This, however, was not usually possible because sperm volumes were seldom sufficient. Values for (1) and (2), together with spermatocrit values, were therefore derived from experiments performed on two series of suspensions made up in 0.154 and 0.191 M-NaCl solutions, respectively. The trapped extracellular medium was then determined on a third series of suspensions (see below) and the values obtained were used to correct the spermatocrits obtained in the first and second series.
Spermatocrit determinations

These were performed in calibrated tubes of the type described by Drevius (1971) on suspension aliquots of 0.800 g in duplicate at 3°C for 50 min at 4000 g, the g number most commonly applied by different authors in separations of spermatozoa and seminal plasma for ion-content determinations (see below). The spermatocrits were read at x20.

Trapped extracellular medium in spermatocrit columns

This was determined by analogy with the method developed for red cells by Chaplin & Mollison (1952). A preliminary test was performed in each experiment. In these tests, the spermatocrit of a 0.3-ml sample of the experimental suspension made up in 0.154 m-NaCl was recorded. From this value, calculation was made of the volume of suspension which, after dilution with a 0.1-vol. of Evans Blue (EB) solution containing 0.5 g of EB/20 ml of 0.154 m-NaCl, would yield a spermatocrit of approximately 0.075 ml, i.e. a volume somewhat less than the volume, 0.080 ml, of the graded parts of the tubes (cf. Drevius, 1971). The final suspension, containing dye, was divided into two equal parts. To one of these was added NaCl to increase the salt concentration from 0.154 to 0.191 m. After gentle shaking for 15 min, the calculated volumes of suspension were transferred in duplicate to spermatocrit tubes and packed cell volumes were recorded after 50 min at 4000 g at 3°C.

A supernatant volume of 0.02 ml was then diluted to 5.0 ml in calibrated volumetric flasks, giving ‘standard’ solutions. The remaining supernatant in each tube was removed. The insides of the tubes above the cell columns were rinsed with saline and finally dried with filter paper. The spermatocrit columns were then transferred quantitatively by micropipette to 5.0-ml volumetric flasks, using saline as wash fluid and also for bringing the volume to 5.0 ml. The cells were spun down, and the supernatants (‘test’ solutions) were removed. ‘Standards’ and ‘test’ solutions were gently shaken for 30 min and then were centrifuged at 4000 g for 30 min. This procedure removed the slight opalescence, most probably caused by cytoplasmic droplets shed during the previous centrifugation.

The optical densities of ‘standards’ and ‘test’ solutions were read on a Zeiss spectrophotometer (Model PMQ II) at 620 nm (Shohl & Hunter, 1941). The ‘blank’ was a solution composed of 0.02 ml of the supernatant of an unstained suspension similarly centrifuged and diluted to 5.0 ml. In all experiments, the final concentration of EB fell within the concentration range of the dye in which, according to Shohl & Hunter (1941), there is a linear relation between optical density and concentration. The trapped extracellular medium was then calculated in accordance with the example given by Chaplin & Mollison (1952).

Determination of the fractional water content and calculation of the specific gravity of epididymal bull spermatozoa

The fractional water content of the spermatozoa, \( V_w/V_c \), was derived from the equation

\[
V_w/V_c = \rho_s(1-M_s)/h - \rho_m(1-h)(1-M_m)/h
\]

This formula is identical with that used by Savitz, Sidel & Solomon (1964),
in determining the fractional water content of human erythrocytes except that the parameters \( \rho_m \) and \( M_m \) (\( m \) indicating 'medium') replace \( \rho_p \) and \( M_p \) (\( p \) indicating blood plasma). In the formula, \( \rho_s \) and \( \rho_m \) express the sp. gr. and \( M_m \) the dry- to wet-weight ratios for suspensions and suspending medium, respectively, while \( h \) is the relative volume of the spermatozoa in the suspensions (ml of spermatozoa/ml of suspension). The volume \( V_s \) of the centrifuged 0·800-g aliquots (p. 17) was obtained from 0·800/\( \rho_s \) and \( h \) by dividing the spermatocrit values, corrected for trapped extracellular medium, by \( V_s \).

In the different experiments, the triplicate determinations of \( M_s \) agreed to within 0·5% and the duplicate determinations of the spermatocrits to within 1·5%.

The sp. gr. of the spermatozoa in the different suspensions was calculated from the equation

\[
\text{h} \times (\text{sp. gr.)}_{\text{spermatozoa}} + (1 - \text{h}) \times \rho_m = 1 \times \rho_s
\]

**Calculation of the ionic concentrations in spermatozoa and epididymal and seminal plasma**

The ion content of spermatozoa and epididymal and seminal plasma, as determined by different investigators, has been expressed in different terms. For purposes of comparison and discussion, mean values obtained by the authors listed in Tables 3 and 4 have been transformed into millimolar concentrations. In the transformation of values originally expressed as mg/100g of spermatozoa or epididymal or seminal plasma, the sp. gr. of epididymal spermatozoa was taken as 1·096, i.e. the mean of the results of Lavon, Volcani, Amir & Danon (1966b), 1·0817, and of Lindahl & Thunqvist (1965), 1·10 to 1·12. The sp. gr. of ejaculated spermatozoa was taken as 1·175 (1·16 to 1·19) (Benedict, Schumaker & Davies, 1967) (Table 3, column b) and also as 1·07 (Lavon, Volcani, Amir & Danon, 1966a) (Table 3, column a). The sp. gr. of epididymal and seminal plasma were taken as 1·032 which is the mean value found by Anderson (1946) for seminal plasma.

**RESULTS**

**Influence of medium tonicity upon the spermatocrit**

The spermatocrit values for 2·5-ml aliquots of suspensions of identical cell concentrations (0·25 ml of stock suspension + 10·0 ml of medium) were decreased in the range 0·154 m-NaCl to 0·764 m-NaCl (Table 1).

**Table 1**

| Spermatocrit values (uncorrected for trapped extracellular medium) for identical suspensions of epididymal bull spermatozoa in saline solutions of increasing tonicities (4000 g for 50 min) |
|---|---|---|---|
| Concentrations of NaCl | 0·154 m | 0·191 m | 0·382 m | 0·764 m |
| Spermatocrits | \( \text{1} \cdot \text{0} \) (\( n = \text{8} \)) | \( 0 \cdot 907 \pm 0 \cdot 0054 \) (\( n = \text{8} \)) | \( 0 \cdot 796 \pm 0 \cdot 0124 \) (\( n = \text{5} \)) | \( 0 \cdot 793 \pm 0 \cdot 0176 \) (\( n = \text{5} \)) |

The values recorded in 0·154 m-NaCl are taken as unity. The errors are standard errors. For further information, see text.
Water content, sp. gr. and electrolytes of bull sperm.

Fractional water content of epididymal spermatozoa suspended in 0·154 and 0·191 m-NaCl solutions

Table 2 (A and B) gives data from one experiment on spermatozoa suspended in 0·154 and 0·191 m-NaCl solutions, respectively, and Table 2 C, the mean values of $V_w/V_c$ obtained in the different experiments.

**Table 2**

<table>
<thead>
<tr>
<th></th>
<th>Series I (0·154 m-NaCl)</th>
<th>Series II (0·191 m-NaCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\rho_s$</td>
<td>$M_s$</td>
</tr>
<tr>
<td><strong>A</strong></td>
<td>1·0111</td>
<td>0·0368</td>
</tr>
<tr>
<td></td>
<td>1·0128</td>
<td>0·0403</td>
</tr>
</tbody>
</table>

* Values used in the determination of all $V_w/V_c$ values of Series I and II, respectively. The errors are standard errors. For further information, see text. TEM = trapped extracellular medium.

Influence of centrifugation on subsequent swelling of the spermatozoa in hypotonic media

Spermatozoa from aliquots of five different stock suspensions were diluted 1:6 with 0·154 and 0·191 m-NaCl solutions, respectively, and centrifuged for 50 min at 4000 $g$. After resuspension, the spermatozoa were observed microscopically. It was found that a high percentage of the cells had lost their cytoplasmic droplets. After moderate centrifugation (300 $g$ for 10 min) in ordinary centrifuge tubes, these cells (A) as well as a sample of a previously non-centrifuged control suspension (B) were suspended in 0·032 m-saline containing 5% of Ficoll and studied under the phase-contrast microscope. About 95% of the cells of (B) were coiled and their cytosol showed bright contrast with the medium, indicating that the cells were swollen (cf. Drevius & Eriksson 1966; Drevius, 1968, 1972a, b). The corresponding percentages for cells of (A), which had been centrifuged previously in 0·154 and 0·191 m-NaCl, were 85·8 (range 78·2 to 89·6) and 85·4 (range 74·8 to 90·3), respectively. Obviously, the strong centrifugation (or this in combination with the removal of the spermatoctrit columns by means of a micropipette) had had a negative effect upon the membrane integrity of the cells.
Table 3

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**IONIC CONCENTRATIONS IN MMOL/LITRE IN BULL SPERMATOZOA FROM CAPUT EPIDIDYMIS (A), CAUDA EPIDIDYMIS (B) AND EJACULATES (C), CALCULATED FROM MEAN VALUES EXPRESSED IN MEquiv./LITRE, MG/100 G OR MG/100 ML BY THE AUTHORS LISTED BELOW THE TABLE**

<table>
<thead>
<tr>
<th>Investigation</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Ca⁺⁺</th>
<th>Mg⁺⁺</th>
<th>Na⁺/K⁺ ratio</th>
<th>Original values in:</th>
</tr>
</thead>
<tbody>
<tr>
<td>A { 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>75</td>
<td>6</td>
<td></td>
<td></td>
<td>1-0</td>
</tr>
<tr>
<td>B { 2 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Means (2 and 3)</td>
<td>21-45</td>
<td>48-75</td>
<td>3-15</td>
<td>7-4</td>
<td>(16-5)</td>
<td>0-44</td>
</tr>
<tr>
<td>C { 4 5 6 7 8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Means (4 to 8)</td>
<td>62-6</td>
<td>66-1</td>
<td>53-4</td>
<td>56-5</td>
<td>6-3</td>
<td>6-8</td>
</tr>
</tbody>
</table>

The original values are given within parentheses. In section (C), the values of the A columns were calculated by considering the sp. gr. of the spermatozoa as 1-07, in the B columns as 1-75 (cf. Material and Methods). For further information, see text.

References to original values. (1) and (2): Crabo (1965), Figs. 27 to 30; (3) and (4): Quinn, White & Wirrick (1965), Table 7, means of six replicates, and Table 3, means of twenty replicates, respectively; (5): Salisbury & Cragle (1956), Table 3, sixteen ejaculates; (6): Quinn & White (1966), Table 1, four replicates; (7): Quinn, White & Wirrick (1966), calculated mean from mean values given in Tables 1 to 3; (8): Quinn & White (1968), Table 1, six replicates.
TABLE 4
IONIC CONCENTRATIONS IN MMOL/LITRE IN BULL SEMINAL PLASMA FROM CAPUT EPIDIDYMIS (A), CAUDA EPIDIDYMIS (B) AND EJACULATES (C), CALCULATED FROM MEAN VALUES EXPRESSED IN MEquiv./LITRE, MG/100 G OR MG/100 ML BY THE AUTHORS LISTED BELOW THE TABLE

<table>
<thead>
<tr>
<th>Investigation</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Ca⁺⁺</th>
<th>Mg⁺⁺</th>
<th>Cl⁻</th>
<th>Na⁺/K⁺ ratio</th>
<th>Original values in:</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>121.2 (121.2)</td>
<td>28.6 (28.6)</td>
<td>1.55 (3.1)</td>
<td>—</td>
<td>3.15 (6.3)</td>
<td>81 (81)</td>
<td>mequiv./litre</td>
</tr>
<tr>
<td>2</td>
<td>57.4 (57.4)</td>
<td>43.7 (43.7)</td>
<td>0.35 (0.7)</td>
<td>3.15</td>
<td></td>
<td>92.1 (92.1)</td>
<td>mequiv./litre</td>
</tr>
<tr>
<td>Means (1 and 2)</td>
<td>89.3</td>
<td>36.15</td>
<td>0.95</td>
<td>3.15</td>
<td></td>
<td>86.5</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>36.1 (36.1)</td>
<td>27.9 (27.9)</td>
<td>0.35 (0.7)</td>
<td>1.35 (2.7)</td>
<td>65.3 (65.3)</td>
<td>mequiv./litre</td>
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</tr>
<tr>
<td>4</td>
<td>32.9 (32.9)</td>
<td>27.9 (27.9)</td>
<td>1.1 (2.2)</td>
<td>—</td>
<td>14.3 (14.3)</td>
<td>mequiv./litre</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>39.0 (87)</td>
<td>23.0 (84)</td>
<td>2.4 (9.2)</td>
<td>1.3 (3.0)</td>
<td>—</td>
<td>39.8</td>
<td>mg/100 g</td>
</tr>
<tr>
<td>Means (3 to 5)</td>
<td>36.0</td>
<td>26.3</td>
<td>1.28</td>
<td>1.32</td>
<td>39.8</td>
<td>1.37</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>6</td>
<td>101 (225)</td>
<td>41.0 (155)</td>
<td>10.2 (39.6)</td>
<td>3.4 (8.1)</td>
<td>—</td>
<td>—</td>
<td>mg/100 g</td>
</tr>
<tr>
<td>7</td>
<td>112.2 (258)</td>
<td>44.0 (175)</td>
<td>9.2 (37)</td>
<td>3.5 (8.4)</td>
<td>49.4 (175)</td>
<td>mg/100 ml</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>126.1 (290)</td>
<td>41.2 (161)</td>
<td>7.0 (28)</td>
<td>—</td>
<td>43.4 (154)</td>
<td>mg/100 ml</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>105.3 (242)</td>
<td>44.0 (172)</td>
<td>6.1 (24.6)</td>
<td>4.0 (9.8)</td>
<td>93.0 (330)</td>
<td>mg/100 ml</td>
<td></td>
</tr>
<tr>
<td>Means (6 to 9)</td>
<td>111.2</td>
<td>42.6</td>
<td>8.1</td>
<td>3.6</td>
<td>61.9</td>
<td>2.61</td>
<td></td>
</tr>
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</table>

The original values are given within parentheses. For further information, see the text.

References to original values. (1) and (4): Crabo (1965), means of average ionic concentrations in epididymal plasma from segments B and F, respectively, in Group I and II bulls in Tables VIII to XIV; (2) and (3): Wales et al. (1966), means of three replicates, each pooled from ten to fifteen bulls; (5) and (6): Quinn et al. (1965), means of six and twenty replicates, respectively; (7): Rothschild & Barnes (1954), mean of plasma values from twenty to thirty ejaculates; (8): Salisbury & Crangle (1956) and Crangle, Salisbury & VanDemark (1958), mean of plasma values from sixteen and four ejaculates, respectively; (9): Crangle, Salisbury & Muntz (1958), mean of fourteen determinations, each on seminal plasma pooled from several ejaculates.
Ionic concentrations in spermatozoa and ‘sperm plasma’ from different levels of the bull’s reproductive tract

The results of the calculations, performed as stated on p. 18, are shown in Tables 3 and 4. The centrifugal force applied in the original experiments for the separation of spermatozoa and plasma varied somewhat. In Table 3, g values were 4000 in investigations (3), (4), (6) and (8), and 700 in (5), whereas in investigations (1) and (2), centrifugation was carried out at 13,000 rev/min in a ‘microfuge’. In Table 4, centrifugations were as follows: 13,000 rev/min in a ‘microfuge’ in (1) and (4), 18,000 g in (2) and (3), 4000 g in (5) and (6), 4000 rev/min in a refrigerated centrifuge in (7), and 700 g in (8) and (9).

DISCUSSION

Fractional water content of bull spermatozoa from the cauda epididymidis

In planning the present study, it was considered desirable to determine the water content of the spermatozoa in media of osmolalities which corresponded to those of their natural milieu (the fluids from the cauda epididymidis) and of the seminal plasma into which they are transferred at ejaculation. The value obtained in the latter medium could then be compared with that determined for ejaculated spermatozoa. Unfortunately, difficulties in obtaining fresh ejaculated semen in adequate quantities precluded such a comparative study.

The spermatoocrit values show that epididymal bull spermatozoa shrink when the osmolality of the medium is increased (Table 1) as ejaculated spermatozoa were found to do when studied by electronic sizing (Bredderman & Foote, 1969; Foote & Bredderman, 1969). On the other hand, spermatoocrit experiments and microscopical observations indicate that ejaculated bull spermatozoa do not shrink significantly in hypertonic media (Pursley & Herman, 1950; Lindahl & Kihlström, 1952; Rothschild, 1959). The shrinking of epididymal bull spermatozoa at strong hypertonicity is also apparent under the microscope: the volume of the cytoplasmic droplet is greatly decreased, its contents being finally tightly pressed against the motor apparatus, from which it can frequently be observed to be shed into the medium.

The $V_w/V_c$ value of the spermatozoa was 12.6% lower in 0.191 M-NaCl than in 0.154 M-NaCl (Table 2 C). Since the osmolality of 0.191 M-NaCl (0.353 osmol) is approximately 19% greater than that of 0.154 M-NaCl (0.286 osmol), the $V_w/V_c$ value in the 0.191 M-NaCl solution should theoretically have been 19% lower than in the 0.154 M-NaCl solution, i.e. 0.474.

This discrepancy may be due to the following factors. (1) The tendency of the dense sperm suspensions, if not carefully handled, to exhibit a certain degree of ‘foaming’, may make the pyknometrical sp. gr. determinations somewhat uncertain. (2) The presence of spermatozoa which become leaky during the centrifugation procedure (p. 19), as well as of the low percentage of cells which are dead and leaky already in the stock suspensions. The leaky spermatozoa will not respond to osmotic gradients. It may be mentioned that Mann (1964) has demonstrated an escape of cytochrome c from spermatozoa in strongly centrifuged suspensions. The presence of leaky spermatozoa may also
Water content, sp. gr. and electrolytes of bull sperm.

affect trapped extracellular medium values; furthermore, these values may be influenced by a binding of the indicator substance to the spermatozoa. It has not been possible to evaluate and correct for the influence of these factors.

If the volume of the spermatozoa is regarded as unity in 0·154 m-NaCl solution, the $V_w/V_c$ value established for cells in 0·191 m-NaCl (Table 2 C) will predict a cell volume in this medium of (in relative units) $1 - (0·586 - 0·512) = 0·926$, implying a volume decrease of 7·4%. If the theoretically expected $V_w/V_c$ value in 0·191 m-NaCl is considered, the cell volume expected in this medium should be $1 - (0·586 - 0·474) = 0·888$, i.e. it should decrease with 11·2% when the spermatozoa are transferred from a 0·154 m- to a 0·191 m-NaCl solution. The actual spermatocrit values recorded in these solutions (which are uncorrected for trapped extracellular medium) gave a difference of 9·3% (Table 1).

If the $V_w/V_c$ values of spermatozoa in 0·382 and 0·764 m-NaCl solutions (cf. Table 1) are calculated on the basis of a $V_w/V_c$ value of 0·586 in 0·154 m-NaCl solution and of the simplifying assumption that the osmotic coefficients in all media are 1·0, the $V_w/V_c$ values of the spermatozoa when in 0·382 and 0·764 m-NaCl solutions should be 0·236 and 0·118, respectively. According to these values, the relative volumes of the spermatozoa in these media should be $1 - (0·586 - 0·236) = 0·650$ and $1 - (0·586 - 0·118) = 0·532$, respectively, i.e. they should be 35 and 47% less, respectively, than in 0·154 m-NaCl solution. As is evident from Table 1, the spermatocrits recorded show instead differences of 20·4 and 20·7%, respectively. These discrepancies may be explained by the fact that, when mammalian spermatozoa become dehydrated beyond a certain extent, no further volume decrease will be registered in spermatocrit experiments which are not associated with determinations of trapped extracellular medium. This is because the apparent packed cell volume will probably be determined by the main outline of the formed rigid elements of the individual spermatozoa (nucleus with membranes, mitochondria and fibrous sheath). However, the true cell volume, representing the sum of volumes of individual cells, may be considerably less. The true cell volume will be determined by the conformation of the cell membrane which, under strong cellular dehydration will probably become invaginated within the spaces between the intracellular structures, e.g. between mitochondria and between tail fibres. The tension then being exerted on the membrane may, at a certain degree of cellular dehydration, induce membrane rupture. This idea is supported by the fact that when bull spermatozoa were suspended in hypertonic sodium citrate, electron microscopical examination revealed that both cell membranes and mitochondrial cristaee were ruptured, whereas the spermatozoa appeared quite normal under the light microscope (Saacke, 1962, cited by Benedict et al., 1967). In these experiments, fresh bull ejaculates were diluted 1:5 with 29% sodium citrate before fixation for electron microscopy (Saacke, personal communication). If a 3·8% solution of this salt is regarded as isotonic to the spermatozoa, the osmotic pressure of the hypertonic solution should have been approximately seven times that of seminal plasma.

Furthermore, there is the possibility that, with increased hypertonicity, a fraction of the water contained in the spermatozoa may be transformed into a 'bound' state (cf. Lindahl & Kihlström, 1952).
The fractional water content of epididymal spermatozoa in 0·191 M-NaCl, 0·512 ml/ml of spermatozoa (Table 2 C), i.e. in a medium with an osmolality corresponding to that of the fluids from the cauda epididymidis (p. 19), is not consistent with determinations by Lavon, Volcani & Danon (1968) of the dry-matter of spermatozoa concentrated by means of 'differential flotation' in hydrophobic phthalate esters. For epididymal spermatozoa, these authors obtained a dry-matter percentage of 31·5 and, for ejaculated spermatozoa, of 29·7, i.e. the water content of the two kinds of spermatozoa was 0·685 and 0·703 ml/ml of cells, respectively. On the other hand, on the basis of dry-matter determinations, O'Donnell (1969) determined the mean water content of ejaculated bull spermatozoa in a medium equivalent to seminal plasma as 53·4%. This value is rather close to that (58·6%) obtained in the present study for epididymal spermatozoa in 0·154 M-NaCl, which has the same osmolality as seminal plasma (Table 2 C).

It is improbable that the types of error associated with the experiments of the present study could alone account for the differences between the results of Lavon et al. (1968) and of the present author. Possibly, there are real physiological differences in the hydration states of spermatozoa from different breeds of bulls. The values obtained by Lavon et al. (1968), however, may be somewhat high, since there is a great tendency for water to become associated with hydrophilic particles when such particles are spun down from a water phase into and through a hydrophobic phase (Berthet & Baudhuin, 1967).

Concentrations of ions and other osmotically active particles in spermatozoa and epididymal and seminal plasma

The importance of ionic quantities in spermatozoa and extracellular liquids for the constitution of osmotic-equilibrium states of mammalian spermatozoa has received little attention. For an introductory analysis of this problem, mean values of the concentrations (in mmol/litre) of the different cations in bull spermatozoa and epididymal and seminal plasma were taken (Tables 3 and 4), and transformed into mmol/litre equivalents of Na⁺. With this simplification, a concentration of cations is obtained for spermatozoa from the cauda epididymidis, which, if associated with Cl⁻, would be equivalent to 21·5 + 48·8 + 3·15 x 3/2 + 7·4 x 3/2 = 86·1 mmol NaCl/litre of spermatozoa (see Tables 3 and 5). Analogously, the actual values for epididymal plasma from the cauda region (Table 4) will be 66·2 mm (see Table 5), for ejaculated spermatozoa—134·5 to 142·5 (values under A and B columns in Table 3) and for seminal plasma—171·4 (see Table 5, column A, where the concentration for ejaculated spermatozoa, 139 mm, is the mean of the values 134·5 and 142·5).

The concentrations in the sperm cytosol (Table 5, column B) were obtained by dividing the values of Table 5, column A, by the fractional water content of the spermatozoa, 0·512 for epididymal cells (see Table 2 C) and 0·534 for ejaculated cells (O'Donnell's value, 1969) (see above).

Rothschild & Barnes (1954) found that the osmotic pressure of seminal plasma, calculated on the basis of its cationic content (cf. Table 4), was greater than that calculated from its freezing-point depression, −0·53°C. These authors assumed, therefore, that all the cations in seminal plasma are not free but that
fractional amounts of Mg\(^{++}\) and Ca\(^{++}\), for example, may be bound to citrate and phosphate ions as well as to anionic sites in the proteins.

The osmolality of epididymal plasma calculated as previously stated is 126 mosmol (Table 5). However, epididymal plasma also contains 22 mmol NH\(_4\)\(^+\)/litre and 378 mg of total carbohydrates/100 ml (Wales, Wallace & White, 1966) as well as 68 mmol glycercylphosphorylcholine/litre (Crabo, 1965). If the ammonium ions are assumed to be balanced by univalent anions, the carbohydrates to be free hexoses and all osmotic coefficients to be unity, the concentrations mentioned will provide an osmotic concentration of \((2 \times 22 + 21 + 68) = 133\) mosmol, i.e. the calculated osmolality of the epididymal plasma will be \(126 + 133 = 259\) mosmol (Table 5) which is \((353 - 259) = 94\) mosmol less than the osmolality empirically determined (Table 5). The background to this fact is uncertain. One explanation is that the value for the freezing-point

<table>
<thead>
<tr>
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<th>Concentrations of 'salts' expressed as equivalents of NaCl in mmol/litre of A Sperm. and extracellular fluids</th>
<th>B Sperm cytosol*</th>
<th>C Osmalities (mosmol) of sperm cytosol and extracellular fluids</th>
<th>D Calculated†</th>
<th>Established†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epididymal spermatozoa</td>
<td>86.1</td>
<td>168</td>
<td>311</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Epididymal plasma</td>
<td>66.2</td>
<td>—</td>
<td>126</td>
<td>353</td>
<td></td>
</tr>
<tr>
<td>Ejaculated spermatozoa</td>
<td>139</td>
<td>260</td>
<td>+133 = 259‡</td>
<td>480</td>
<td>—</td>
</tr>
<tr>
<td>Seminal plasma</td>
<td>171.4</td>
<td>—</td>
<td>317</td>
<td>286</td>
<td></td>
</tr>
</tbody>
</table>

Differences in osmalalities between calculated and established intra- and extracellular liquids:
- Epididymal spermatozoa—epididymal plasma = 311 – 353 = –42 mosmol
- Ejaculated spermatozoa—semenial plasma = 480 – 286 = +194 mosmol

The calculations were made on the basis of the mean concentrations of the major cations in spermatozoa and plasma listed in Tables 3 and 4 and of a fractional water content of epididymal spermatozoa of 0.512 ml (cf. Table 2 C) and of ejaculated spermatozoa of 0.534 ml/ml of cells (O'Donnell, 1969). For further information, see text.

* All ions assumed to be 'free'. The osmalalities are derived with the aid of osmotic coefficients for NaCl solutions of the actual molalities, the osmotic coefficients being calculated from data in Robinson & Stokes (1949) and Giese (1960).
† See Material and Methods.
‡ See text.

depression –0.66°C (Salisbury & Cragle, 1956) of the epididymal fluids consisting of spermatozoa and epididymal plasma, may be greater than the value for epididymal plasma per se because of a leakage of solutes from spermatozoa which may have been injured by the low temperature.

There is a difference between the calculated osmalality of the cytosol of epididymal spermatozoa and the established osmalality of their natural medium
of $353 - 311 = 42$ mosmol (Table 5). This discrepancy cannot be regarded as great in considering the errors which are no doubt implicated in the determinations of ion-content and water-content of the spermatozoa. Furthermore, unidentified compounds could raise the intracellular osmolality above 311 mosmol.

As regards ejaculated spermatozoa, the problem is apparently quite different. In these cells, the calculated value for the cytosol, 480 mosmol, is about 1-7 times greater than that established for seminal plasma, 286 mosmol (Table 5). Here, it must be assumed that a large fraction of the intracellular ions are 'bound', i.e. osmotically inactive. This fraction, equivalent to an intracellular excess of 194 mosmol, corresponds approximately to 100 mmol NaCl/litre. This implies that about 38\% ($100/260 = 0.38$) of the intracellular ions are bound (cf. Table 5, column B).

Thus, the accumulated experimental data indicate that the event of ejaculation in bulls is associated with a series of striking physiological changes in the spermatozoon and their environment (Crabo, 1965): a significant decrease in the osmotic pressure of the medium; a shift in the Na$^+$/K$^+$ quotient in the medium from 1-37 to 2-61; a parallel shift in the Na$^+$/K$^+$ quotient of the spermatozoon from 0-44 to 1-17; a significant increase in the spermatozoon not only of Na$^+$ but also of K$^+$ (Table 3), and a binding of approximately 38\% of the intracellular ions.

**Specific gravity of bull spermatozoa**

The values for the sp. gr. of epididymal bull spermatozoa recorded by different authors are shown in Table 6. The average sp. gr. of spermatozoa in

<table>
<thead>
<tr>
<th>Investigation</th>
<th>Specific gravity</th>
<th>Methods and authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0817 ± 0.002</td>
<td>GC (phthalate esters); Lavon et al. (1966b)</td>
</tr>
<tr>
<td>2</td>
<td>1.10 to 1.12</td>
<td>GC (Ficoll); Lindahl &amp; Thunqvist (1965)</td>
</tr>
<tr>
<td>3a</td>
<td>1.11 to 1.14</td>
<td>Centrifugation in saline-Ficoll solutions. Concentrations of NaCl: in (a) and (c) 0.17 M, in (b) the spermatozoon were swollen, 0.031 M. In (c), the spermatozoon had been previously lysed in distilled water (Drevius &amp; Eriksson, 1966)</td>
</tr>
<tr>
<td>3b</td>
<td>1.03 to 1.07</td>
<td>0.191 M-NaCl solution (4a) and 0.154 M-NaCl solution (4b). Calculated values. The calculations were performed as described in Material and Methods. (Drevius, this paper.)</td>
</tr>
<tr>
<td>3c</td>
<td>&gt; 1.14</td>
<td>(mean ± S.E.; n = 8)</td>
</tr>
<tr>
<td>4a</td>
<td>1.113 ± 0.002</td>
<td>(mean ± S.E.; n = 8)</td>
</tr>
<tr>
<td>4b</td>
<td>1.095 ± 0.004</td>
<td>(mean ± S.E.; n = 8)</td>
</tr>
</tbody>
</table>

GC = gradient centrifugation.
same osmolality as seminal plasma, falls between those of investigations (1) and (2).

As regards ejaculated bull spermatozoa, published sp. gr. values show a considerable variation, from approximately 1.02 to 1.3 (Lindahl & Kihlström, 1952; Lavon et al., 1966b; Benedict et al., 1967; O’Donnell, 1968). According to Benedict et al. (1967), the true physiological sp. gr. of ejaculated bull spermatozoa falls within the range 1.16 to 1.19. The sp. gr. of rabbit spermatozoa has been reported to vary from less than 1.11 to more than 1.13 with a mean of 1.132 (Beatty 1964 a, b, 1969).

Since bull spermatozoa are osmotically active (Drevius & Eriksson, 1966; Drevius, 1968, 1972a, b; Bredderman & Foote, 1969; Foote & Bredderman, 1969), reliable determinations of their sp. gr. are possible only if (a) the suspending medium has the physiological osmolality and (b) the integrity of the cell membrane is preserved. Thus, the sp. gr. is decreased in hypotonically swollen spermatozoa (Table 6, 3 b) but increased in spermatozoa whose membrane integrity has been upset by hypotonic lysis (Table 6, 3 c) or by treating the cells with surface-active substances or freezing and subsequent thawing (Benedict et al., 1967). When membrane integrity has been upset, the sp. gr. will be determined by the ‘sperm skeleton’ which has a greater sp. gr. than the intact spermatozoa (Kihlström, 1958; Benedict et al., 1967). In hypertonic media, the spermatozoa become dehydrated (Bredderman & Foote, 1969; Foote & Bredderman, 1969; cf. Table 1, this study) and therefore exhibit a greater sp. gr. than the true, physiological one. Furthermore, in strongly hypertonic media in which the membranes of the spermatozoa become ruptured or leaky (Saacke, 1962, cited by Benedict et al., 1967), the sp. gr. of the spermatozoa, determined by their ‘skeleton’, will be high, as shown in experiments by Benedict et al. (1967) in which the spermatozoa were suspended in strongly hypertonic solutions of different univalent salts.

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