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Surface Area and Volume Measurements for Ram and Human Spermatozoa[1]

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ABSTRACT

Surface area and volume measurements were made for ram and human spermatozoa. Measurements were made using different techniques in an attempt to confirm estimates arrived at by independent methodologies. Sperm head projected surface areas were calculated from published formulae using linear micrometry measurements or were obtained directly by image analysis. Total surface areas were calculated as twice the projected head area plus the flagella area, estimated from published dimensions. Spermatozoon surface area was also measured from electron micrographs using a stereological method. Micrometric methods gave values of 135 μm² for ram spermatozoa and 106 μm² for human spermatozoa. Stereology methods gave values of 142 μm² for ram spermatozoa and 106.5 μm² for human spermatozoa, offering good agreement between the two methods. Sperm volumes were estimated by stereology and by radiolabel volume exclusion methods. From stereology, ram sperm volume was 31.3 μm³ and human sperm volume was 22.2 μm³. From the volume exclusion method, ram sperm volume was estimated as 25 μm³. Attempts to measure total volume and water volume simultaneously using an adaptation of this method were unsuccessful. Separate estimation of water volume for ram spermatozoa gave a value of 12.8 μm³, suggesting a 50% water space. Results from this study are compared with existing published values.

INTRODUCTION

Surface area, volume, and aqueous volume are basic cell parameters that have been measured for a wide variety of cell types. These are required measurements if established models of cell responses to anisosmotic conditions and particularly to different cryopreservation protocols are to be applied to spermatozoa. Since Leeuwenhoek's original observations in 1677 [1], sperm structure has been investigated in considerable detail, resulting in a good understanding of the anatomy of the spermatozoon [2, 3]. There is also an extensive literature dealing with the linear dimensions of spermatozoa from numerous species [4]. However, the characteristic sperm morphology and the very small size of some of the sperm dimensions has made it difficult either to convert linear measurements into surface area and volume estimates or to establish these parameters using other methods. Consequently, reliable values for sperm surface area and volume have remained elusive.

Considering the numerous studies concerned with the linear dimensions of spermatozoa, there are remarkably few estimates of sperm surface area in the published literature. A number of authors have considered the projected surface area of the bull sperm head either by planimetric methods or by calculation from optical or electron microscopical measurements [5]. However, these studies have only rarely included measurements of flagellar area or total surface area. Early estimates of sperm volume were also calculated in the main from optical or electron microscopy measurements, whereas later estimates have most commonly been made using electronic sizing techniques [5].

In the study reported in this paper, we attempted to measure ram and human sperm surface areas and volumes and to estimate the percentage of water space of ram spermatozoa. As far as possible we sought to confirm our estimates by the use of different methodologies and by comparison with published values. Surface areas were calculated from optical measurements and by analysis of electron micrographs using a stereological method. Volume measurements were made stereologically and by the use of a radiolabel volume exclusion method, and ram sperm water volume was measured using a volume exclusion method.

MATERIALS AND METHODS

Semen Collection

Ram semen was obtained by artificial vagina from Friesland rams held at the Royal Veterinary College (London, UK). Only ejaculates with high wave motion and a high percentage of motility were used. Human semen was obtained by masturbation from healthy donors; all samples were judged normo-spermic by World Health Organization criteria [6].

Micrometry

The mean projected head area of ram spermatozoa was calculated according to van Duijn [7], using an approximation of the Folium of Descartes:

$$A = \{(1.05 - 0.225\, B_{basis}/B_{max}) \times (0.36\, B_{basis} + 0.69\, B_{max})L\} \quad (1)$$

where A = projected head area, B_{max} = maximal head width, B_{basis} = head width at point of connection to midpiece, L = head length. Measurements were made using a ×100 oil immersion objective lens and a calibrated, eyepiece screw micrometer. Cells were examined as a wet preparation immobilized in one of four different ways. Semen was diluted 1:200 (v:v) in a modified Tyrode's diluent (NaCl 137 mM, KCl 1 mM, $Na_2H_2PO_4$ 0.4 mM, $MgCl_2$ 0.5 mM, glucose 5.5 mM, HEPES 20 mM, pH 7.4, 300 mOsm) containing 1) 70 mM NaF, 2) 70 mM NaN_3, 3) 0.4% formaldehyde. Semen was also diluted 1:200 (v:v) in Tyrode's diluent and attached to polylysine (1 mg/ml) coated slides.

Alternatively, head area was measured directly using the C-Scan image analysis system (C-Scan Imaging plc, Cambridge, UK) using cells immobilized with either NaF or NaN_3 prepared as for micrometer measurements.

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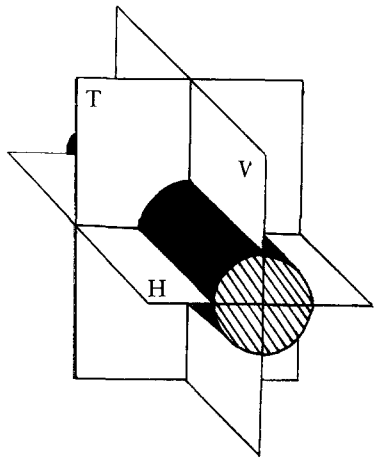

FIG. 1. Diagram illustrating the three mutually perpendicular planes—horizontal (H), vertical (V), and transverse (T)—used for cutting ultrathin sections from the cylindrical sperm pellets for transmission electron microscopy.

Human sperm projected head area was calculated according to van Duijn [8] using the formula:

$$A = 0.73 \times L \times B_{max} \quad (2)$$

where A = projected head area, L = head length, B_{max} = maximal head width. Spermatozoa were washed using a discontinuous Percoll gradient [9]. Cells were immobilized with either NaF or NaN_3 and prepared as described previously.

Electron Microscopy

Spermatozoa were fixed in 5% glutaraldehyde in 175 mM cacodylate buffer and postfixed in 1% osmium tetroxide. Fixed cells were counted using a hemocytometer, loaded into microcapillary tubes of known radius (diameter/2), and centrifuged to produce a cylindrical pellet of cells. Pellet length (l) was measured using a traveling microscope, and pellet volume was calculated as r^2l. Pellets were removed intact from the capillary tubes and dehydrated through ten steps of alcohols to propylene oxide in 10-min stages and embedded in epoxy resin before ultrathin sectioning for transmission electron microscopy.

A necessary condition for all stereological analysis is random orientation of structures with respect to the section plane. Centrifuging spermatozoa within microcapillary tubes results in an anisotropic arrangement of the cells within the pellet. It has been demonstrated that in homogeneous anisotropic specimens, the precision of estimation is considerably augmented if isotropic uniform random orientated (IUR) sets of three mutually perpendicular sections (orthogonal triplet probes) are used instead of three independent IUR sections [10], and this approach was adopted here.

Fixed and embedded cylindrical pellets were cut into three parts for sectioning in three perpendicular planes (Fig. 1). Three grids containing sections were prepared for each orientation, of which one was selected at random and stained with uranyl acetate and lead citrate. For each orientation, eight micrographs were taken from a single section at a magnification of ×5 000 according to a prearranged pattern. Thus a set of 24 photographs was produced from each sample for analysis.

Stereological Estimation of Surface Area

Surface area was estimated from the surface density (S_v) of the sections [11]. Surface density was represented by the contour length density of the sperm profiles within the section, which in turn was estimated by placing a grid of test lines of known length over the section and counting the number of intersections formed by these lines with the profile border, so that $S_v = 2 \times I_l$ where I_l = number of intersections per unit length of test lines. Sperm surface area (A_{sp}) was calculated as $A_{sp} = (S_v \times V_p)/n$, where V_p = pellet volume, n= number of cells [11].

Stereological Estimation of Volume

Volume was estimated from the volume density (V_v) on the sections, obtained by using an overlay grid with a number of sampling points and estimated as the proportion of sampling points overlying sperm profiles on the section. Individual sperm volume (V_{sp}) was calculated as $V_{sp} = (V_v \times V_p)/n$, as previously described [11].

Ram Sperm Water Volume

Water space was measured using the method of Ford and Harrison [12], using tritiated water and [^{14}C] mannitol as an extracellular space marker. Semen was diluted 1:10 with PBS (NaCl 160 mM, Na_2HPO_4 8 mM, $NaH_2PO_4 \cdot 2H_2O$ 2 mM) and divided into four replicates. A volume of 50 μl was removed from each replicate and diluted 1:20 with immobilizing fluid for counting in a hemocytometer slide. Diluted semen (270 μl) was spiked with 15 μl of ^3H_2O (10 μCi/ml) and 15 μl of [^{14}C]mannitol (6 mM, 0.02 μCi/μl) and incubated at room temperature for 30 min. Labeled spermatozoa (200 μl) were loaded onto 150 μl of silicon oil (DC 550/1107; Dow Corning, Midland, MI) and centrifuged for 8 min at 520 × *g* at 20°C in an Eppendorf tube. A volume of 10 μl of supernatant was removed and added to 10 ml of scintillation fluid. The sperm pellet was cut out of the tube and added to 10 ml of scintillation fluid. Water volume was calculated as:

$$T - (C \times R)/n \times S \quad (3)$$

where T = ^{3}H dpm in the pellet, C = ^{14}C dpm in the pellet, R = ratio of ^{3}H dpm/ml:^{14}C dpm/ml in the incubation medium, S = ^{3}H dpm/μl water, and n = number of spermatozoa in pellet.

Simultaneous Measurement of Ram Sperm Water Volume and Cell Volume

An attempt was made to measure water volume and total cell volume on the same sample simultaneously using a modification of the method of Ford and Harrison [12]. Semen was diluted 1:5 in PBS, and subsamples were removed for cell counting as described previously. A volume of 360 μl of diluted semen was spiked with 20 μl of [^{14}C]mannitol (6 mM 0.02 μCi/μl) and 20 μl of ^3H_2O (10 μCi/ml) and incubated for 30 min at room temperature. Aliquots (50 μl) of the radiolabeled sample were loaded into each of six capillary tubes of known diameter with 10 μl of silicon oil (DC 550/1107) and centrifuged for 10 min at 1500 × *g* and 20°C. After centrifugation, the pellet and supernatant were measured using the traveling microscope. Tubes were broken at the oil layer separating pellet and supernatant, and the pellet and 10 μl of supernatant were each added to 10 ml of scintillant in separate vials for scintillation counting.

TABLE 1. Measurements of ram spermatozoa head dimensions in micrometers (mean ± SEM).[a]

Immobilizing agent	(n)[b]	B_{basis}	B_{max}	L	Projected area[c]
Polylysine	(50)	2.52 ± 0.20	5.04 ± 0.26	8.45 ± 0.32	34.81
Formaldehyde	(50)	2.52 ± 0.20	4.96 ± 0.26	8.31 ± 0.37	33.67
NaF	(50)	2.46 ± 0.33	4.77 ± 0.24	8.29 ± 0.36	32.32
NaN_3	(50)	2.60 ± 0.27	4.95 ± 0.32	8.47 ± 0.39	34.21

[a] B_{basis} = head width at midpiece junction (μm), B_{max} = maximum head width (μm), L = head length (μm).
[b] Ten measurements were made on each of five ejaculates.
[c] Calculated from van Duijn's formula [7].

Cell water volume was calculated as previously described. Total cell volume was calculated as:

$$(r^2l) - (C \times R)/n \times S \quad (4)$$

where r = radius of capillary tube, l = pellet length, C = ^{14} dpm in the pellet, R = ratio of ^{3}H dpm/ml:^{14}C dpm/ml in the incubation medium, S = ^{3}H dpm/μl water, and n = number of spermatozoa in pellet.

RESULTS

Micrometry: Ram Spermatozoa Surface Area

Ten measurements were made on each of five ejaculates derived from four rams (n = 50 spermatozoa, one ram used twice) for each of four different immobilizing agents. There were no significant differences in the sperm dimensions between rams or between treatments with the different immobilizing agents (Table 1). The overall mean value for projected head area obtained using van Duijn's formula [7] was 33.8 ± 0.3 μm^2 (mean ± SEM). Direct measurement of projected head area according to the C-Scan image analyzer using either NaF or NaN_3 to immobilize cells gave values of 32.0 ± 0.3 μm^2 and 32.1 ± 0.3 μm^2 (mean ± SEM, n = 100), respectively, showing good agreement with the calculated values. If a mean is taken from all the different estimates, a value of 33.2 μm^2 is obtained.

Total surface area is given by 2 × projected head area + midpiece and principal piece area. Duncan and Watson [13] modeled the ram sperm principal piece and midpiece as two cylinders and calculated a total flagella area of 69 μm^2 using diameter measurements made from electron micrographs and published values for flagellar length [4]. If this value is used with the above head area, the total surface area is estimated as 135.4 μm^2.

Human Spermatozoa

Ten measurements were made from single ejaculates for each of seven different donors, and areas were calculated using van Duijn's formula [8] (Table 2). The overall mean value for projected head area was 20.5 ± 2.3 μm^2. If the flagellar area is modeled as above using published values for midpiece and principle piece dimensions [4], an estimate of 65 μm^2 for flagellar area is obtained, giving a total surface area of 106 μm^2 using the above model.

Stereology: Surface Area

Estimation of surface density requires that sperm profiles are randomly arranged in each electron micrograph. However, for spermatozoa pelleted in the capillary tubes there was a tendency for the long axes of the sperm heads to align parallel to each other (Fig. 2), which would, for instance, result in a greater number of interceptions being recorded with use of a grid with vertical lines than with one with horizontal lines. To overcome this problem it is necessary to use isotropic probes. For this study, counts were made using a test grid consisting of a series of semicircular test lines (Fig. 3a). With use of this grid, the mean (± SEM, n = 6) ram sperm surface area was calculated as 142.3 ± 10.8 μm^2 (Table 3). For human spermatozoa, the equivalent estimate was 106.5 ± 6.2 μm^2 (Table 3).

Volume

Volume density was calculated from point counts using either the ends of the semicircular test lines (Fig. 3a) or a grid composed of squares with intersections used as test points (Fig. 3b), resulting in grids with either 84 or 1271 test points, respectively. For ram spermatozoa, mean sperm volume (± SEM, n = 6) using the 84-point grid was 30.9 ± 2.2 μm^3 and using the 1271 point grid was 31.7 ± 2.2 μm^3 (Table 4). A paired *t*-test showed these values to be not significantly different ($t_{(5)} = 1.01$, $p = 0.357$), and the overall mean value was 31.3 ± 3.5 μm^3. For human spermatozoa, the respective values were 24.2 ± 0.9 μm^3 and 20.1 ± 1.3 μm^3 (Table 4). Because of the small sample size, these results were tested by the nonparametric Wilcoxon test and found to be not significantly different; the overall mean value was 22.2 ± 1.2 μm^3. Although the

TABLE 2. Measurements of human spermatozoa head dimensions in micrometers (mean ± SEM).

Donor identity	(n)[a]	Length (μm)	Maximum width (μm)	Projected area[b] (μm^2)
5	(10)	6.96 ± 0.46	4.53 ± 0.50	23.02
16	(10)	7.15 ± 0.40	4.49 ± 0.28	23.44
24	(10)	6.80 ± 0.43	4.24 ± 0.55	21.05
25	(10)	6.07 ± 0.59	3.92 ± 0.45	17.37
23	(10)	6.63 ± 0.40	3.87 ± 0.56	18.73
18	(10)	6.68 ± 0.45	4.27 ± 0.32	20.82
17	(10)	6.36 ± 0.57	4.10 ± 0.51	19.04
Mean ± SEM	(70)	6.66 ± 0.58	4.20 ± 0.52	20.50 ± 2.25

[a] Ten measurements were made on single ejaculates from each donor.
[b] Calculated from van Duijn's formula [8].

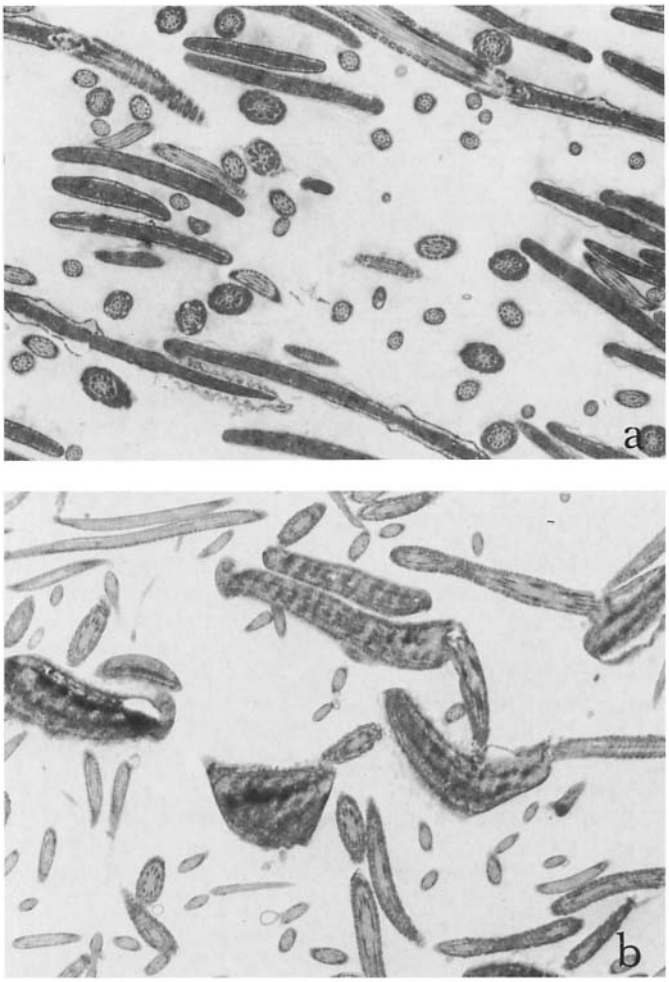

FIG. 2. Electron micrographs (×10 000) showing the alignment of sperm profiles in (**a**) the vertical and horizontal planes in contrast to the more random appearance of (**b**) the transverse plane.

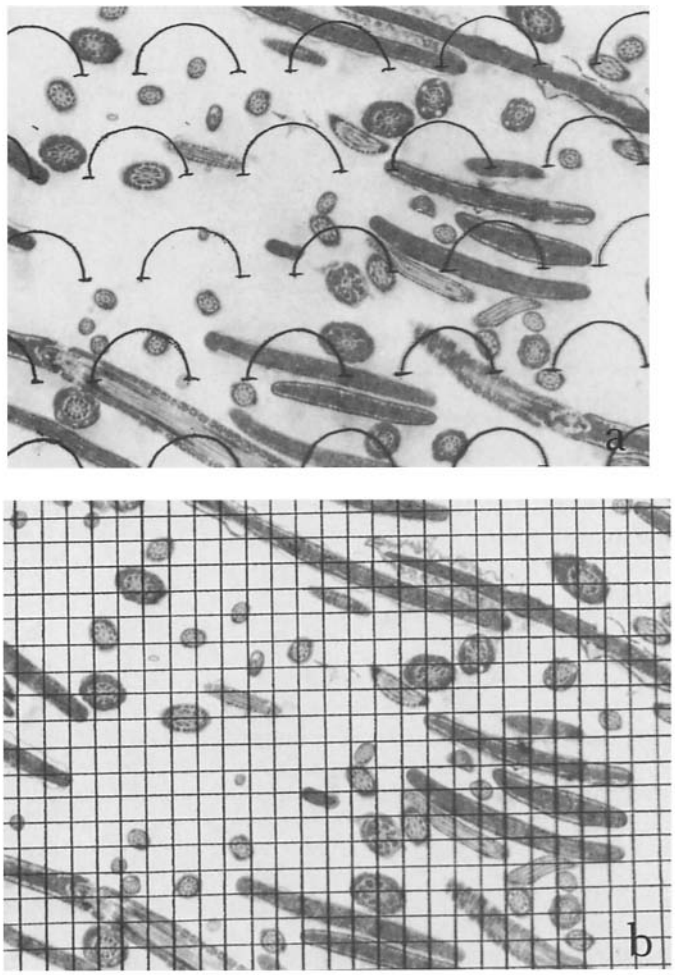

FIG. 3. **a**) Electron micrograph with overlying semicircle grid used for surface area and volume estimates. **b**) Electron micrograph with overlying square grid used for volume estimates. **a** and **b**, ×10 000.

1271-point grid provides a much more thorough probe for micrograph analysis, essentially the same result is achieved more easily, using many fewer points, with the semicircle grid. In order to increase accuracy with stereological methods, it is generally better to increase the sample size rather than to use a more intensive probe with the existing sample.

Volume Exclusion: Cell Water Volume

Three experiments were performed to measure cell water volume. Experiments 1 and 2 used ejaculates from four rams, and experiment 3 used ejaculates from three further rams. In each case, four replicate measurements were made for each ejaculate, and mean values (± SEM) are given in Table 5. Where different ejaculates from the same ram were used in separate experiments, there was generally good agreement between the values obtained with the exception of ram 088 (Table 5), for which values of 13.08 ± 1.40 and 28.82 ± 7.15 were obtained in experiments 1 and 2, respectively. The high mean value and large standard error in experiment 2 represents replicate values of 44.43, 37.46, 15.90, and 17.49 μm^3. The reason in this case for such a large spread of replicate values from a single ejaculate is unknown; it seems unlikely that the mean water volume of spermatozoa from different ejaculates from the same animal would naturally vary by more than 100%, although it should be noted that ram 088 also showed the largest amount of inter-ejaculate variation in the attempts to measure volume and water volume simultaneously (Table 6). However, since no methodology problems were noted for this sample, the values as recorded have been used in cal-

TABLE 3. Estimation of surface area for ram and human spermatozoa.

Spermatozoa	Surface area (μm^2)
Ram[a]	
007	120.5
007	137.6
008	129.4
001	126.9
024	192.9
055	146.6
Mean ± SEM	142.3 ± 10.8
Human[b]	
Pool 3	114.8
Pool 4	110.2
Pool 5	94.5
Mean ± SEM	106.5 ± 6.2

[a] Values are given for six single ejaculates collected from five different rams.
[b] Values are given for pooled ejaculates, with each pool containing 3 ejaculates from different donors.

TABLE 4. Ram and human spermatozoa volume estimates using two different test grids.[a]

Spermatozoa	Sperm volume (μm^3) 1271-Point grid	84-Point grid
Ram[b]		
007	26.4	22.8
007	30.9	32.4
008	33.6	33.9
001	24.8	25.7
024	38.8	37.2
055	35.8	33.2
Mean ± SEM	31.7 ± 2.2	30.9 ± 2.2
Human[c]		
Pool 3	18.1	23.5
Pool 4	19.6	23.2
Pool 5	22.6	26.0
Mean ± SEM	20.1 ± 1.3	24.2 ± 0.9

[a] Mean values are not significantly different between the two grids; overall mean values were 31.3 ± 3.5 μm^3 for ram and 22.2 ± 1.2 μm^3 for human.
[b] Values are given for six single ejaculates collected from five different rams.
[c] Values are given for pooled ejaculates, with each pool containing 3 ejaculates from different donors.

culating the overall mean value. The overall mean value for ram sperm water volume = 12.8 ± 1.15 μm^3 (mean ± SEM).

Water Volume with Cell Volume

Three experiments were performed in an attempt to simultaneously measure total cell volume and cell water volume for the same sample. Each experiment used ejaculates from four rams with either 4 or 6 replicates for each ejaculate. In one case, no usable results were obtained (ram 62, experiment 2). Individual replicate mean values for total cell volume ranged from 13.3 ± 1.9 to 36.0 ± 3.0 μm^3 (ram 88, experiments 1 and 3; Table 6). The overall mean value for total cell volume was 25.10 ± 0.76 μm^3. Individual replicate mean values for cell water space ranged from 20.2 ± 5.3 to 37.3 ± 4.1 μm^3 (Table 6). A large degree of variation between replicates resulted in very high standard errors for some of these values, and one obviously erroneous mean value has been omitted from the analysis (ram 32, experiment 1). The overall mean value for water volume was 24.84 ± 1.64 μm^3, giving a percentage of water space for ram spermatozoa of 99%, a clearly impossible figure suggesting that this technique is not capable of distinguishing between total cell volume and cell water volume. The failure to discriminate between cell and water volume is compounded by the poor repeatability of the technique, with high standard errors for mean values both for individual rams and for individual experiments.

DISCUSSION

Surface Area Measurements

The linear dimensions of spermatozoa have been of interest to researchers over a number of years, with a variety of studies designed to correlate sperm morphology with potential fertility and attempts to demonstrate morphological differences between X and Y spermatozoa. These studies date back at least as far as 1925 [14] and have identified many of the problems associated with making these types of measurements (reviewed by van Duijn [7]). Estimates of the projected area for the bull sperm head have appeared in the literature since the 1950s based on calculations from linear dimensions (van Duijn, 1975 [5]: 40.8 μm^2; van Duijn and van Voorst, 1971 [15]: 34.2 μm^2), planimetry (Bahr and Zeitler, 1964 [16]: 31.3 μm^2), or graphical integration (van Duijn, 1960 [7]: 40.6 μm^2). These surface area estimates are limited both by the accuracy with which measurements can be made and by the formula used to calculate the area of what is an irregular shape from linear dimensions.

Optical limitations make the resolution obtainable using a micrometer eyepiece 0.2 μm at best, and more generally only 0.4–0.5 μm is obtained [8]. Resolution can be increased approximately 10-fold (0.02 μm) by the use of the Dyson image splitting eyepiece [17]. Even errors of this small magnitude can lead to considerable variation in calculated values after formulae for surface area or volume have been applied.

Van Duijn [7] derived a formula based on the Folium of Descartes to calculate the projected head area of bull spermatozoa, which gave close agreement between his calculated area values and those produced by graphical integration. Ram and bull spermatozoa are morphologically similar to each other, both having a flattened paddle-shaped head of similar dimensions [4], and the same formula has been shown to be equally useful for ram spermatozoa [46]. The bull model was used by Duncan and Watson [13] to determine the projected area of the ram sperm head. Values in this study calculated using van Duijn's formula show close agreement with Duncan and Watson's earlier estimate [13] and with the direct measurement of projected head area using the image analyzer. The use of image analysis does not solve many of the problems inherent in using linear measurements to estimate sperm surface area, and indeed

TABLE 5. Measurement of ram sperm water volume (μm^3) by radiolabeled volume exclusion method (mean ± SEM).[a]

Ram identity	Exp. 1 (n = 4)	Exp. 2 (n = 4)	Exp. 3 (n = 4)
032	13.31 ± 2.28	15.19 ± 1.43	
033	6.44 ± 1.77	7.73 ± 1.55	
062	9.78 ± 2.32	12.94 ± 3.93	
088	13.08 ± 1.40	28.82 ± 7.15	
001			10.35 ± 2.27
024			12.28 ± 0.84
079			10.86 ± 1.15
Mean ± SEM	10.65 ± 1.14	16.17 ± 0.84	11.17 ± 1.15

[a] Four replicate measurements were made for each ejaculate; experiments 1 and 2 used separate ejaculates from the same four rams and experiment 3 used ejaculates from three additional rams.

TABLE 6. Simultaneous measurement of ram sperm total volume and water volume (μm^3; mean ± SEM).

	Exp. 1 (n = 4)		Exp. 2 (n = 6)		Exp. 3 (n = 6)	
Ram identity	Total vol.	Water vol.	Total vol.	Water vol.	Total vol.	Water vol.
032	17.68 ± 2.15	160.49 ± 124.37[a]	28.48 ± 7.28	26.67 ± 6.44	25.90 ± 4.25	22.66 ± 1.25
033	24.75 ± 0.65	27.94 ± 3.95	29.42 ± 4.35	37.26 ± 4.06	26.88 ± 2.75	21.79 ± 1.53
062	23.76 ± 1.52	20.24 ± 5.25			27.54 ± 1.68	20.74 ± 2.22
088	13.28 ± 1.91	20.33 ± 23.32	21.86 ± 1.15	26.52 ± 3.00	35.98 ± 3.00	24.26 ± 0.86
Mean ± SEM	19.87 ± 1.42	24.84 ± 1.64	26.59 ± 2.80	30.15 ± 2.84	29.07 ± 1.66	22.36 ± 0.77

[a] Value omitted from calculation of mean.

estimated values are subject to new measurement errors associated particularly with image analysis. However, results from this study suggest that image analysis is capable of producing values consistent with, but not necessarily more accurate than, those gained from linear measurement. Image analysis is less laborious to perform than micrometry, and the agreement between results serves to give a degree of confidence in the models used to calculate surface area from linear dimensions. For human spermatozoa, van Duijn [8] used scale models empirically to derive a formula for projected head area. Van Duijn's [8] values of 4.4 and 3.2 μm for L and B_{max}, respectively, are somewhat smaller than the values obtained in this study (6.7 and 4.2 μm) and those obtained by Katz et al. [18] (5.3 and 3.4 μm). Using van Duijn's [8] formula, projected head area for the human sperm is 10.3 μm and 13.2 μm [18], and 20.5 μm (present study). In part this variation may be explained by the pleomorphism of human spermatozoa, but our own estimate remains double that of van Duijn.

Estimates for the surface area of the sperm flagellum are scarce in the literature. Bull and ram sperm flagellar area have been estimated from light and electron microscopy measurements using different models to approximate the flagellar area. For bull spermatozoa, Drevius and Eriksson [19] took data from Bahr and Zeitler [16] and used a very simple flagellar model that considered the midpiece and principal piece as two cylinders to arrive at a flagellar area of 92 μm^2. Drevius [20] used measurements from hyposmotically swollen bull spermatozoa and calculated flagellar area as the area of an oblate spheroid, obtaining a value of 114.3 μm^2. Duncan and Watson [13] used the two-cylinder model for ram spermatozoa and estimated flagellar area as 69 μm^2. The few published estimates for total sperm surface area represent combinations of the head and flagellar estimates above.

The stereology values for ram and human sperm surface areas given in this paper represent the first published estimate for sperm surface area not dependent on measurement of linear dimensions and not requiring a model to approximate shape. Stereology is able to accurately estimate surface area and volume for complex shapes from two-dimensional images. The technique as used here, however, is limited by errors implicit in measuring the size of the sperm pellet and in counting sperm number using the hemocytometer. The method also assumes that there is no volume change during processing of the sperm for electron microscopy [21]. The value for ram spermatozoa (142.3 μm^2) is very close to both Duncan and Watson's [13] value (139 μm^2) and the value calculated from the linear dimensions quoted here (135.4 μm^2), and is in fairly close agreement with published bull spermatozoa values (145 μm^2 [20]; 154 μm^2 [19]). (We have taken total area as equal to twice the projected head area plus flagella area, not projected head area plus flagella as quoted by Drevius and Eriksson [19]). The stereology value for human sperm surface area (106.5 μm^2) is in complete agreement with our value calculated from linear measurements (106 μm^2), despite our high value for projected head area, but is somewhat lower than the frequently used value of 120 μm^2 [22–24]. The 120-μm^2 value is quoted by Du et al. [24] as based on van Duijn's [8] measurements, although it is not stated how the flagellum was modeled for the calculation.

Volume Measurements

Sperm volume has been more extensively investigated than has surface area, although again perhaps the majority of the published data concern bull spermatozoa. The earliest estimates are based on the same optical measurements used for surface area [8], but with the added complication of having to measure the thickness of the head. For direct micrometry of thickness, sperm would need to be measured in side view, an orientation not usually available under the microscope. Therefore, a number of alternative indirect methods for measuring cell thickness have been developed. Measurements have been made using differential focusing with interference microscopy [25–27] and contrast matching with phase contrast microscopy [28]. However, use of these methods additionally requires knowledge of the sperm refractive index, which is in itself difficult to estimate [15]. Because of the problems outlined above together with the added difficulties in measuring cell thickness and the small number of cells that can practically be measured, sperm volumes calculated from linear dimensions can really only be considered as an approximation of the true sperm volume. Van Duijn empirically derived a formula for the volume of the human sperm head and modeled the midpiece as a cylinder, the principal piece as a ribbon, and the terminal piece as a cylinder and calculated a total sperm volume of 15.2 μm^3. This value is now widely considered to underestimate the true volume, although the dimensions from which it is calculated are the same as those used to derive the quoted value of 120 μm^2 for human sperm surface area [23, 24].

Volume estimations have also been made using indirect methods such wet weight and density measurements, which have generated a value of 33 μm^3 for human spermatozoa [25] assuming a density of 1.12 g/cm^3 for hydrated spermatozoa [29].

However, the great majority of values for sperm volume appearing in the literature have been obtained using electronic particle counters, most frequently the Coulter counter. Electronic sizers measure volume as a function of the change occurring in electrical conductivity in a circuit filled with an electrolyte solution when a small particle passes through an orifice. However, these volume measurements are subject to a number of assumptions concerning the particles being measured. It is assumed that particles behave

TABLE 7. Published volume estimates for bull, boar, and ram spermatozoa.

Reference	Method	Volume (μm^3)				Ref.
		Bull	Boar	Ram	Human	
van Duijn (1957)	Mensuration				15.4	[8]
Segal, Laurence (1962)	Coulter				56.0	[30]
Gordon et al. (1965)	Coulter				15.0	[38]
Iversen (1965)	Coulter	23.27	29.41			[45]
O'Donnell (1969)	Coulter	19–25	21–29	25–29		[39]
Bredderman, Foote (1969)	Coulter	25.2				[40]
Brotherton, Bernard (1974)	Coulter				25.6	[31]
van Duijn, van Voorst (1971)	Mensuration	38.95[a]				[15]
Laufer et al. (1977)	Coulter				17.4	[32]
Hammerstedt et al. (1978)	Coulter	25	22	26		[36]
Jeyendran et al. (1987)	Coulter				16.25	[33]
Smith et al. (1988)	Stereology				21.04	[35]
Turner et al. (1989)	Coulter				18.58	[41]

[a] Head and midpiece only.

according to Stokes' law when streaming in a fluid, and have no capacitative effect, and that there will be no effects due to conductivity of the particle itself and no effect due to surface charges on the particle, none of which are safe assumptions in the case of spermatozoa. Furthermore, the resistance caused by a particle in a fluid, and hence its perceived volume, depends on its shape and its orientation in the electric field. Different authors have used different shape correction factors or none in their estimations of sperm volume, and it is far from clear how significant an effect this may have on results. These problems are aggravated by the lack of a calibration standard more appropriate to spermatozoa than the plastic spheres currently available. Use of the Coulter counter has produced a wide range of volume values for the domestic species and for human spermatozoa (Tables 7 and 8). An early attempt with human spermatozoa by Segal and Laurence [30] resulted in a high value of 56 μm^3, but this is now generally attributed to faulty calibration; other values for human spermatozoa have ranged between 15 and 25 μm^3. A further problem with many of these Coulter counter estimates is the use of Zaponin (Coulter Electronics Ltd., Luton, UK) to lyse contaminants in sperm suspensions [31–33]. Zaponin will progressively lyse the sperm plasma membrane and peripheral cytoplasm; hence Brotherton [34] reported the volume of human sperm to be 22.54 μm^3 after Zaponin treatment but 46.25 μm^3 before treatment. Estimates for the domestic species are also variable (Table 7), ranging from 17 to 29 μm^3. Thirty years of electronic sizing studies have produced a reasonably narrow volume range that encompasses the true values for spermatozoa from different species, but have not with any confidence yet been able to provide absolute figures.

Smith et al. [35] used a stereological method to measure the volume of human spermatozoa, which yielded a value of 30.5 μm^3, including the cytoplasmic droplet, estimated at 9.46 μm^3, giving a sperm without droplet a value of 21.04 μm^3. In this study, human sperm were subject to a Percoll wash before processing, giving a population largely free of droplets. Therefore, the estimate of Smith et al. [35] may be considered to be in good agreement with our own figure of 22.2 μm^3.

Water Volume Measurements

For many purposes a more useful value than total volume is the sperm water volume. Water volume has been measured for spermatozoa from a number of species using volume exclusion methods (with either colorimetric or radiolabels) and using electron paramagnetic resonance (EPR) techniques (Table 8). In this study, the method of Ford and Harrison [12] gave a value of 12.82 μm^2, in good agreement with Hammerstedt et al.'s [36] value of 12 μm^3 for ram spermatozoa using EPR, but somewhat lower than the 23 μm^3 reported by Hammerstedt et al. [37]. EPR values have generally fallen in the range of 20–25 μm^3 (Table 8). Examination of Table 6 indicates that our attempts to measure both water volume and total cell volume in the same semen sample resulted in very unstable estimates related neither to ejaculates within individuals nor to between-experiment variation. Thus, although the mean cell volume from these experiments (25.1 μm^3) was a little lower than the volume estimated by stereology (31 μm^3), the mean estimate of cell water volume constituted 99% of the estimated cell volume, a clearly erroneous figure. We believe the failure of this technique is a consequence of the very small total intracellular water space as compared with a relatively large extracellular water space within the sperm pellet. Both the EPR method and the volume exclusion method are subject to the limitations due to cell counting;

TABLE 8. Published water volume estimates for bull, boar, ram, and human spermatozoa.

Reference	Method[a]	Volume (μm^3)				Ref.
		Bull	Boar	Ram	Human	
Hammerstedt et al. (1978)	ESR	25	20	12		[36]
Hammerstedt et al. (1979)	ESR			23		[37]
Du et al. (1991)	ESR	21				[42]
Du et al. (1994)	EPR	22–25.4				[43]
Ford, Harrison (1983)	Vol. excl.				22.1	[12]
Kleinhans et al. (1992)	ESR				20.0	[44]

[a] ESR, Electron Spin Resonance; EPR, Electron Paramagnetic Resonance (synonyms).

total cell volume is dependent upon the accuracy of measuring the pellet volume in the same way as are the stereology values.

In summary, we suggest that for ram spermatozoa a cell surface area of 142 μm^2 and a volume of 31 μm^3 with a 50% cell water space be used, and that for human spermatozoa values of 106.5 μm^2 for surface area and 22.2 μm^3 for total volume be adopted.

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