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A Comparison of Critical Osmolality and Hydraulic Conductivity and Its Activation Energy in Fowl and Bull Spermatozoa

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ABSTRACT: Measurements were made of critical osmolality, the osmolality at which 50% of the cells are lysed, and of the permeation time, the time taken to lyse 50% of the cells in an osmotic solution lower than the critical osmolality, for fowl and bull spermatozoa. Cell lysis was determined by means of fluorescent viability stains (carboxyfluorescein diacetate and propidium iodide) using a flow cytometer. The advantages and pitfalls of this approach are addressed. The values obtained have been used to compute the water permeability, or hydraulic conductivity, of the plasma membrane and its activation energy for each species. Fowl spermatozoa were found to have a lower critical osmolality (17 mOsm) than bull spermatozoa (36 mOsm), and this is discussed in relation to the differences in cell shape and size. The hydraulic conductivities of fowl and bull spermatozoa were 2.1 and 10.8 μm × atmosphere × minute, respectively, and the respective activation energies were 4.4 and 3.0 kcal/mol. The relevance of these findings to cryopreservation of spermatozoa is considered.

Key words: Water permeability, plasma membrane, flow cytometry, osmotic swelling, sperm compartments.

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The cryoperservation of spermatozoa results in a considerable loss of cell viability, with severe reductions in fertility in many species. Even in those species where fertility is apparently unaffected or only slightly impaired, such as cattle and human, the survival of the cells rarely exceeds 50%. Although much has been done empirically to improve survival, a plateau of success has now been reached; further studies probably can have only a limited impact.

Although spermatozoa were among the first cell types to be successfully cryopreserved, the science of cryobiology has been developed by research on other cell types. High survival percentages have been achieved, and the critical events involved in cryopreservation (eg, cryoprotectant permeability, cell dehydration as a function of cooling rate, and the like) have been modeled mathematically (Mazur, 1984). Estimates are needed of hydraulic conductivity (water permeability), its activation energy, and the surface area and aqueous volume of the cell. Due to difficulties associated with shape and size of the cells, these approaches have only recently begun to be applied to spermatozoa (Duncan and Watson, 1992).

In this study we report a novel and sensitive approach to the estimation of hydraulic conductivity and activation energy in spermatozoa using fluorescent membrane permeability probes and flow cytometry. The results are discussed with reference to their use in determining optimal cryoprotection protocols.

Materials and Methods

Semen Collection and Treatment

Fowl semen (*Gallus domesticus*) was obtained by digital pressure (Burrows and Quinn, 1937) from hybrid males housed singly in batteries under controlled lighting and fed a pellet diet *ad libitum*. Depending on the experiment, either individual ejaculates or pools of semen representing at least three individual ejaculates were used. Only semen exhibiting >90% motility was considered acceptable. Bull semen (*Bos taurus*) was obtained, using an artificial vagina, from animals held at the Dairy Breeding Research Center of the Department of Dairy and Animal Science of The Pennsylvania State University. Only ejaculates with >80% motility were accepted for study.

Fowl semen was initially diluted four-fold with Lake's high-temperature (HT) diluent, 365 mOsm, pH 7.4 (Chaudhuri and Lake, 1988). Semen was subsequently washed twice in the same solution. Bull semen was initially diluted five-fold with a modified Tyrode's medium (Graham et al, 1986) containing 2 mg/ml bovine serum albumin (TALP), and was subsequently washed twice by five-fold dilution and gentle centrifugation in phosphate-buffered saline (PBS) containing egg yolk (20%). The latter was obtained as the supernatant from a 1:1 dilution of egg yolk in distilled water and centrifuged at 10,000 × g for 20 minutes. The final osmolality

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was 300 mOsm; pH was 7.4. After washing, samples were adjusted to approximately 5×10^8/ml spermatozoa, and sperm concentrations were measured by light absorption using a previously calibrated spectrophotometer.

Fluorescent Stain Preparation

A stock solution of carboxyfluorescein diacetate (CFDA; Calbiochem, San Diego, CA) was prepared in dimethyl sulfoxide (1 mg/ml). Propidium iodide (PI; Sigma Chemicals, St. Louis, MO) was dissolved in distilled water (1 mg/ml). Stock solutions were protected from light and stored at 4°C for no more than 7 days.

Determination of Hydraulic Conductivity (L_p)

A method that has been used successfully with other cell types (Mazur, 1963) was used, which involves estimating critical osmolality and permeation time. Critical osmolality is defined as that osmolality at which 50% of the cells have swollen by osmosis to the point of rupture of the plasma membrane. Permeation time is the time taken for the cells to swell to the point of rupture when placed in a solution of osmolality less than the critical osmolality. Hydraulic conductivity is calculated from these parameters, together with estimates of the cell surface area and osmotically active cell water content (Mazur, 1963; Mazur et al, 1984).

Determination of Critical Osmolality

Approximately isosmotic PBS (without egg yolk) was prepared (fowl: 350 mOsm, bull: 300 mOsm), and portions were diluted with distilled water, producing progressively hyposmotic steps down to zero. Actual osmolalities were measured with a freezing point depression osmometer (Model 3W2; Advanced Instruments, Inc., Needham Heights, MA). Carboxyfluorescein diacetate stock solution was added (fowl: 10 μl/ml, bull: 20 μl/ml) to a known volume of the suspension of washed spermatozoa. The sample was mixed and allowed to stand at room temperature for 10 to 15 minutes. Volumes of the semen containing approximately 10^7 spermatozoa (typically 5 to 10 μl) were then added to 1-ml portions of each of the osmolarity steps and mixed thoroughly. Propidium iodide stock solution (10 μl) was added to each tube. Cell suspensions remained at the indicated osmolality for approximately 30 minutes before analysis by flow cytometry. A preliminary experiment showed no difference in result if the cells were returned to isosmolal conditions before flow cytometry, so this step was omitted from subsequent experiments. Isosmolal solutions were used for calibration of the flow cytometer containing 10^7 spermatozoa alone, or with CFDA or PI.

Determination of Permeation Time

The time to cell rupture was determined by exposing cells for fixed periods to hyposmotic conditions, which would cause cell rupture before equilibrium is reached. To do this accurately, 1-ml hyposmotic solutions (double distilled water, fowl: 0 mOsm, bull: 25 mOsm PBS) were prepared. Washed spermatozoa containing CFDA were added to each of the tubes on a vortex mixer, and the hypotonic swelling of the cells was arrested at a precisely timed interval by addition of 0.1 ml hyperosmotic solution. The concentration of the hyperosmotic solution was calculated to return the environment of the cells to isosmolar. Propidium iodide was then added and the fluorescence determined on the flow cytometer.

This procedure was repeated at 0, 15, 24, and 37°C (fowl), and at 5, 15, 23, and 39°C (bull). To avoid cold shock in the 5°C study of bull spermatozoa, semen was slowly cooled after the addition of CFDA by transferring the sample in a 200-ml beaker of water at room temperature to the refrigerator (5°C) for 2 hours. The treatments were rapidly returned to room temperature for flow cytometry.

To obtain the data for relative permeability, two different methods were used. Since the time intervals for fowl spermatozoa were longer, each data set was plotted, and the time at which 50% of the cells were intact was interpolated from the individual graphs. Mean time values for a given temperature were calculated from the individual determinations. For bull spermatozoa, for which the time intervals were shorter, the mean survivals at each time interval were calculated and used to estimate the time at which 50% survival occurred.

Differences Between Bulls and Ejaculates

Two ejaculates, collected from each of a number of bulls within a 5-minute period, were washed and resuspended to 500×10^6 spermatozoa/ml. Twenty microliters of CFDA stock solution was added and mixed. After 30 minutes, chosen because this interval results in maximal carboxyfluorescein release within cells, samples were dispersed in isotonic PBS and analyzed on the flow cytometer. Results were subjected to analysis of variance using the SAS General Linear Models program (Spector et al, 1985).

Flow Cytometry

Samples were analyzed on an EPICS 753 flow cytometer (Coulter Electronics, Inc., Hialeah, FL) fitted with a bevelled tip (Johnson and Pinkel, 1986). Cells were excited at 488 nm by an argon laser (100 mW power). The emitting light was passed through a 515-nm long pass filter and a 457–502-nm laser blocker before a 590-nm dichroic beam splitter. The filter set-up for the detection of carboxyfluorescein (CF) was a combination of a 515-nm long pass filter and a 525-nm band pass filter. The photomultiplier tube high voltage was set at 1,100 volts. For the detection of PI, a combination of 610-nm and 630-nm long pass filters was used, with the photomultiplier tube high voltage set at 900 volts. Sperm samples were filtered through a 44-μm nylon mesh immediately before analysis to remove agglutinated cells and large debris.

A forward angle light-scattering gate was set to allow determination of the total cell population, excluding debris and cell aggregates. Upper gates were set high enough to include variations due to osmotically induced changes in cell size. Peak and mean fluorescence channels for each distribution were determined using the EPICS 753 MDADS II computer.

Calculation of Hydraulic Conductivity and Activation Energy

Hydraulic conductivity (L_p) was determined from the value for critical osmolality and the permeation time using Equation (4) of Leibo (1980). Values for the cell surface area, A, and the osmotically active intracellular water volume, V_1, for fowl (117.21 μm^2 and 5.51 μm^3, respectively) were taken from Ravie and Lake (1982), and those for bull (145 μm^2 and 14.85 μm^3, respectively) from Drevius (1972b).

A value for activation energy (E_a) was derived from the slope of a plot of the $\log_e$ relative water permeability at different tem-

peratures multiplied by a constant appropriate to the temperature interval over which the permeability was measured (1.65×10^5 degrees × cal/mol; Mazur et al, 1984).

Results

Fowl spermatozoa showed a discrete change from green to red on membrane rupture, whereas bull spermatozoa changed to a predominantly mixed fluorophore population. Typical distributions of fluorescent particles are shown (Fig 1) for the two species. Under isosmotic conditions, fowl spermatozoa showed a very high percentage of carboxyfluoroscein-positive (CF + ve) and propidium iodide-negative (PI − ve) cells, and, as osmotic lysis occurred, the predominant group became CF − ve/PI + ve; dual staining was seen in less than 7% of the samples. In contrast, bull spermatozoa showed a greater and more variable proportion of dual-stained or CF − ve/PI + ve cells under isosmotic conditions, and the CF + ve/PI − ve cells displayed a greater range of intensity of fluorescence. In severely hyposmotic conditions, the predominant group became CF + ve/PI + ve. Under microscopic evaluation, these cells were identified as showing a strong or weak green fluorescence over the acrosome region together with a red fluorescent nucleus region, most intense at the base of the head. Even in profoundly hyposmotic conditions, many of these cells did not lose their weak green fluorescence. Differences were seen between ejaculates. Intact spermatozoa were taken as the CF + ve/PI − ve group.

In an attempt to explore these observations further, a series of ejaculates was processed simultaneously and examined for differences in CF intensity (Table 1). There were no significant differences between bulls, but when bull 547 (with the very unusual and excessive values for the second ejaculate) was removed, second ejaculates were of significantly poorer fluorescence intensity than were first ejaculates (mean, $P < 0.01$; peak, $P < 0.05$).

The results of the critical osmolality experiments are shown in Figure 2, normalized to the survival under isosmotic conditions. Fowl spermatozoa showed little change in the percentage of CF + ve/PI − ve cells until the osmolality reached less than 25 mOsm, when there was an abrupt rupture of the membranes of the entire population. Bull spermatozoa were more sensitive to dilution and storage at ambient temperature for 30 minutes in hyposmotic media. A heterogeneous response was observed for the population, as evidenced by the gradual decline in percent intact cells over a wide range of osmolalities, and the variability between ejaculates in their sensitivity to hyposmotic condi-

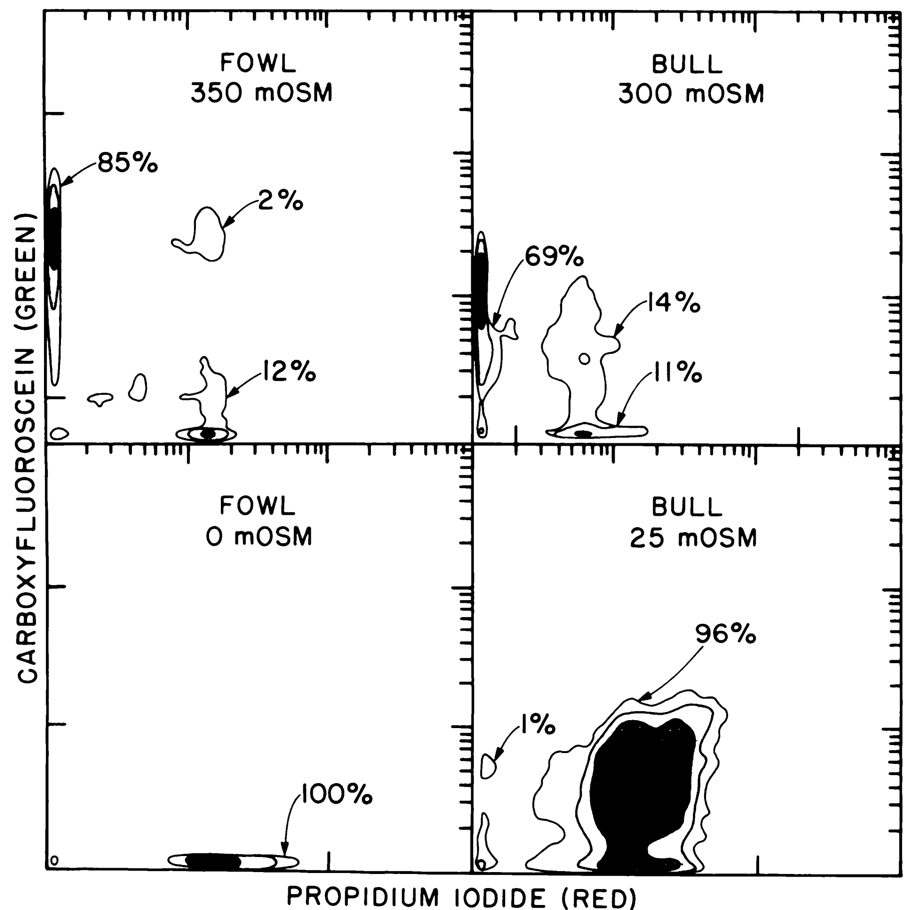

FIG. 1. Effect of hyposmotic stress on membrane integrity of fowl and bull spermatozoa, as revealed by the fluorescent dyes, carboxyfluorescein diacetate (green, intact membranes) and propidium iodide (red, damaged membranes), using a flow cytometer. These representative topographical displays use numbers (in parenthesis) to indicate total percentage of cells within any boundary and gray shading to indicate peak positions within that boundary. In all subsequent figures, intact cells are those cells having green fluorescence and no red fluorescence.

Table 1. A comparison between first and second ejaculates of bull spermatozoa on the development of carboxyfluorescein fluorescence

	Fluorescence intensity (photomultiplier voltage)			
	Mean		Peak	
Bull	Ejaculate 1	Ejaculate 2	Ejaculate 1	Ejaculate 2
824	0.121	0.110	0.119	0.126
923	0.091	0.044	0.099	0.052
622	0.162	0.176	0.065	0.082
933	0.123	0.073	0.133	0.089
626	0.121	—	0.140	—
935	0.071	0.044	0.075	0.054
629	0.140	0.058	0.152	0.064
547	0.151	0.362	0.174	0.415
Means (excl Bull 547)	0.118	0.068*	0.126	0.078†

* $P < 0.01$.
† $P < 0.05$.
The second ejaculate was collected within 5 minutes of the first, the spermatozoa washed and exposed to CFDA for 30 minutes. Mean and peak photomultiplier channel number (log fluorescence intensity) on the flow cytometer were recorded, and transformed to photomultiplier voltage to allow direct linear comparison across samples. Bull 547 was eliminated from the analysis because of the excessively high values for ejaculate 2.

tions, as shown by the larger SEMs approaching the critical value. A sudden decline in intact cells to a very low value occurred between 50 and 25 mOsm. Critical osmolality was defined as the osmolality at which 50% of those spermatozoa intact immediately before the sudden reduction were ruptured (ie, from Fig 2, 46% of those fowl spermatozoa, and 30% of those bull spermatozoa intact under isosmotic conditions). This value for fowl spermatozoa was 17 mOsm, whereas that for bull spermatozoa was 36 mOsm.

Spermatozoa were exposed to severely hyposmotic conditions to estimate the time to rupture for 50% of cells that were intact at osmolalities just above the critical osmolality (as defined above). For fowl spermatozoa, survival remained high until sufficient water had entered the cells to cause membrane rupture, whereupon the percentage of intact cells fell abruptly. Values for these rupture times were highly repeatable (Table 2), and a consistent increase in water permeability was seen with increasing temperature in the range of 0 to 37°C.

With bull spermatozoa, however, a steady decline in intact cells was seen with increasing time after dilution (Fig 3). The time at which the critical percentage value (30%) was reached increased with decreasing temperature, as shown by the large SEMs; however, individual variation was considerable. Propidium iodide uptake was extensive for cells cooled slowly to 5°C (approximately 80%; Fig 3), probably reflecting some modification of sperm membrane permeability to fluorescent stains, since cell viability estimated by motility, in samples cooled to 5°C and then returned to 37°C, was frequently much greater than that predicted by membrane permeability assays alone. Thus, data for bull spermatozoa, especially at 5°C, reflect the response of only a fraction of the total population.

Hydraulic conductivity values were calculated as follows. Assuming certain simplifying concepts, the osmotically active volume of the cell at cell rupture is increased by the ratio of isosmolal:critical osmolality. Thus, osmolalities of seminal plasma together with critical osmolalities from this study can be used to calculate this ratio (for fowl spermatozoa 325/17 = 19.1—isosmotic value taken from freezing point depression of fowl semen; Ravie and Lake, 1982), while that for bull spermatozoa is 285/36 = 7.9 (freezing point depression of bull seminal plasma = 0.53; Rothschild and Barnes, 1954). In addition, values for the osmoles of solute in the isotonic cell, N, were calculated, following Leibo (1980), as 1.79×10^{-15} (fowl) and 4.23×10^{-15} (bull) osmoles. These values, together with the mean permeation times at room temperature found by experiment (Table 2, Fig 3), were substituted into Equation (4) of Leibo (1980) to give values of hydraulic conductivity, L_P. They were calculated as 2.1 and 10.8 $\mu m^3/\mu m^2$/minute/atmosphere for fowl and bull spermatozoa, respectively. The permeation times at various temperatures were plotted as the natural log of the reciprocal of the relative permeability (permeability at room temperature = 1, Fig 4). The slopes of the curves were 0.0269/°C (fowl) and 0.0184/°C (bull), giving E_a values of 4.4 kcal/mol (fowl) and 3.0 kcal/mol (bull).

Discussion

Advantages and Limitations of this Method

The shape and regionalization of the sperm cell makes the acquisition of data relating to water permeability very difficult. Changes in cell volume cannot be measured simply by microscopy, and more complex methodology must be used. Duncan and Watson (1992) used a method for estimating the critical osmolality of ram spermatozoa that depended on counting the proportion of the spermatozoa with looped tails in hyposmotic conditions, and this resulted in a much higher critical value than we report here. However, given that their method did not directly determine cell rupture, and that a different species was studied, the discrepancy between the current values and those of Duncan and Watson (1992) is not surprising.

If PI gains access to the interior of the cell, it binds tightly and essentially irreversibly to DNA. This stain intercalates with double-stranded nucleic acids, and, when excited with blue light, produces a red fluorescence proportional to nucleic content (Steinkamp et al, 1982). Both DNA and RNA can be detected, but the minimal RNA content of sperm and the observation that red fluorescence is localized in the head allow it to be used for a marker of

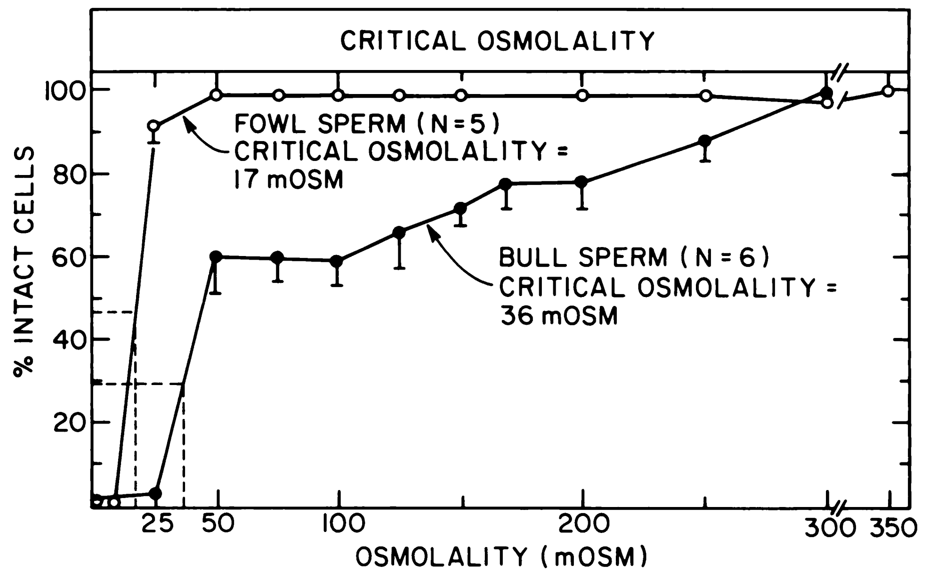

FIG. 2. Determination of critical osmolality of fowl and bull spermatozoa by fluorescent viability stains and flow cytometry. Values represent means (±SEM) of replicates from different individuals (where error bars are less than the extent of the symbol they are omitted). Data are presented as percentage of those intact at isosmotic pressure. The dashed lines indicate the estimated osmolality at which 50% of the cells, which were intact at the next highest osmolality, ruptured.

the head compartment in these studies. In contrast, CFDA passes freely across the membranes of intact cells and is accumulated in a low-permeability form, the free acid (CF), in proportion to the activity of available nonspecific esterases (Thomas et al, 1979). Loss of CF fluorescence was taken to indicate a breach of a membrane barrier. Thus, a single-compartment model is predicted to show coincident loss of CF and uptake of PI. Dual staining might indicate a multiple-compartment model, with extent of labeling dependent on the relative kinetics of stain passage across membranes. However, this simple view is confounded by the observations of bull spermatozoa at low osmolality and at 5°C. Dual staining was retained in profoundly hyposmotic conditions, in which no intracellular compartment bounded by a semipermeable membrane would be expected to remain intact. At low temperature the percentage of CF + ve/PI − ve bull spermatozoa was found to be very low, even in circumstances designed to avoid cold shock. It is possible that at low temperature the increase in ion permeability known to occur, at least in ram spermatozoa (Robertson and Watson, 1987), also allows the ingress of PI into otherwise intact cells. For these reasons, we decided to take

Table 2. The mean times to cell lysis of 50% of fowl spermatozoa exposed to distilled water at different temperatures.

	Temperature (°C)			
	0	15	24	37
Mean lysis time (seconds)	62	42	31	23
SEM	3.1	2.1	1.4	1.3
Number of replicates	5*	6	4	5

* An additional three samples failed to show more than 15% lysis after 72 seconds at this temperature and were excluded from the data. Samples analyzed represented pools of semen from at least three males.

a conservative view and define as intact only those cells that were CF + ve/PI − ve.

Although the results are presented as the proportions of CF + ve/PI − ve cells, the combined use of the two stains was particularly useful in determining the appropriate end point for bull spermatozoa. Whereas fowl spermatozoa generally behaved as a single compartment (losing CF and taking up PI), bull spermatozoa retained CF staining in the acrosome after they had clearly begun to take up propidium iodide. A likely explanation is the firm attachment of the plasma membrane at the base of the nucleus (Fawcett, 1975; Drevius 1972b), thus creating two adjacent plasma membrane-bound compartments. When the tail compartment swells and ruptures, CF is retained in the head regions, but PI penetrates the base of the nucleus through the neck, and spreads forward. An appearance consistent with this explanation was seen with the fluorescence microscope, with the red fluorescence being most intense at the base of the head and weakening more anteriorly. The loss of tail membrane integrity probably also allows for some solute leakage by the reverse route, preventing further swelling and rupture of the plasma membrane in the head region. As CF tends to follow the solutes, intense green fluorescence would be retained only in the last intact membrane-bounded organelle, namely the acrosome.

An important source of variability between ejaculates is the differences in CF fluorescence intensity (Table 1). Since high percentages of cells were intact, it is unlikely that variable subpopulations were being compared. The most likely explanation is that intracellular esterase activity varies with ejaculate, but this was not further explored.

Differences Between Bull and Fowl Spermatozoa

With bull spermatozoa in the critical osmolality experiments, there was a difficulty in determining the 50% cell rupture point, since they show evidence of heterogeneity in

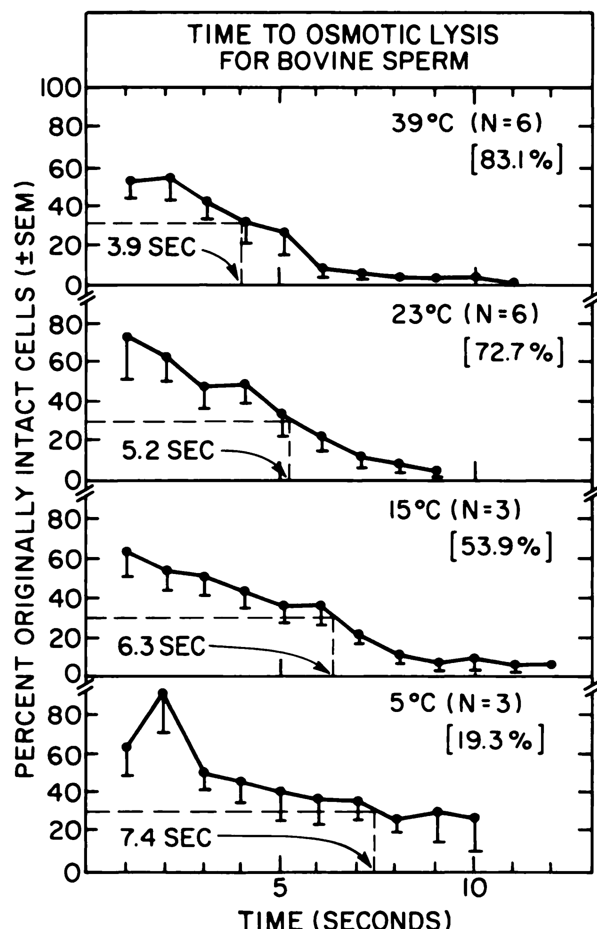

FIG. 3. Effect of temperature of incubation on the water permeation time for bull spermatozoa. Washed spermatozoa were subjected to a 25 mOsm solution for fixed time periods and the percentage of intact cells were determined. The data are presented as mean percentage (±SEM) of those intact at time 0, which varied with temperature (see values given in parentheses). The reference for determining the time to lysis was determined from Figure 2, ie 30% intact. Where error bars are less than the extent of the symbol they are omitted.

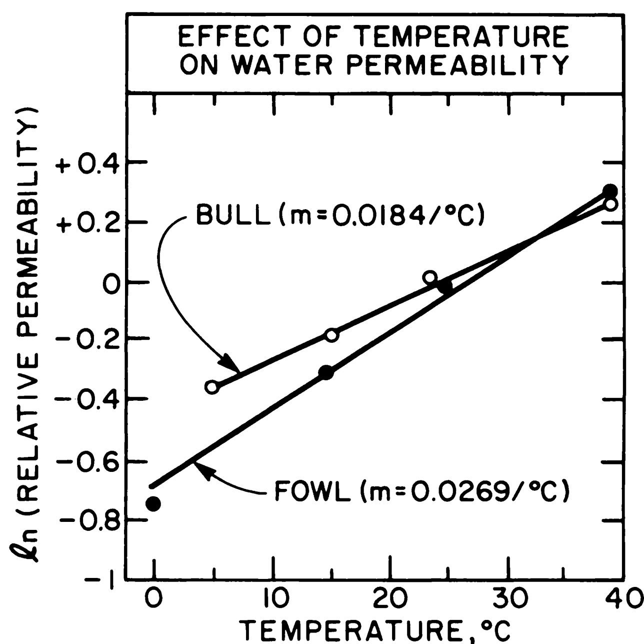

FIG. 4. Effect of temperature on the water permeability of the plasma membrane of fowl and bull spermatozoa. Water permeability constants were determined for each temperature and each cell type as described in the text. Values were normalized to room temperature (24°C for fowel, 23°C for bull). The data are plotted as the $\log_e$ relative permeability against temperature, and regression analysis was used to determine a line of best fit.

the ejaculate. Some cells seemed far more sensitive to disruption than others, as indicated by the progressive decrease in intact cells as osmolality was decreased from 300 mOsm to 100 mOsm (Fig 2). Presumably they are disrupted by the osmotic force across the membrane or by the passage of water itself, rather than by exceeding a given surface area. The larger standard errors in the bull data also reflect the variability in the magnitude of this effect between bulls. Fowl semen was much more consistent, and did not show this variability.

The sudden reduction in survival at a particular low osmolality was taken as the point at which the cells had reached a limit beyond which their volume could not increase because of limitations in surface area. At this point, any further inflow of water resulted in rupture of the cell membrane.

Potential Relationship of Critical Osmolality and Cell Shape

Critical osmolality was lower for fowl spermatozoa than for bull spermatozoa, indicating a lesser capacity to increase in volume before the limit was reached in the latter species. Drevius and Eriksson (1966) showed that spermatozoa accommodate an increase in volume under hyposmotic conditions largely by swelling in the midpiece and tail region, with concomitant coiling of the axoneme within the swollen membrane. By this means, the cell is transformed to a more spherical structure. Providing membrane rupture is avoided, the cell can adopt its normal configuration and motile function on return to isosmotic conditions (Drevius and Eriksson, 1966).

The greatest increase in volume for a given surface area can be achieved by a change from a lanceolate structure to a sphere. Fowl spermatozoa, with a lanceolate head as well as midpiece and tail, are theoretically able to accommodate a greater change in volume without rupture of the plasma membrane than bull spermatozoa. Bull spermatozoa, with a bilaterally flattened paddle-shaped head, would be expected to rupture at a lower degree of swelling (ie, at a higher critical osmolality). An unstated assumption in this concept is that the internal components offer no resistance to the adoption of a spherical shape. This is certainly not the case for the nucleus, composed of condensed nucleoproteins bonded with disulfide bridges. Membrane rupture might be

expected to occur before a spherical shape was reached because of membrane tensions resulting from resistance to distortion in the nucleus. Moreover, as has been suggested, the head and the tail in the bull spermatozoon act as separate compartments; although both swell, the change of shape of the tail is more pronounced, yielding an oblate spheroidal structure (Drevius, 1972a). In contrast, Bakst (1980) observed the membrane distension of fowl and turkey spermatozoa under hypotonic conditions to involve plasma membrane over both head and tail.

The change in cell water volume is calculated on the assumption that spermatozoa behave as perfect osmometers with no transmembrane solute movement under these conditions, and with no intracellular water volume that does not participate in osmotic events. While this may not be absolutely true, the discrepancy is only minor for most cells, and was previously estimated as a Ponder's R factor of 0.86 for epididymal bull semen (Drevius, 1972b). All cell volume calculations in the current study involve this correction factor. The isotonic water volume is assumed to be equivalent to 53% to 58% (average, 55%) of the cell volume (Drevius, 1972a). The equation used to convert change in cell water volume to change in cell volume is

$$V/V_{iso} = ((Osm^{i}_{iso} = Osm^{e}_{crit})(0.55)(0.86)) + (0.45) + ((0.55)(0.14))$$

where V = cell volume, V_{iso} = isotonic cell volume, Osm $^{i}_{iso}$ = isotonic cell osmolality (assumed equal to osmolality of seminal plasma), and Osm $^{e}_{crit}$ = osmolality of external medium at critical cell rupture. Thus, the increase in cell volume before rupture of the plasma membrane of fowl and bull spermatozoa was 9.6 and 4.3 times, respectively. The figure for bull spermatozoa is slightly greater than the figure of 3.78 to 3.94 times published by Drevius (1972b).

If the surface area of fowl sperm is taken as 117.2 μm^2 and the volume as 9.2 μm^3 (Ravie and Lake, 1982), and assuming no change in surface area and the adoption of a perfectly spherical shape at critical osmolality, the volume becomes 119.3 μm^3. The cell volume has increased 13.0 times, which computes to a critical osmolality of 12.4 mOsm. For bull sperm (surface area, 145 μm^2, and volume, 27 μm^3; Drevius, 1972b), the spherical volume of identical surface area is 164 μm^3. The volume has increased 6.1 times, which computes to a critical osmolality of 24.3 mOsm, somewhat below that measured experimentally. Drevius (1972b) found that the volume did not increase to this extent before rupture, that is, the cells did not reach a perfectly spherical shape.

The lack of correspondence of these computed critical osmolality values with the observed values prompts consideration of an alternative model. The simplest assumption, based on measurements of ram spermatozoa (Duncan and Watson, 1992), which indicated approximately equal surface area for head and tail regions, is that the cell surface area is distributed between two equal spheres. In this case, for fowl spermatozoa, the combined volume of total surface area equivalent to that of the cell is 84.4 μm^3, a volume increase of 9.2 times, which computes to a critical osmolality of 17.8 mOsm. For bull spermatozoa, a similar calculation yields a total volume of 116 μm^3, representing an increase of 4.3 times, which computes to a critical osmolality of 36 mOsm. The correspondence with measured values is much closer, lending support to the belief that spermatozoa indeed swell as two separate compartments. The lack of fluorescence evidence for this in fowl spermatozoa is perhaps due to the relatively small acrosomal compartment of the fowl spermatozoon (Lake et al, 1968).

Similar observations for critical osmolality were made for bull spermatozoa (Ruffing et al, 1990) and for human spermatozoa (Noiles et al, 1991), when computed in a comparable way to our figures (50% of those cells intact at osmolalities immediately above the abrupt decline in percentage excluding PI), suggesting that mammalian spermatozoa may have similar critical osmolalities. It would be interesting to repeat these observations on spermatozoa of a rodent species to examine the effect of the markedly different shape of the head. From a cryobiological standpoint these values are of interest during thawing and removal of cryoprotectant, when differentials between permeability constants of glycerol and water at any temperature might constitute conditions for cells to swell temporarily (Hammerstedt et al, 1990). The ability to withstand a greater degree of swelling theoretically would improve chances of survival.

Determination of Water Permeability in Sperm Plasma Membranes

Our values for hydraulic conductivity compare very favorably with the value of 1.94 $\mu m^3/\mu m^2 \times$ atmosphere $\times$ minute for fowl spermatozoa calculated using fowl sperm dimensions and other parameter values for nonsperm cells (Ravie and Lake, 1982), and that of 10.4 $\mu m^3/\mu m^2 \times$ atmosphere $\times$ minute for bull spermatozoa calculated using bull sperm dimensions and the time to tail coiling on exposure to hypotonic media (Drevius, 1971). Our methodology appears to be reliable for the fowl spermatozoa, and this gives us confidence in our value for bull spermatozoa, even though it suggests an extremely permeable membrane (Dick, 1966). The L_p values represent average values for the cell type. Cells with such a striking degree of regional specialization in terms of membrane structure and function, in addition to regional compartmentalization, make such estimates difficult to interpret. However, it could be argued that the measured L_p, depending as it does on loss of plasma membrane integrity in any region of the cell, reflects the most vulnerable part of the cell. Since in cryobiology the concern is for the survival of fully functional cells, a value

of L_p revealing the most readily disrupted part of the cell is a valid parameter value.

The activation energy measurements were clearly indicative of a greater temperature dependence of L_p in fowl spermatozoa than in bull spermatozoa. However, the relatively poor survival of bull spermatozoa at 5°C raises the question of the validity of L_p measurements at these temperatures. Species in which spermatozoa have a strong susceptibility to cold shock have sperm membrane compositions very different from those of less susceptible species, which results in different sperm membrane lipid phase behavior (Watson, 1981; Watson and Morris, 1987; J. Parks, personal communication). Hydraulic conductivity at low temperatures could well be expected to be significantly altered in those species with marked sperm membrane lipid transition behavior. Moreover, the extrapolation of temperature dependence estimates below 0°C is required for calculations of water loss during freezing, which has been found to be valid for other cell types (Mazur, 1984). For fowl spermatozoa, which do not exhibit marked phase transitions, this approach may also be valid, but for bull spermatozoa it may be more suspect. However, until methods become available to measure water movement in spermatozoa below 0°C, it cannot be avoided.

Implications for Understanding Cryosurvival of Spermatozoa

The results raise some interesting questions in relation to cryopreservation. The L_p and E_a values predict an optimal cooling rate of several thousand degrees Celsius per minute, but in practice the optimal cooling rate is <100°C/minute. This suggests that L_p may not be as important in determining the optimum cooling rate as in other cell types.

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References

Bakst MR. Fertilizing capacity and morphology of fowl and turkey spermatozoa in hypotonic extender. *J Reprod Fertil*. 1980;60:121–127.

Burrows WH, Quinn JP. The collection of spermatozoa from the domestic fowl and turkey. *Poultry Sci*. 1937;16:19–24.

Chaudhuri, Lake PE. A new diluent and methods of holding fowl semen for up to 17 hours at high temperature. In: *Proceedings of the 18th World Poultry Congress*. London: Butterworth-Heinemann Ltd, 1988;591–596.

Dick DAT. *Cell Water*. Washington, DC: Butterworths; 1966:89–92.

Duncan AE, Watson PF. Predictive water loss curves for ram spermatozoa during cryopreservation: comparison with experimental observations. *Cryobiology*. 1992;29:95–105.

Drevius L-O. Permeability coefficients for water and polyhydric alcohols. *Exp Cell Res*. 1971;69:212–216.

Drevius L-O. Water content, specific gravity and concentrations of electrolytes in bull spermatozoa. *J Reprod Fertil* 1972*a*;28:15–28.

Drevius L-O. Bull spermatozoa as osmometers. *J Reprod Fertil*. 1972*b*; 28:29–39.

Drevius L-O, Eriksson H. Osmotic swelling of mammalian spermatozoa. *Exp Cell Res*. 1966;42:136–156.

Fawcett DW. The mammalian spermatozoon. *Dev Biol*. 1975;44:394–436.

Graham JK, Foote RH, Parrish JJ. Effect of dilauroylphosphatidyl choline on acrosome reaction and subsequent penetration of bull spermatozoa into zona-free hamster eggs. *Biol Reprod*. 1986;35:413–424.

Hammerstedt RH, Graham JK, Nolan JP. Cryopreservation of mammalian sperm: what we ask them to survive. *J Androl*. 1990;11:73–88.

Johnson L, Pinkel D. Modification of a laser-based flow cytometer for high resolution of DNA analysis of mammalian spermatozoa. *Cytometry*. 1986;7:268–273.

Lake PE, Smith W, Young D. The ultrastructure of the ejaculated fowl spermatozoon. *Q J Exp Physiol*. 1968;53:356–366.

Leibo SP. Water permeability and its activation energy of fertilized and unfertilized mouse ova. *J Membrane Biol*. 1980;53:179–188.

Mazur, P. Kinetics of water loss from from cells at sub-zero temperatures. *J Gen Physiol*. 1963;47:347–369.

Mazur P. Freezing of living cells: mechanisms and implications. *Am J Physiol*. 1984;247:C125–C142.

Mazur P, Rall WF, Leibo SP. Kinetics of water loss and the likelihood of intracellular freezing in mouse ova. *Cell Biophys*. 1984;6:197–213.

Noiles EE, Ruffing NA, Kleinhans FW, et al. Critical tonicity determination of sperm using fluorescent staining and flow cytometry. In: Perry P, ed. *Boar Semen Preservation* II. Berlin: Berleu, 1991; 359–364.

Ravie O, Lake PE. Prediction of ice formation in fowl spermatozoa at particular cooling rates. *Cryo-Lett*. 1982;3:91–100.

Robertson L, Watson PF. The effect of egg-yolk on the control of intracellular calcium in ram spermatozoa cooled and stored at 5°C. *Anim Reprod*. 1987;15:177–187.

Rothschild L, Barnes H. Osmotic pressure of bull semen diluents. *Nature*. 1954;173:636–637.

Ruffing NA, Critser JK, Mazur P, Watson PF. Determination of the critical tonicity of bovine spermatozoa using dual fluorescent staining. *Cryobiology*. 1990;27:648.

Spector PC, Goodnight JH, Sall JP, Sarle WS. The GLM procedure. In: *SAS Users Guide: Statistics*. Version 5 Edition. Cary, NC: SAS Institute Inc; 1985:433.

Steinkamp JA, Stewart CC, Crissman HA. Three-color fluorescence measurements on single cells excited at three laser wavelengths. *Cytometry*. 1982;2:226–231.

Thomas JA, Buchsbaum RN, Zimniak A, Racker E. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry*. 1979;18:2210–2218.

Watson PF. The effects of cold shock on sperm cell membranes. In: Clark A, Morris GJ, eds. *Effects of Low Temperatures on Biological Membranes*. London: Academic Press; 1931:189–218.

Watson PF, Morris GJ. Cold shock injury in animal cells. In: Bowler K, Fuller BJ, eds. *Temperature and Animal Cells*. Cambridge: The Company of Biologists, 1987;311–340.