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# Motility of bovine spermatozoa studied by laser light scattering

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**Abstract.** Laser light scattering has been employed to determine the swimming speed distribution and the fraction of motile cells in samples of bovine spermatozoa. As predicted from theory, average trajectory velocities determined by laser light scattering were approximately four times the average translational speed estimated using light microscopy. The proportion of motile spermatozoa decreased with time at the same rate when samples were prepared in either HEPES or phosphate buffers. However, whereas the mean swimming velocity declined slowly in HEPES buffer, it dropped rapidly when phosphate buffer was used. Dilution (in the range $40 - 0.4 \times 10^6$ spermatozoa $\cdot$ ml$^{-1}$) in either of these two buffers reduced the fraction of motile spermatozoa in the sample, but the mean swimming velocity of the remaining active spermatozoa was unchanged. Lowering the temperature from 37° C to 15° C reduced the mean swimming speed by a factor of 2–3 and the fraction of motile cells by a factor of 4–5.

**Key words:** Laser light scattering, motility, spermatozoa

## Introduction

Laser light scattering has been applied in quantitative studies of the motility of bacteria (Nossal and Chen 1972, 1973; Schaefer et al. 1974; Holz and Chen 1978; Wang and Chen 1981; Chen and Wang 1982) and spermatozoa (Bergé et al. 1967; Nossal 1971; Dubois et al. 1975; Lee and Verdugo 1976; Matsumoto et al. 1977; Hallett et al. 1978; Finsy et al. 1979; Craig et al. 1979; Harvey and Woolford 1980; Racey et al. 1981; Craig et al. 1982; Hallett 1982; Lee 1982). Methods of analyzing laser light scattering data from swimming spermatozoa and micro-organisms have been reviewed (Chen and Hallett 1982). The analysis of sperm motility employed by F. R. Hallett and collaborators (Craig et al. 1979; Racey et al. 1981; Hallett 1982), which incorporates information on swimming patterns of individual spermatozoa determined by microcinematography, has proved particularly successful. This method is adopted in an investigation of the effects of temperature and dilution on the motility of bull spermatozoa in vitro.

## Theory

The initial theory for laser light scattering by motile organisms stems from the proposal of Nossal (1971) that motile particles be considered as point scattering sources, for which the velocity is constant during the decay of the autocorrelation function of the fluctuations in the scattered electric field. For such particles the normalized first-order correlation function of the scattered electric field $g^1(\tau)$ is given by

$$g^1(\tau) = 4\pi \int_0^\infty \frac{\sin(kv\tau)}{kv\tau} P(v)\, dv\,, \tag{1}$$

where $P(v)$ is the velocity distribution, $k$ is the scattering vector and $\tau$ is the correlation delay. Experimentally, $g^1(\tau)$ is obtained from the measured intensity autocorrelation function $C(\tau)$, so that

$$g^1(\tau) = A[C(\tau) - B]^{0.5}\,, \tag{2}$$

where $A$ is a normalizing constant and $B$, the baseline, is obtained from the square of the average intensity. Although in principle $P(v)$ may be determined directly by Fourier sine transformation of the correlation data $g^1(\tau)$ [see Eq. (1)], this method

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suffers from the truncation of $g^1(\tau)$ (Cummins 1977). Two alternative approaches for extracting $P(v)$ are the method of moments (cf. Chen and Hallett 1982) and the method of splines (Stock 1978). Both these methods require data of extremely high accuracy and consequently do not distinguish between the several velocity distributions, $P(v)$, that have been applied to sperm motility (cf. Cummins 1977). Number fluctuation spectroscopy (Schaefer and Berne 1975) has not been applied extensively to light scattering by spermatozoa.

Hallett et al. (1978) found that the intensity autocorrelation function derived from laser light scattering experiments on normal, swimming spermatozoa was best fit by a Lorentzian:

$$L(\tau) = \frac{1}{1 + (k\bar{v}\,\tau/2)^2}\,. \tag{3}$$

An average trajectory speed $\bar{v}$ can be obtained from the fitted half-width of this Lorentzian. From Eqs. (1) and (3), Fourier inversion of $L(\tau)$ provides the distribution of trajectory speeds, $P(v)$, as a gamma distribution with two degrees of freedom (Hallett et al. 1978):

$$P(v) = \frac{v}{\pi\bar{v}^2}\, e^{-2v/\bar{v}}\,. \tag{4}$$

Equation (4) may be differentiated to find the most probable speed ($v_m$) at the maximum of the speed distribution:

$$v_m = \frac{\bar{v}}{2}\,. \tag{5}$$

Analysis of cinematographic data for the helical path of normally swimming bull spermatozoa relates this instantaneous velocity ($v_m$, which is tangential to the trajectory) to the average linear translational velocity ($v_t$), obtained from light microscopy (Hallett et al. 1978) so that:

$$v_t = 0.5\, v_m\,. \tag{6}$$

Hence Eqs. (5) and (6) predict the average velocity $\bar{v}$ determined by laser light scattering to be approximately a factor of four greater than the corresponding microscope-derived speed ($v_t$).

Since the original Nossal theory assumes scattering from a point source, an assumption not satisfied by sperm, a refined ellipsoidal Rayleigh-Gans-Debye model has been developed for bull spermatozoa (Craig et al. 1979). The model indicates that at low angles the empirically-determined Lorentzian function in Eq. (3) provides the rotation frequency $\bar{w}$ of the head, the major scattering element of the spermatozoan. This result is consistent with the experimentally observed linear relationship between mean head rotation frequency and mean translational velocity ($\bar{v}$) (Rikmenspoel et al. 1960):

$$\bar{v} = c\bar{w}\,, \tag{7}$$

where $c$ is the proportionality constant.

## Materials and methods

### *Living material*

Ejaculates from bulls were maintained at 20° C until required for experimentation. Light scattering measurements commenced within 60 min of ejaculation. Ejaculates were diluted with either phosphate-buffered saline (composition in mM: NaCl, 138; KCl, 2.7; $Na_2HPO_4$, 8.1; $KH_2PO_4$, 1.5; glucose, 10; pH 7.3) or HEPES buffer (composition in mM: NaCl, 134; KOH, 2.5; $MgCl_2$, 4.0; NaOH, 7.5; $Na_2HPO_4$, 2.0; glucose, 10.0; HEPES, 20.0; + bovine serum albumin 4 mg · $ml^{-1}$; pH 7.55). Buffers were filtered through a 0.45 μm Millipore filter before use. Unless otherwise stated all samples were maintained at 30° C during the experiments.

### *Laser light scattering*

*(a) Apparatus.* Samples were examined in the temperature-controlled compartment of a spectrometer described in detail elsewhere (Sattelle et al. 1982). The 488-nm line from an unfocussed Argon ion laser (Spectra Physics 164), attenuated to 20 μW, illuminated the temperature-controlled sample of bull spermatozoa. To avoid complications due to internal sperm structure (cf. Holz and Chen 1978; Craig et al. 1979), the scattered light was detected at a scattering angle ($\theta$) of 15°, using a photomultiplier tube (EMI 9863) mounted on the rotatable arm of the spectrometer. The scattered light was detected through two 200 μm diameter pinholes resulting in a scattering volume of $1.0 \times 10^{-4}$ ml, which was sufficiently large to avoid number fluctuations (Schaefer and Berne 1975) at the cell concentrations used. The entire spectrometer was mounted on an air table with a resonant frequency of 2.0 Hz.

Photon pulses were passed to an amplifier-discriminator (Princeton Applied Research, 1140A) and fed into a 64-channel Langley-Ford correlator. The autocorrelation function was displayed on an oscilloscope and stored on magnetic tape cassettes of a

computer terminal (Texas Instruments Silent 700) for later analysis by computer (IBM 370/165). Data collection times were typically 50 s at a sample time ($\tau$) of 100 μs. Three correlation functions were obtained and the three fits were averaged for each reported measurement. Total scattered intensity was calculated from the recorded number of photopulses, the number of correlation samples and the sample time ($\tau$).

Prior to each measurement, the sample was shaken gently to resuspend settled cells. Approximately 3 min were allowed for swirling to decay. The sample temperature was monitored using a calibrated thermocouple.

*(b) Data analysis.* Both Gaussian and Lorentzian scattering functions for normal swimmers were tested. In agreement with Hallett et al. (1978), the Lorentzian provided better fits to the data and resulted in higher motile fractions and lower swimming speeds. Unlike the samples used by Hallett et al. (1978), defective swimmers were not observed with the buffers used in the present studies. Consequently a term for circular swimmers was not included in the fit to the autocorrelation data. Since an unfocussed beam was used and since most samples contained a large fraction of motile cells, sedimentation of spermatozoa through the Gaussian profile of the beam was minimal. Therefore, a single exponential was used in the present investigation to approximate diffusion of non-motile sperm (Finsy et al. 1979; Stock 1978). Hence autocorrelation data were fitted to an equation of the form:

$$g^1(\tau) = \frac{l}{1 + (k\bar{v}\,\tau/2)^2} + (1 - l)\, e^{-DK^2\tau}, \tag{8}$$

where $l$ is the live fraction, $K\left(= \frac{4\,\pi n}{\lambda} \sin\frac{\theta}{2}\right)$ is the scattering vector and $D$ is the diffusion coefficient for non-motile spermatozoa. Data were fitted to the above equation using a least-squares, gradient-search, computer programme employing the Marquardt algorithm (Bevington, 1969).

Any of the three parameters $l$, $\bar{v}$ and $D$ in Eq. (8) could be fixed in the programme if an independent estimate was available. In this study $D$ was obtained from formaldehyde-killed spermatozoa by fitting autocorrelation data to a third-order polynominal of the form:

$$\ln\,[g^1(\tau)] = -\bar{\Gamma}\tau + \frac{\mu_2\,\tau^2}{2!} - \frac{\mu_3\,\tau}{3!}, \tag{9}$$

where $\bar{\Gamma}$ is the first moment or average decay, $\mu_2$ is the second moment or variance and $\mu_3$ is the third moment or skewness of the distribution of the exponential decays in the correlation function (cf. Palmer et al. 1982). Subsequently, a z-averaged diffusion coefficient for dead spermatozoa was obtained from the first moment according to:

$$D = \frac{\bar{\Gamma}}{\overline{K^2}}. \tag{10}$$

*Microscopy*

The sperm density was estimated by counting samples diluted in formol saline on a haemocytometer slide.

The motility of spermatozoa in undiluted semen was assessed by area change frequency (ACF) (Dott and Foster 1979). The mean velocity of spermatozoa in diluted samples was estimated by number flux counting (Katz and Dott 1975) using an image analyzing computer (Quantimet 720 with frame smasher) to count the number ($N$) of spermatozoa crossing the line. The computer also provided the surface density ($\bar{n}$) of motile spermatozoa and the proportion of motile (live) spermatozoa.

The mean velocity ($v_t$) was determined from:

$$v_t = \frac{\pi}{2}\frac{N}{Lt\bar{n}}, \tag{11}$$

where $L$ is the length of line and $t$ is the time.

Ten estimates of mean velocity were made in 30 s and the average and coefficient of variation were calculated. If the coefficient of variation was > 10% then a new average was calculated omitting the highest and the lowest estimate. If the coefficient of variation was still > 10% the average was considered to be invalid. This occasionally happened because of a drift affecting all the spermatozoa in the field of view despite the 3-min equilibration time allowed before measurements began.

Both ACF and mean velocity were estimated at 30° C.

## Results and discussion

*Autocorrelation functions from laser light scattered by bull spermatozoa*

As shown in Fig. 1, autocorrelation functions derived from laser light scattered by swimming bull spermatozoa were Lorentzian in shape. Rapid killing of the sperm by adding formaldehyde (to a final concentration of 0.05%) resulted in an autocorrelation function which decayed much more slowly. This would be predicted (cf. Chen and Hallett 1982) for a system of motile scatterers which, following formal-

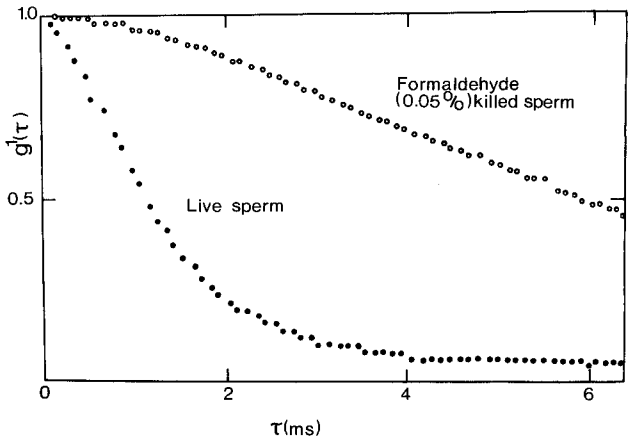


**Fig. 1.** Typical autocorrelation functions for bull spermatozoa (●) and the same sample killed by the addition of 0.05% formaldehyde (○). Each function was derived from approximately $10^6$ photopulses in a 50-s period at a sample time ($\tau$) of 100 μs. The scattering angle was 15°, the sample temperature was 30° C, and the sperm concentration was $1 \times 10^7$ ml$^{-1}$

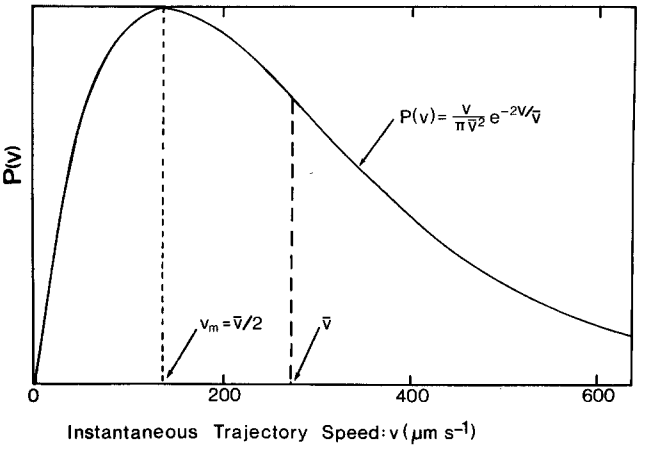


**Fig. 2.** Swimming speed distribution for the autocorrelation function derived from live sperm in Fig. 1, calculated using Eq. (4). From 2-parameter fits, (cf. Eq. (8) and taking $D = 0.04 \times 10^{-7}$ cm$^2 \cdot$ s$^{-1}$) the average speed ($\bar{v}$) was determined. Both the average ($\bar{v}$) and most probable ($v_m$) speeds of the calculated distribution are shown

**Table 1.** Comparison between motility parameters for spermatozoa determined simultaneously by laser light scattering and light microscopy approximately 1 hr after collection. Microscope measurements (ACF, velocity, live fraction) were made at 30° C at the original ejaculate concentration. Laser measurements were made at 30° C at optimal sperm concentrations of $4-40 \times 10^6$ ml$^{-1}$. The laser-determined average trajectory velocity ($\bar{v}$) is approximately a factor of four greater than the microscope-derived translational velocity [see Eqs. (5) and (6)]

| Day | Bull | Buffer | Microscope | | | | Laser | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Ejaculate concentration ($\times 10^6$) | ACF | Velocity (μm · s$^{-1}$) | Live fraction (%) | Concentration for $\bar{v}$ ($\times 10^6$) | $\bar{v}$ (μm · s$^{-1}$) | Live fraction (l) (4) |
| 1 | Frosty | PBS | 850 | 44 | – | – | 42.5 | 291 | 61 |
| 2 | Badger | PBS | 875 | 50 | – | – | 3.8 | 247 | 24 |
| | Lady's man | PBS | 930 | 37 | – | – | 4.0 | 141 | 21 |
| 3 | Lady's man | PBS | 950 | 20 | – | – | 15.8 | 186 | 60 |
| | Lady's man | HEPES | 950 | 20 | – | – | 15.8 | 317 | 88 |
| 4 | Lady's man | PBS | 1,680 | 51 | 81 | 71 | 21.0 | 298 | 90 |
| | Lady's man | HEPES | 1,680 | 51 | 95 | 61 | 21.0 | 369 | 88 |
| | Frosty | PBS | 1,370 | 10 | 61 | 64 | – | – | – |
| | Frosty | HEPES | 1,370 | 10 | 52 | 75 | – | – | – |
| | Hamlet | PBS | 765 | 2 | – | 15 | – | – | – |
| | Hamlet | HEPES | 765 | 2 | 66 | 63 | – | – | – |
| 5 | Lady's man | PBS | 1,870 | 43 | 50 | 26 | 23.4 | 268 | 90 |
| | Lady's man | PBS + glucose | 1,870 | 43 | 111 | 49 | 23.4 | 200 | 84 |
| | Lady's man | HEPES | 1,870 | 43 | 97 | 27 | 23.4 | 248 | 76 |

dehyde treatment, undergo only Brownian motion. Cumulants fits of correlation functions from dead sperm yielded a value for $D$ of $0.04 \times 10^{-7}$ cm$^2 \cdot$ s$^{-1}$ [cf. Eq. (10)]. Fixing $D$ in Eq. (8) to this value, two-parameter fits were then applied to the autocorrelation function for each spermatozoa sample, from which an average trajectory speed ($\bar{v}$) and live fraction (l) were determined. Using Eq. (4), a swimming speed distribution [$P(v)$] was calculated, with a most probable speed ($v_m$) as shown in Fig. 2.

Parameters determined for spermatozoa samples from four bulls obtained on five different occasions over a 2.5-month period are summarized in Table 1. At the time of measurement (normally 2 h following ejaculation), the microscope-velocities were 50–110

$\mu m \cdot s^{-1}$ whereas the microscope-live fraction was in the range 60%–75%. Laser-determined live fractions (1) were slightly higher than those estimated by light microscopy on the same samples. As predicted by Eqs. (5) and (6), the laser-derived average trajectory velocity $\bar{v}$ was approximately a factor of four greater than the corresponding microscope-determined velocity. These laser and microscope velocity measurements were made simultaneously in two separate laboratories, to eliminate differences due to ageing of the samples.

There are two factors in particular which may influence the estimate of the mean velocity of spermatozoa by the methods used in these experiments, (i) the chamber in which the spermatozoa were swimming at the time of the measurement, and (ii) the part of the spermatozoa from which the signal(s) originated.

The chamber used in the laser experiments was infinitely large in relation to both the size of the spermatozoa and the distance they move during the measurement. However, under the microscope the spermatozoa were constrained to swim in a 2-dimensional plane because the third dimension between the slide and the square cover-slip (22 × 22 mm) was c. 15 μm. The effect on the resulting estimates is unknown.

*Number flux counting by light microscopy*

Number flux counting uses phase-contrast illumination to provide the image. The computer uses only the signal from the head of the spermatozoa, which in these preparations did not rotate, but was free to move from one side of the trajectory to the other. However, unless the trajectory is coincident (or nearly coincident) with the line, the estimated mean velocity will be the velocity of the spermatozoa along the trajectory. Occasional 'high' estimates can be explained by such a coincidence and were excluded as explained in the Methods.

Spermatozoa scatter light from the head, midpiece, and main piece of the tail. Changes in light scattered will be most abrupt when the orientation of the head relative to the incident illumination alters. The movement of all elements of the sperm will have one vector in common (that of the whole spermatozoan). In addition each element will have vectors peculiar to itself: the head may go from side to side of the track or rotate; the midpiece/main piece elements will also have components perpendicular to the track and of varying magnitude, depending on the distance from the head and the type of motility. The calculation of the mean velocity of spermatozoa makes allowance for these factors but the estimate is higher than for most other methods.

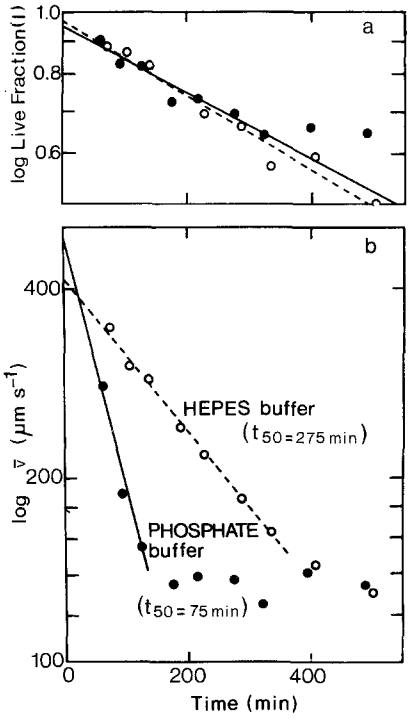


**Fig. 3a and b.** Comparison of the capacity of HEPES (○) and phosphate (●) buffers to maintain (**a**) the live fraction (l) and (**b**) the swimming (average trajectory) speed ($\bar{v}$). The concentration of the sample (Lady's man; day 4 – Table 1) was 21 × $10^6$ $cm^{-3}$. The half decay times of $\bar{v}$ ($t_{50}$) are calculated for both buffers

**Table 2.** Swimming speeds determined by microscopy using different buffers for dilution of spermatozoa. PBS = phosphate buffered saline. Values are the mean ± SEM, each based on six determinations

| | HEPES buffer | PBS buffer | PBS buffer + glucose |
|---|---|---|---|
| Swimming speed ($\mu m \cdot s^{-1}$) (Sample from Lady's man 15.4.81) | 97.0 ± 11.0 | 50.2 ± 7.2 | 111.4 ± 8.4 |

*Comparison of HEPES and phosphate buffers in the maintenance of motility*

Samples of semen were diluted in HEPES and phosphate buffers and the average trajectory velocity ($\bar{v}$) was determined together with the fraction (1) of live spermatozoa. Samples were maintained in optical cuvettes at 30° C and were transferred periodically, after resuspending settled cells, to the temperature-controlled sample holder of the light scattering spectrometer. In this way the relative effectiveness of the two buffers in maintaining motility was assessed. As Fig. 3 shows, an exponential decrease with time was observed for both ($\bar{v}$) and (l) in agreement with

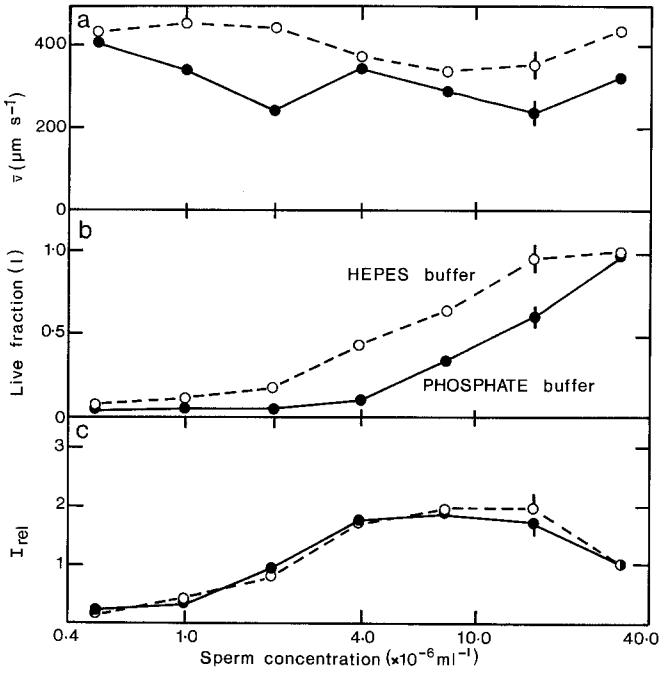


**Fig. 4a–c.** Effects of dilution on spermatozoa in HEPES (○) and phosphate (●) buffers. Swimming speed (**a**) and live fraction (**b**) have been corrected for decay with time throughout the measurements, according to the exponential decay curves of Fig. 3. Relative scattered intensity (**c**) is normalized to the intensity scattered by the highest concentration ($32 \times 10^6$ $ml^{-1}$) of spermatozoa. Error bars are the standard deviation of four measurements at a particular concentration, and for clarity are shown only at a single concentration

Cooke et al. (1976). Higher values for $\bar{v}$ were maintained for longer ($t_{50}$ = 275 min) in HEPES buffer compared to phosphate buffer ($t_{50}$ = 75 min). However, the decay with time of the fraction of live spermatozoa in the sample was indistinguishable for the two buffers. Thus although there is no difference between the ability of the two buffers to maintain the ratio of motile to non-motile cells, HEPES buffer results in significantly higher swimming speeds over a 5-h period. Microscope measurements showed that phosphate buffer with added glucose (10.0 mM) appeared as effective as HEPES buffer in maintaining swimming speeds over a 4.5-h period (see Table 2).

*Effects of dilution on sperm motility*

As a preliminary to studies of motility as a function of sperm concentration, we investigated the effects of dilution on formaldehyde-killed sperm. Ejaculates were diluted 1 : 40 (to $42 \times 10^6$ $ml^{-1}$) in either phosphate or HEPES buffer and thereafter diluted by stages to 1 : 320. With both buffers, diffusion coefficients varied by less than 15% over the concentration range tested. An average diffusion coefficient ($D$) of approximately $0.04 \times 10^{-7}$ $cm^2 \cdot s^{-1}$ was obtained at spermatozoa concentrations of $10^7$ $ml^{-1}$. Using the Stokes-Einstein relation ($D = K_B T/6 \pi\eta r$, where $K_B$ = Boltzman's constant; $T$ = absolute temperature; $\eta$ = viscosity of the medium; r = radius of spherical scatterer), this value of $D$ corresponds to a hydrodynamic diameter of 1.0 μm for equivalent spheres. Measurements on 1.1 μm diameter latex spheres yielded an average diffusion coefficient in agreement with that obtained for killed sperm and also showed very little dependence of $D$ on concentration.

Similar dilution experiments were performed on spermatozoa not treated with formaldehyde. As the sperm concentration was lowered from 40.0 to $0.40 \times 10^6$ $ml^{-1}$, changes in total scattered intensity were observed (Fig. 4c). From a peak at around $10.0 \times 10^7$ cells $\cdot$ $ml^{-1}$, the intensity progressively declined as the concentration was reduced. The drop in intensity on increasing the concentration from 10 to $40 \times 10^6$ $ml^{-1}$, probably reflects multiple scattering. Therefore, to avoid complications associated with multiple scattering (Berne and Pecora 1976), sperm concentrations should be kept below $20.0 \times 10^7$ $ml^{-1}$. Harrison et al. (1982) provide evidence which suggests that the deleterious effect of dilution on the motility of spermatozoa (Mann 1964) might be due to the tendency of motile spermatozoa in a suspension containing little or no protein in solution to stick to glass. The results of the experiments on diluted bull semen could be explained in the same way. The velocity of cells in suspension was not affected by dilution (Fig. 4a) but the proportion of motile cells in suspensions decreased with increasing dilution (Fig. 4b).

*Effects of temperature on motility of spermatozoa*

As the temperature was reduced from 37° C to 15° C, a 4-fold drop in live fraction from an initial value of 63% was observed (Fig. 5b). Over the same temper-

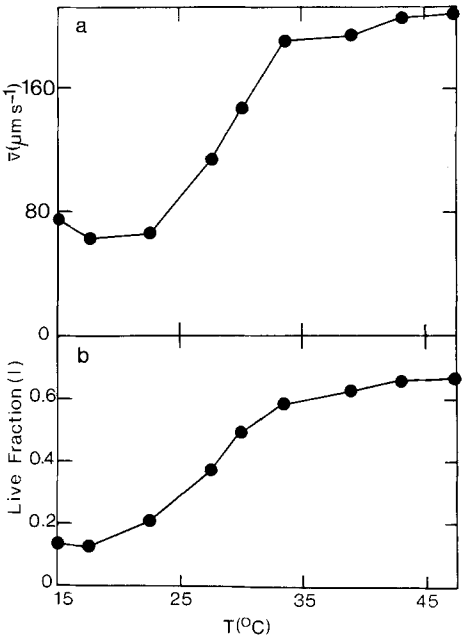


**Fig. 5a and b.** Effects of temperature on swimming speed (**a**) and live fraction (**b**) of spermatozoa in HEPES buffer. Sample concentration was 23 × $10^6$ ml$^{-1}$ and measurements were obtained 2.5–3 h after ejaculation. Results were obtained using an increasing temperature scan. Cells were resuspended at each temperature. Average trajectory velocities ($\bar{v}$) were not corrected for viscosity changes (the small decrease in buffer viscosity from 15° C to 45° C would attentuate the response of $\bar{v}$ to temperature by an amount within the error of the measurement). Also, the decay with time of $\bar{v}$ (which from Fig. 3 would be small between 2.5–3 h and would tend to enhance the effect of temperature on $\bar{v}$) was not taken into account

ature range the average swimming speed ($\bar{v}$) decreased from 200 μm · s$^{-1}$ to 76 μm · s$^{-1}$. Clearly motility is maintained over a wide temperature range and there was no significant change in velocity in the temperature range 33–45 °C.

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