Behaviour of ejaculated spermatozoa from bull, boar and ram during thin-layer countercurrent partition in aqueous two-phase systems

R. A. P. HARRISON1,* M. L. JACQUES1, M. L. PASCUAL MINGUEZ2 and N. G. A. MILLER1

1Department of Biochemistry, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge CB2 4AT, UK
2Depto de Bioquimica y Biologia Molecular y Celular, Facultad de Veterinaria, Universidad de Zaragoza, Miguel Servet 144, 50010 Zaragoza, Spain

*Author for correspondence

Summary

Ejaculated spermatozoa from bulls, boars and rams were subjected to thin-layer countercurrent partition in aqueous two-phase systems composed of dextran T500 and polyethylene glycol 6000 (PEG) in sucrose-based Hepes-buffered media. In the basal system, the great majority of spermatozoa tended to partition with the dextran-rich bottom phase; however, by including very low levels of phosphate, they could be made to partition increasingly with the PEG-rich top phase (complete at 10 mM phosphate). A procedure was developed for carrying out four separations simultaneously under identical conditions, whereby it could be shown that distribution varied with the number of spermatozoa in the sample. In the case of bull, the effect of cell number could be reduced considerably by inclusion of small quantities of seminal plasma in the phase system, but no such effect was found for ram or boar. Considerable variation in distribution pattern was seen between samples, which did not appear to be due to technical inconsistency. Livability in the phase systems was also variable, and we believe that PEG may exert a detergent-like effect on the sperm surface that is exacerbated in highly defined media free of protective proteins.

Key words: sperm populations, aqueous two-phase systems, thin-layer countercurrent partition.

Introduction

In recent years, partitioning in aqueous two-phase systems formed from dextran and poly(ethylene)glycol species has been developed as a mild separation procedure of high selectivity (Albertsson, 1986). Although it has mostly been applied to molecular species and to cellular organelles, the procedure has also been used successfully with cell populations (see Walter et al., 1991). To improve resolution, repeated (or countercurrent) partition is generally used, and, because separation of the rather viscous phases can be a time-consuming stage of each partition cycle, special procedures have been adopted to minimize this separation time. One method is to allow phase separation to take place in thin layers of liquid, when there is a large area of bulk interface relative to total phase volume; the separation technique is then known as thin-layer countercurrent partition (TLCCP; Treffry et al., 1985).

The heterogeneity of sperm cell populations is well recognised. Apart from morphologically damaged or abnormal cells, half the sperm population in every sample of semen carries an X-chromosome while the other half carries a Y-chromosome. However, there is also heterogeneity amongst apparently intact normal cells, due to differences in degree of maturity, capacitation, senescence etc. (see Luderer et al., 1982; Villarroja and Scholler, 1985; Oshio, 1988; Robertson et al., 1988; Cuasnicu and Bedford, 1989). In studying sperm samples from different provenances or following different treatments, this latter heterogeneity causes great problems because clear-cut differences in function are rarely observed. The synthetic capabilities of a spermatozoon are very limited (see Harrison, 1977), and its behaviour is essentially controlled by external effectors acting through cell surface and plasma membrane components. Functional heterogeneity within a sperm population is therefore likely to be reflected in differences between individuals in surface/membrane characteristics. As partition behaviour of cells and particles in aqueous two-phase systems is largely dependent on their surface characteristics, TLCCP would therefore seem to be an ideal method for separating sub-populations of functional spermatozoa.

This paper describes the application of TLCCP to ejaculated sperm from boar, ram and bull.

Materials and methods

Spermatozoa

Ejaculates were obtained from rams (Welsh-Suffolk cross-
breds and Clun Forest and boars (Large White) from the Institute's colonies, and from bulls (Friesian and Limousin) on loan to Animal Breeding Technology, Cambridge Ltd from the Milk Marketing Board; all donors had produced offspring. Semen from rams and bulls was collected using an artificial vagina, and the sperm-rich fraction of semen from boars was collected using the "gloved hand" technique. Suitable care was taken to avoid "cold-shock" damage to the samples during collection; afterwards, they were allowed to cool slowly to the normal ambient temperature (20-22°C) at which all preparative manipulations were performed.

Suitable care was taken to avoid "cold-shock" damage to the spring. Semen from rams and bulls was collected using an extender (Revell and Glossop, 1989) to a final concentration of about 1.5 x 10⁸ cells/ml and stored overnight at ambient temperature in the dark, before washing.

**Counting of spermatozoa**

Sperm concentrations in semen samples were estimated using a haemocytometer, while sperm concentrations in TLCCP media were estimated using a Coulter Counter Model ZF (Coulter Electronics Ltd., Harpenden, Herts). For the latter, samples were diluted (optimally to about 3 x 10⁶ cells/ml) in Isoton (Coulter Electronics Ltd.) containing 0.1% (w/v) Nonidet P40 (Brotherton, 1975, 1977) and 0.1% (w/v) formaldehyde; the counting solution had been passed through a 0.22 µm filter. To avoid sample losses on pipette tips etc, the samples were diluted by "rinsing out" the pipette into the counting medium. The spermatozoa were counted in 0.1 ml sub-samples using a 70 µm aperture, an aperture current setting of 4, an attenuation setting of 0.707, and a threshold setting of 26.

**Batch partition experiments**

Five-ml volumes of selected phase systems were prepared by weighing out suitable quantities of stock solutions of dextran T500 (Pharmacia Biosystems Ltd, Milton Keynes, Bucks), PEG 6000 (BDH Ltd, Poole, Dorset), sucrose, buffer, etc (see section on preparation of phase systems below for details of medium composition) into individual 12-ml conical-bottom screw-cap tubes (Type 144S, Sterilin). The components were mixed thoroughly in a separating funnel, and left to settle twice at about 3-hourly intervals before being left to settle overnight. The separated phases were then drawn off carefully (the interface and associated material being discarded), after which they were stored frozen at −20°C until required. The resultant phase pairs were always used together.

All stock solutions except the dextran were assumed to have the composition expected; the composition of the dextran solutions was estimated through their optical rotations and the specific optical rotation factor of 1.99% (w/v)/dm (Albertsson, 1986, p. 266). The relative amounts of dextran and PEG were adjusted to yield about 300 ml of PEG-rich top phase and 200 ml of dextran-rich bottom phase; not only did this accord with the relative rate of use of the two phases, but it also resulted in a relatively fast rate of phase separation (Albertsson, 1986, p. 29).

The phase pairs from each batch were characterized as follows: (1) the dextran content was estimated by polarimetry, using a Bellingham and Stanley Model A polarimeter (Bellingham and Stanley Ltd., Tunbridge Wells, Kent, UK) and subtracting the optical rotation of the polymer-free sucrose-based medium from the measured optical rotation of the phase sample. (2) The freezing-point depression was measured (as "osmolality" equivalent) using an Advanced Micro-Osmometer (Model 3MO; Advanced Instruments Inc., Needham Heights, Mass, USA); the presence of the hydroxylated polymers resulted in very high false "osmolality" readings, which were nevertheless sufficiently repeatable to be a valuable characteristic. (3) The conductivity was measured at 25°C using a Philips PW9501 Conductivity Meter (Pye Unicam, Cambridge, UK). (4) The pH was measured at 25°C using a Philips PW9420 pH Meter fitted with a Philips CE2 semi-micro electrode. (5) The inorganic phosphate was measured according to Rouser et al. (1970), using as "blanks" samples of top and bottom phases not containing added phosphate.

Characteristics so obtained for the standard pH 6.5 and pH 7.5 phase systems used in the present work are given in Table 1. According to these parameters, phase composition varied very little between batches.

**Preparation of sperm suspensions for TLCCP**

Samples of about 4 x 10⁶ cells were layered over two-step cushions of 4 ml 35% Percoll-saline on 2 ml 70% Percoll-saline in round-bottomed 100 mm x 16 mm glass tubes, and centrifuged for 5 min at 200 g max, followed by 15 min at 900 g max. The Percoll layers were made up in the saline medium described above, from an iso-osmotic "100% Percoll-saline" prepared from Percoll suspension (Pharmacia) and a "10-fold saline stock", according to Vincent and Nadeau (1984). After centrifugation, the supernatant layers were aspirated off to

Dextran and 3.8% (w/v) PEG in the sucrose medium; because this mixture did not form two phases, the sperm concentration detected in it was used to estimate the total number of cells added to each tube. Partition was then expressed as the percentage of the added cells that remained in the bulk top phase.
leaves in each tube 1 ml of the 70% Percoll in which most of the spermatozoa were loosely pelleted and could be readily resuspended. This procedure removed cytoplasmic droplets, cell debris and, in the case of boar semen, gel particles that often contained trapped spermatozoa. Recovery of spermatozoa during this washing procedure was high (mean of 77.0% ± 8.3; s.e.m.; n=4, from different boars) and there was no evidence that the recovered population did not represent the starting population.

The resultant sperm suspensions in 70% Percoll-saline were then diluted with 3 volumes of saline medium, layered over 3 ml of "top-phase", and centrifuged for 5 min at 400 g max to transfer all the cells into the top-phase layer. The supernatant Percoll-saline layer was carefully aspirated off, to leave as much of the top-phase layer as possible, in which the spermatozoa were gently but thoroughly resuspended. Losses of spermatozoa during this stage were very low.

**TLCCP**

Thin-layer countercurrent partition was carried out on a Bioshef apparatus (Department of Biochemistry, The University of Sheffield, Sheffield, UK); the essential features of this apparatus were described by Treffry et al. (1985). The Bioshef was controlled from a BBC Microcomputer and run inside a large domestic refrigerator (Sadia Model L2) that had been fitted with a thermostatic control unit to maintain a desired temperature (between 0 and 22°C) to within ± 0.1 deg.C; unless otherwise stated, the temperature in the refrigerator was maintained at 17°C. Two rotors could be run simultaneously; each rotor contained 60 chambers, the lower disk cavities of which had a volume of 0.75 ml.

Before a run, the desired phases (prepared as described above), together with one or two rotors, were placed in the refrigerator to equilibrate to the set temperature. The rotor was then positioned on the machine, and each chamber loaded with 0.68 ml of bottom phase; next, 0.75 ml of the sperm samples in top phase (see preparation procedure above) were added to the starting chambers. The run was immediately initiated, with 30 s of shaking and 7 min of resting time before each transfer. Either 29 or 59 transfers were performed (two samples or one sample per rotor, respectively). If two rotors were run simultaneously, loading was completed in the first before it was begun in the second. Generally, runs were begun at about 5 p.m. and the rotors unloaded the following morning.

To unload, 0.8 ml of polymer-free sucrose-based medium (temperature-equilibrated) was added to each chamber, and the rotor shaken for 3 min. The rotor was then inverted to allow drainage of each chamber into a tube (LP4; Luckham) positioned against the chamber entrance by means of a spring-loaded frame. The tubes had been thoroughly and carefully pre-washed to reduce possibilities of contamination with plastic micro-particles; such particles were shown in preliminary experiments to produce spurious counts in the Coulter Counter during analysis of the fractions for sperm content.

**Results**

**Initial experiments to select phase systems**

It was decided at the outset to found the phase system on a sucrose-based medium known to be suitable for sperm survival (Harrison et al., 1978), because the sodium chloride usually present in cell media might itself influence partition (Albertsson, 1986, p. 334).

Small quantities of EDTA were included, in order to reduce both cell-cell agglutination (Harrison et al., 1978) and the possibilities of oxidative damage (by chelating contaminating heavy metals; see Halliwell and Gutteridge, 1990); EDTA does not affect sperm viability adversely at normal ambient temperatures (RAPH, unpublished results).

In preliminary batch partition experiments carried out in capped tubes, various concentrations of dextran and PEG were tested. It was found (Table 2) that, even at the lowest concentrations at which two phases were formed, a very significant proportion of the sperm population showed a high affinity for the interface (see Table 1).

**Table 1. Characteristics of phase systems used in TLCCP of bull, boar and ram sperm populations**

<table>
<thead>
<tr>
<th>pH</th>
<th>Upper phase</th>
<th>Lower phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>1.142±0.130</td>
<td>10.099±0.182</td>
</tr>
<tr>
<td>6.5</td>
<td>1.055±0.163</td>
<td>10.083±0.168</td>
</tr>
</tbody>
</table>

Values are means±s.e.m. for 5 separate batches.

**Table 2. Partition behaviour of ram and boar spermatozoa in phase systems composed of different concentrations of dextran and polyethylene glycol 6000**

<table>
<thead>
<tr>
<th>Dextran (% w/v)</th>
<th>PEG (% w/v)</th>
<th>% of cells in bulk top phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.4</td>
<td>3.9</td>
<td>34.5</td>
</tr>
<tr>
<td>5.6</td>
<td>4.0</td>
<td>15.5</td>
</tr>
<tr>
<td>5.8</td>
<td>4.1</td>
<td>11.7</td>
</tr>
<tr>
<td>6.0</td>
<td>4.3</td>
<td>8.7</td>
</tr>
<tr>
<td>6.2</td>
<td>4.4</td>
<td>6.7</td>
</tr>
<tr>
<td>6.4</td>
<td>4.5</td>
<td>4.7</td>
</tr>
</tbody>
</table>

Sperm samples were applied to phase systems prepared in tubes, and partitioned as described in Materials and methods. After the phases had settled out, samples were removed from the upper phase for counting, and the observed number of sperm compared with that in a control polymer mixture that did not form two phases. The counts were corrected for the relative volumes they were found in, as described in Materials and methods.
systems that included a final concentration of either no (○), 1 mM (●) or 10 mM (▲) Na phosphate (previously buffered to pH 7.5). In the separations illustrated in this and all subsequent figures, the number of cells recovered from each chamber has been expressed as a percentage of the total number recovered.

Treffry et al., 1985). However, when low concentrations of phosphate were included (Albertsson, 1986, p. 334), essentially all the spermatozoa remained in the top phase (data not shown), even at higher concentrations of polymers (when phase systems are more stable). As a consequence of these experiments, it was decided to use the phase systems described in Materials and methods (characteristics in Table 1), and to modify partition by means of small changes in phosphate concentration and pH.

Testing of basic TLCCP methodology

Initially samples were subjected to 59 transfers on TLCCP. A typical pattern of distribution seen with boar sperm samples is illustrated in Fig. 1, in which the effect of varying the phosphate composition is also shown. In the presence of 1 mM phosphate, most of the spermatozoa were found near the middle of the fraction train, as predicted from the conclusions of the batch experiments, whereas omission of phosphate caused the bulk of the spermatozoa to be found at the beginning of the fraction train (i.e. to show a high affinity for the interface). Increasing the concentration of phosphate to 10 mM, on the other hand, increased the tendency of the spermatozoa to remain in the top phase, whence they were found at the end of the fraction train.

Using the phosphate-free system, experiments were carried out on the effect of changing the settling time; with insufficient settling time, droplets of the bottom phase would be included with the bulk top phase at each transfer, with the result that associated spermatozoa would be found further along the fraction train than with sufficient settling time. In fact, results using settling times of either 4 or 10 min (data not presented) were indistinguishable from those for 0 mM phosphate shown in Fig. 1, showing that the 7 min settling time used in Fig. 1 was more than sufficient. (It was of interest that distribution patterns did not differ with the settling times we used, for Fisher and Walter (1984) and Heywood-Waddington et al. (1986) found that partition of cells and organelles was affected by settling time; it may well be that phase separation in thin layers (as in our Bioshef rotor chambers) proceeds differently from phase separation in tubes (as used by Fisher and Walter, 1984, and Heywood-Waddington et al., 1986).)

The use of 59 transfers, however, only allowed the running of one sample at a time in each rotor. Although characterization of the different batches of phase system did not reveal any obvious variations from batch to batch (see Table 1), it was decided to use 29 transfers routinely, to allow direct comparison of two samples or treatments under identical conditions on a single rotor. A preliminary experiment, in which four replicate samples were run in pairs simultaneously on two rotors, showed that intrinsic repeatability was good; a slight difference was seen between rotor 1 and rotor 2, but there was very little difference between the two halves of each rotor. Resolution after 29 transfers was as good as after 59.

Effect of sample concentration

While trying to establish reliable TLCCP methodology, we frequently observed variations in distribution pattern between samples. Experiments were therefore carried out to see if distribution depended on sample cell numbers. In each experiment, four samples, containing between 5 × 10⁸ and 4 × 10⁶ spermatozoa in 0.75 ml, were prepared from a single ejaculate by diluting the washed sperm suspension in top phase serially into further top phase. The set of four samples was run simultaneously in pairs on two rotors, using a single phase batch for the entire run. Three experiments were performed with boar, two with ram and two with bull; one of the boar experiments is shown in Fig. 2, one of the ram experiments is shown in Fig. 3, and Fig. 4C and D illustrate the effect of concentration on bull sperm distribution. In all cases, considerable variation in distribution with sperm concentration was observed. Individual fractions were checked under the microscope to ensure that the peaks recorded by the Coulter Counter were due to spermatozoa; moreover, once proper precautions had been taken to eliminate as far as possible spurious particulate matter from the resultant TLCCP fractions and then to subtract an average “background” due to this material from the Coulter Counter results, the sperm numbers recovered related closely to the numbers applied at all dilutions. Variations in distribution with sperm concentration could not therefore be accounted for by losses within the rotor.

Effect of seminal plasma

When spermatozoa come into contact with seminal
Fig. 2. The effect of sperm numbers on thin-layer countercurrent partition of boar spermatozoa. Four sub-samples from a boar sperm population were diluted to different extents in top phase, and loaded into chambers 1 and 31 of two rotors; all four sub-samples were then subjected to 29-transfer TLCCP simultaneously in standard pH 6.5 system. (A) $5.2 \times 10^8$ cells loaded (108% recovered); (B) $1.2 \times 10^8$ cells loaded (135% recovered); (C) $0.28 \times 10^8$ cells loaded (144% recovered); (D) $0.10 \times 10^8$ cells loaded (130% recovered).

Fig. 3. The effect of sperm numbers on thin-layer countercurrent partition of ram spermatozoa. Four sub-samples from a ram sperm population were diluted to different extents in top phase, and loaded into chambers 1 and 31 of two rotors; all four sub-samples were then subjected to 29-transfer TLCCP simultaneously in standard pH 7.5 system. (A) $2.2 \times 10^8$ cells loaded (94% recovered); (B) $0.60 \times 10^8$ cells loaded (125% recovered); (C) $0.17 \times 10^8$ cells loaded (117% recovered); (D) $0.050 \times 10^8$ cells loaded (118% recovered).
plasma, they are known to adsorb seminal plasma proteins onto their surfaces (see, for example, Metz et al., 1990; Miller et al., 1990). It seemed possible that the effect of sperm concentration on partition behaviour was due to surface components being "extracted" from the spermatozoa by differential partition; spermatozoa with modified surface characteristics might result from this process, and the efficiency of removal would be likely to depend on sperm concentration. Sperm samples were therefore run at two different concentrations in the presence or absence of seminal plasma. This condition was achieved by adding seminal plasma both to the top phase into which the sperm samples were first transferred and to the upper and lower phases in the rotor. Calculation suggested that as little as 0.1% (v/v) seminal plasma (1 μl/ml) was likely to reveal significant effects.

The results of one of two experiments with bull spermatozoa are shown in Fig. 4; similar results were obtained in the other, although a different animal was involved. These appeared to show that the presence of seminal plasma can prevent variations in partition behaviour due to sperm concentration. However, the results obtained with ram and boar samples were disappointing: no clear-cut effects were observed in three experiments with ram (2 animals) and two experiments with boar (2 animals, 2 levels of seminal plasma).

**Effect of cold-shock**

During the experiments with seminal plasma on ram and boar mentioned above, considerable variation in distribution was observed between animals as well as that resulting from different sperm concentrations. Cell separations using TLCCP are usually carried out at low temperatures (about 2°C) in order to lower cell metabolism; however, it had been decided in advance that TLCCP of spermatozoa should be carried out at 17°C, the lowest practical temperature commensurate with avoiding low-temperature damage to the unprotected cells (cold shock; Watson, 1981). The possibility that 17°C was still too low for reliable survival was examined using ram spermatozoa: a run was performed at 20°C, in which a normal sperm sample was compared with a sample that had been cold-shocked; each sample was tested at two cell concentrations. Initially, in the normal sample, 62% of the cells were alive, as shown by propidium iodide exclusion (Harrison and Vickers, 1990), compared with only 7% in the cold-shocked sample. The results of this experiment are shown in Fig. 5. It will be seen that cold shock had a profound effect on distribution when the samples were run at the higher concentration, whereas there was little effect at the lower concentration. By comparing the position of the cold-shocked (dead) population with the positions of the two populations in the more concentrated control sample, it might be deduced that the right hand of the latter two represents dead cells, and that the effect of running samples at lower concentrations is to kill them, whence they show greater affinity for the top phase. However, comparison of Fig. 5 with Fig. 3 shows that lowered sample concentration produced opposing partition behaviour in these two experiments. Similar
Aqueous two-phase partition of spermatozoa

Fig. 5. Effect of "cold shock" on the partition behaviour of ram spermatozoa. A sample of ram spermatozoa was transferred to top phase and then divided into two parts. One was incubated at 25°C for 5 min, then at 5°C for 10 min (with agitation), and finally replaced at 25°C for a further 5 min; the other was maintained at 25°C throughout as a control. Following this treatment, the control suspension contained 38% damaged cells (stainable with propidium iodide: Harrison and Vickers, 1990) while the cold-shocked suspension contained 93% damaged cells. Sub-samples of each suspension were subjected to 29-transfer TLCCP simultaneously at two different cell concentrations in standard pH 7.5 phase system. (A) Control population: 2.5 x 10^8 cells loaded (103% recovered); (B) control population: 0.27 x 10^8 cells loaded (99% recovered); (C) cold-shocked population: 2.3 x 10^8 cells loaded (98% recovered); (D) cold-shocked population: 0.24 x 10^8 cells loaded (85% recovered).

conflicting effects of concentration on partition behaviour were noted in both bull and boar.

In another separation carried out at 20°C, samples from two different rams were each run at two different concentrations. The results are shown in Fig. 6. It will be seen that the two samples behaved differently, though the effect of cell number was consistent with earlier observations. The distribution patterns observed were similar to those seen in earlier separations carried out at 17°C, so it was concluded that variations were not related to temperature. Since the differences were seen even within a single phase batch, earlier variations in distribution between runs could not be ascribed to subtle differences between phase batches. Although the same individual (Pig) was used in Fig. 5 as in Fig. 6C, separation patterns did not show consistency in this respect; for example, those illustrated in Fig. 3 involved Sunny, the same individual as in Fig. 6A.

Discussion

Our experiments show that the behaviour of bull, boar and ram spermatozoa during TLCCP in aqueous two-phase systems appears to vary greatly from sample to sample. The reputed sensitivity of these two-phase systems is such that there is a serious possibility of variation in conditions between runs being responsible for the observed differences in distribution behaviour. However, by first showing that replicate samples run simultaneously in the same batch of phase system on two rotors show very similar distributions and then running experimental samples in parallel under identical conditions, we have been able to eliminate this possibility. We believe therefore that the high sensitivity of TLCCP is able to reveal subtle differences between semen samples. It is interesting to note that Magargee et al. (1988) reported similar variation between samples, even from a given animal, in an extensive study of lectin binding by ram spermatozoa.

At present, the difficulty is to interpret the differences seen by TLCCP. The observation that distribution depends strongly on sperm cell numbers and that this dependence can be perturbed (in bull sperm populations, at least) by including seminal plasma in the phase system seems to us very significant. It is well known that mammalian spermatozoa readily adsorb onto their surfaces proteins from body fluids, particularly seminal plasma (see, for example, Dravland and Joshi, 1981; Mann and Lutwak-Mann, 1981; Metz et al., 1990; Miller et al., 1990); the strength of the adsorption varies, some components being removed by simple washing techniques, others by treatment with hypertonic saline, and others requiring detergent treatment (see, for example, Russell et al., 1985). Since PEG, by reducing the polarity and dielectric properties of the aqueous environment, can perturb membrane structure and cause release of constituents (McCammon and Fan, 1979; Arnold et al., 1983; Arnold et al., 1985), it is likely that spermatozoa placed in a PEG-containing two-phase system will release surface-coating components that will then partition independently. Unless
these extracted components partition in parallel with the spermatozoa, they will separate from the main sperm population increasingly as the transfers progress. The degree to which surface components are released/extracted from individual cells will almost certainly affect the cells' partition characteristics, which may actually change during the course of the TLCCP. Obviously, the efficiency of the progressive extraction and separation will be greater at lower sperm concentrations.

Once this possibility is considered, variation between samples becomes easier to explain: TLCCP is revealing differences in the overall surface compositions of the individual cells, both in the membrane surface (protein?) compositions and in the coating compositions. Each ejaculate is indeed essentially unique in terms of the precise relative contributions to it from the different organs of the male reproductive tract, and the results of Magargee et al. (1988) suggest strongly that the intrinsic membrane surface may vary within the population (in their work, samples from opposing epididymides in the same individual showed clear differences in lectin binding).

In the experiments described above, our failure to find any effects of addition of homologous seminal plasma to ram and boar TLCCP separations might be ascribed to not having added enough seminal plasma. However, the problem of increasing the seminal plasma concentration significantly without disturbing the composition and characteristics of the phases was beyond the scope of the present work. We wonder whether our inability to affect the TLCCP behaviour of ram and boar spermatozoa with seminal plasma reflects a greater sensitivity of their cell surfaces to PEG, and whether this in turn is connected with the difficulties of cryopreserving these sperm species as compared to bull spermatozoa (Hammerstedt et al., 1990).

Apart from disturbing its partition characteristics, removal of the spermatozoa's surface components during TLCCP would be likely to have serious repercussions on its viability. This might explain the variable loss in viability that we observed in our separated sperm populations. In early experiments (data not presented), in which spermatozoa were not washed through Percoll layers prior to transfer into top phase, we found that most spermatozoa were viable (by exclusion of propidium iodide) following TLCCP; in ram samples in which two populations were detected, there tended to be differences in viability between the two. However, in the experiment illustrated in Fig. 5, in which spermatozoa had been efficiently washed (through Percoll layers), all cells in the peak fractions of both populations in the control sample run at higher concentration (Fig. 5A) took up propidium iodide (data not presented). A subsequent experiment, in which ram spermatozoa were incubated at 20°C in two-phase mixtures, confirmed the detrimental effect of such conditions on sperm viability (data not presented). This problem remains to be pursued.

Unfortunately, spermatozoa may also die after the TLCCP run has been completed; thus to find dead cells associated with a given partition characteristic does not imply that this characteristic can be ascribed to a state of senescence or death. Moreover, although the cold-shocked sample appeared to have stronger affinity for the PEG-rich top phase (Fig. 5C), the partition
behaviour of cold-shocked sperm would not necessarily be the same as that of cells that have died through extraction of surface components. Despite the viability problems encountered, however, we think that progress has been made in respect of the type of phase system that can be applied to delicate mammalian cells. The classic NaCl/phosphate buffer systems used to control partition of red blood cells (see Walter, 1985) are unsuitable for lengthy incubations, particularly for use with spermatozoa, whose metabolism has long been known to be affected by high levels of phosphate (see Mann, 1964); yet phosphate remains an effective modifier of partition. By using sucrose-based media to maintain osmolality, we were able to control the bulk partition of ram and boar sperm populations between interface and top phase at will with phosphate concentrations of less than 10 mM. As spermatozoa generally remain viable between pH 5.5 and pH 9 (see Mann, 1964), pH control of the medium should also be a useful modifier of partition, since spermatozoa are known to bear a range of negatively charged groups on their surfaces (see, for example, Hammerstedt et al., 1979; Moore, 1979). In fact, we used the system of lower pH for the boar sperm samples, precisely in order to reduce slightly their affinities for the top phase in the presence of 1 mM phosphate.

We thank Tony Tilley and John Doggett for numerous collections of ram and boar semen, Professor Chris Polge of Animal Biotechnology Cambridge Ltd for samples of bull semen, and Dr Manual Lopez Perez (Dept of Biochemistry and Molecular and Cellular Biology, University of Zaragoza) for his considerable encouragement and support. We thank also the Spanish Ministry of Education and Science for travel funds to enable M.L.P.M. to spend a training period at the Institute of Animal Physiology.

References


(Received 4 November 1991 - Accepted, in revised form, 13 February 1992)