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# Thermotropic phase transitions in the plasma membrane of ram spermatozoa

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**Summary.** A steady-state fluorescence polarization technique, using the membrane probe 1,6-diphenyl-1,3,5-hexatriene (DPH), showed that separately detectable transitions occurred in the regions of 17, 26 and 36°C in isolated preparations of ram sperm plasma membrane. An independent technique based on the temperature-related behaviour of calcium- and magnesium-activated ATPase detected a single phase transition in the region of 24°C. Modulation of ATPase by neighbouring lipid composition was inferred from findings that phospholipase $A_2$ caused significant stimulation of the enzyme. Cholesterol-rich liposomes caused an upward shift of the phase-transition temperature from 24°C to 30°C, but the reasons for this are unclear.

It is considered that these phase transitions may have profound effects on sperm survival and physiology, both during normal fertilization processes and in response to cryostorage.

## Introduction

Recent progress in membrane biology has demonstrated that dramatic alterations in the organization of membrane lipids can be induced as a result of temperature-related phase transitions from the gel to the liquid–crystalline state and *vice versa*. Such transitions are also associated with changes in membrane permeability and the induction of membrane fusion.

The mammalian sperm plasma membrane represents the functional interface between the cell cytoplasm and the diversity of environments encountered between spermatogenesis and eventual fusion with the egg. There is therefore a strong likelihood that the occurrence of thermal and isothermal phase transitions in this membrane could be physiologically important during sperm maturation in the epididymis, storage in the slightly cooler cauda epididymidis before ejaculation, and the fertilization process itself. Non-physiological thermal phase transitions might also be implicated as contributory factors in the loss of sperm viability associated with cold-shock and cryopreservation procedures for semen of animals of most species.

The occurrence of lipid phase-transition events in ram sperm plasma membranes has been demonstrated by Holt & North (1984), using freeze-fracture electron microscopy; during that study, however, we were unable to study the response of sperm membranes over a series of temperatures and showed only that phase transitions had occurred once the cells had been cooled to 5°C. The aim of the present investigation was therefore to supplement the previous work by examining the response of ram sperm plasma membranes over a continuous sequence of temperatures. For this purpose we have used two independent assessments of phase-transition behaviour in isolated plasma membrane fractions.

A number of intramembranous enzymes, such as 5′-nucleotidase and alkaline phosphatase of the rat hepatocyte plasma membrane (Livingstone & Schachter, 1980), exhibit kinetic differences associated with lipid phase transitions. In this study the sperm plasma membrane $Ca^{2+}$–$Mg^{2+}$ ATPase (EC 3.6.1.3) was investigated to see whether similar behaviour could be detected, previous

studies by Bradley & Forrester (1980) having already established that this is an integral membrane protein.

Since the possibility exists that temperature-related changes in enzyme activity might be unrelated to alterations in membrane lipid organization, two other means of affecting the lipid environment were examined. The effect of phospholipase $A_2$ upon subsequent membrane ATPase activity was examined, since the consequent release of fatty acids and lysolecithin would probably disrupt membrane structure. Conversely, the possibility that membranes could be loaded with cholesterol, thereby changing the phase transition behaviour, was studied by monitoring membrane ATPase activity in the presence of cholesterol-rich liposomes.

Steady state fluorescence polarization measurements of the lipophilic probe 1,6-diphenyl-1,3,5-hexatriene (DPH) were also used to detect membrane-lipid phase transitions. This technique has been extensively documented (for review, see Shinitzky & Barenholz, 1978), and provides a sensitive means of detecting organizational changes within the membrane.

## Materials and Methods

*Preparation of sperm plasma membranes.* The semen samples used in this study were obtained from Finnish–Landrace rams by the use of an artificial vagina. The samples were washed through ficoll columns (Harrison, 1976) to remove seminal plasma and most of the cytoplasmic droplets, then used for the preparation of a plasma membrane fraction as described previously (Holt & North, 1985). This fraction, produced by discontinuous gradient centrifugation, contains >90% plasma membrane, with about 7–10% contamination by acrosomal outer membrane. The topographical source of the plasma membrane has not been determined.

The membranes were stored at −20°C until required; for the ATPase investigations they were then used without further treatment. Preliminary experiments showed that the frozen storage had no effect upon the behaviour of the ATPases studied subsequently.

The membrane fractions used for fluorescence polarization studies were washed and resuspended in distilled water, whilst those to be incubated with phospholipase $A_2$ were washed and resuspended in Tris–HCl buffer, pH 8·4. The final protein concentrations were determined using a modification of the Lowry procedure (microprotein determination kit; Sigma (London), Poole, Dorset, U.K.).

*Determination of ATPase activity.* ATPase activity was monitored by incubating 0·2 ml of the membrane fraction (25–50 μg membrane protein), together with 1 ml 2 mM-adenosine triphosphate in 50 mM-Tris–HCl buffer, pH 7·4. Estimates of ATPase activity at different temperatures were performed in the presence of 5 mM-calcium chloride; the use of other ions is described as appropriate. After incubation at the required temperature for 10 min, the reaction was stopped by the addition of 0·8 ml cold (4°C) trichloroacetic acid. The amount of phosphate released during the incubation was then assayed by a modification of the Fiske & SubbaRow (1925) technique (kit supplied by Sigma).

To examine the effects of exogenous lipids on ATPase activity at different temperatures, ~100 μg liposomes in 100 μl distilled water were preincubated at 30°C for 10 min with each aliquant of membrane fraction before addition of the ATP solution.

The results of these experiments were examined as Arrhenius plots; data plotted in this way produce a linear response when no changes in enzyme activation energy occur in response to temperature changes. The lines were fitted to the data points by a combination of visual assessment and least squares regression analysis within each data set. Since a consistent feature of the results was the presence of two linear regions with different slopes, the intersection between two such fitted linear regions was taken as the phase transition temperature.

*Effect of phospholipase $A_2$ on ATPase activity.* Membrane fractions, 0·3 ml containing 25–50 μg protein, in 50 mM-Tris–HCl buffer, pH 8·4, were incubated overnight at 37°C in the presence of 50 μg phospholipase $A_2$ (Sigma; prepared from bee venom). Control aliquants lacking phospholipase $A_2$ were also incubated overnight. After incubation, 1 ml 2 mM-ATP solution, containing 0·1 mM-ethyleneglycol tetra-acetic acid (EGTA), 0·1 mM-ouabain and combinations of calcium and magnesium ions, was added to each assay tube. The low level of EGTA was included to provide a calcium-free environment when required and the ouabain was included to inhibit sodium- and potassium-activated ATPases.

After a further 30 min incubation at 37°C, ATPase activity was terminated by the addition of cold trichloroacetic acid. Inorganic phosphate was assayed as described above. Results were corrected for the slight presence of phosphate in the phospholipase preparation used. Each assay was performed in duplicate, and three separate membrane fractions were examined.

*Preparation of liposomes.* Liposomes containing cholesterol (Sigma, chromatography standard) and phosphatidylcholine (PC) (dipalmitoyl-PC, distearyoyl-PC, dioleoyl-PC; Sigma) were prepared by resuspending vacuum-desiccated films of these lipids (molar ratio, 1:1) in 20 ml distilled water at 40°C. The suspensions were sonicated for 12 min, then centrifuged at 50 000 *g* for 15 min to remove undispersed lipids.

To confirm the multilamellar nature of the liposomes formed by this technique, samples were negatively stained with sodium phosphotungstate and examined by electron microscopy. The cholesterol:phospholipid ratios of various liposome preparations were also checked to ensure that a molar ratio of about 1:1 had been achieved. For this purpose, equal volumes of liposome dispersions were dried into paired glass tubes. The cholesterol in one tube was estimated by a ferric chloride technique (Rudel & Morris, 1973), whilst the phosphate content of the other tube was assayed after phospholipid decomposition (Bartlett, 1959).

*Fluorescence polarization measurements.* Suspensions of sperm plasma membranes (200–300 μg protein/ml) and liposomes (100 μg lipid/ml, nominal concentration) were labelled with $10^{-6}$ M-DPH by a rapid dilution of $2 \times 10^{-3}$ M-DPH in tetrahydrofuran, followed by 30 min incubation at 25°C.

The degree of fluorescence polarization of DPH in the samples was measured with an Elscint microviscometer MV-1, using an excitation wavelength of 365 nm. Polarization values (P) for DPH were obtained directly from the instrument. However, to compensate for the depolarization due to light scattering, P was plotted as a linear function of absorbance of the sample at 450 nm; the true value of P for each sample, at 25°C, was then obtained by extrapolating to zero absorbance (Johnson & Nicolau, 1977; Johnson, 1981).

Polarization values for the plasma membrane samples were measured at a series of temperatures (±0·1°C) from 8·7 to ~50°C. Polarization values (P), where P is defined as:

$$(I_v - I_h)/(I_v + I_h)$$

and where $I_v$ and $I_h$ are the intensities of vertically and horizontally polarized emission with respect to vertically polarized excitation, were obtained as direct readings from the microviscometer.

The polarization of fluorescence was also expressed as the fluorescence anisotropy parameter $((r_0/r) - 1)^{-1}$, a relative scale of microviscosity (Shinitsky & Barenholz, 1978); here $r$ is the measured fluorescence anisotropy and $r_0$ is a derived value for DPH (0·362) (Shinitsky & Barenholz, 1978) and represents the fluorescence anisotropy when no rotations take place during the lifetime of the excited state. Arrhenius plots of the anisotropy parameter are linear for lipid systems of invariant phase (Shinitsky & Barenholz, 1978; Brasitus, 1983) and therefore abrupt deviations from linearity are indicative of phase transitions.

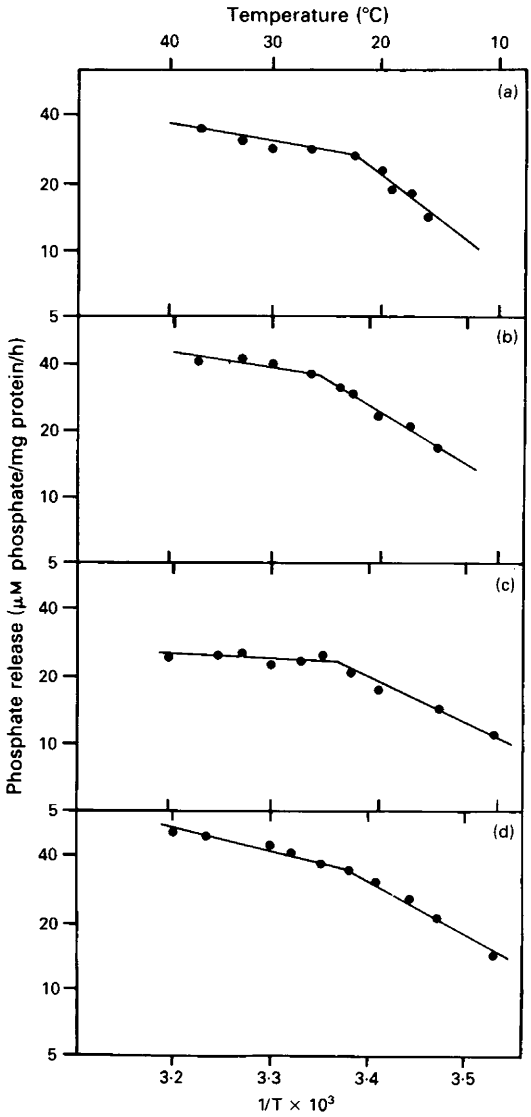


**Fig. 1.** Activity of calcium-stimulated ATPase in isolated sperm plasma membrane vesicles, as a function of temperature; results of four separate experiments are presented as Arrhenius plots. A single discontinuity is apparent in each plot in the region of 22–24°C, giving a mean (±s.e.m.) of 23·8 + 0·82°C.

## Results

*Effects of varying temperature on sperm membrane ATPase activity*

The behaviour of sperm plasma membrane $Ca^{2+}$–$Mg^{2+}$ ATPase activity at a series of temperatures was monitored by measuring the initial velocities of phosphate release, in the presence of 5 mM-$Ca^{2+}$. Observations made by Breitbart *et al.* (1983), and confirmed during this study, indicated that in the absence of magnesium this enzyme could be activated by $Ca^{2+}$ concentrations in the millimolar range, whilst in the presence of 3 mM-$Mg^{2+}$ maximal activity was achieved with only 160–200 μM-$Ca^{2+}$.

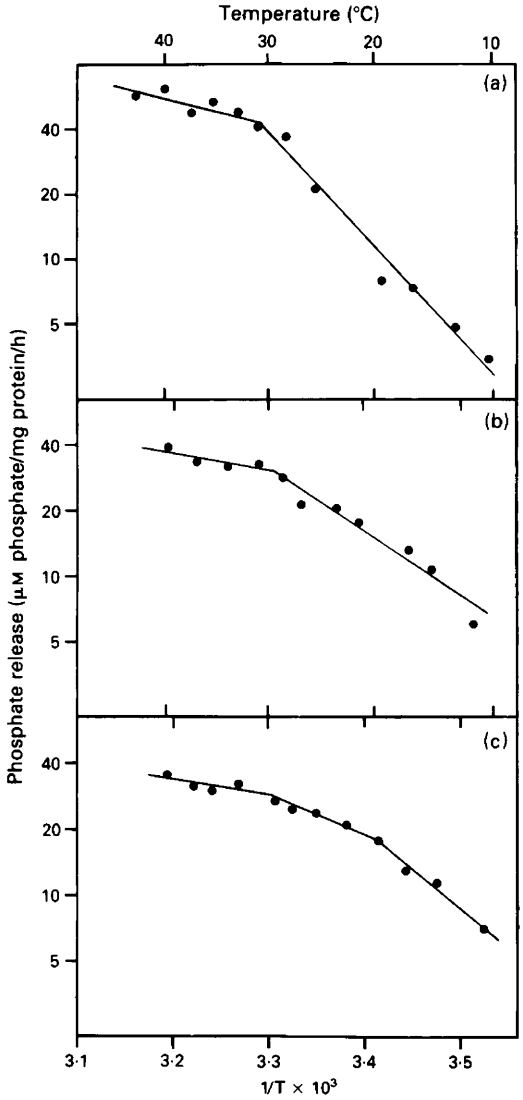


**Fig. 2.** Arrhenius plots of calcium-stimulated ATPase activity in isolated sperm plasma membrane vesicles coincubated with liposomes of distearoyl- (a), dioleoyl- (b) or dipalmitoyl- (c) phosphatidylcholines. In each panel, discontinuities are apparent in the region of 30°C, whilst a second at 20°C is only evident in (c).

Arrhenius plots of the data from four such experiments are shown in Fig. 1. These graphs consistently showed a biphasic relationship between temperature and enzyme activity, although slight variations in specific activity were observed between different membrane preparations. The discontinuities evident in these plots occurred in the range 22·5–25·6°C, with a mean (±s.e.m.) of 23·8 ± 0·82°C.

The raw data from the 4 experiments shown in Fig. 1 were kindly examined by Dr H. M. Dott (AFRC Institute of Animal Physiology, Cambridge, U.K.) using a computerized curve-fitting procedure. This analysis confirmed that there were two algebraically distinct regions to each curve.

In three instances the discontinuities occurred at 20·1°C, whilst in the fourth it was identified at 24°C. These results were therefore useful in confirming the Arrhenius analyses.

*Effects of exogenous lipids upon sperm membrane ATPase activity*

The experiments described above were repeated in the presence of liposomes containing cholesterol and phosphatidylcholine. The liposomes appeared to exert some effect upon the membrane ATPase activity since the resultant Arrhenius plots no longer showed the characteristic discontinuity at 22–25°C. Liposomes containing cholesterol and distearoyl- or dioleoyl-phosphatidylcholine caused the discontinuity to shift into the higher temperature range, 29–30°C (Figs 2a, b), whilst those containing dipalmitoyl-phosphatidylcholine caused not only this effect but also the introduction of another discontinuity at 20°C (Fig. 2c). The mean (±s.e.m.) of the new discontinuity (30·1 ± 0·41°C) was significantly higher than that detected in the absence of exogenous lipids ($P < 0·001$).

*Effect of phospholipase $A_2$ on sperm membrane ATPase activity*

In this experiment, enzyme activity after overnight co-incubation with phospholipase $A_2$ was monitored in the presence of: (a) no added ions, (b) 3 mM-$MgCl_2$, (c) 3 mM-$MgCl_2$ + 0·2 mM-$CaCl_2$, (c) 5 mM-$CaCl_2$. The results of three replicates were analysed using an analysis of variance, and are summarized in Fig. 3. Phospholipase treatment had no effect upon the differential activation requirements of the ATPase enzyme studied in this system, the presence of 5 mM–$CaCl_2$

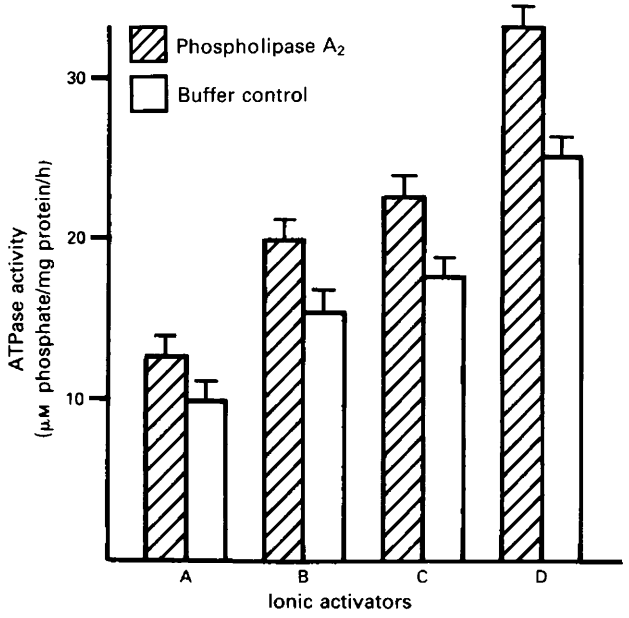


**Fig. 3.** Histogram showing the effects of bee venom phospholipase $A_2$ on ram sperm plasma membrane ATPase activities in the presence of various ionic activators. The ionic compositions of the incubation media were: A, no added ions; B, 3 mM-$MgCl_2$; C, 3 mM-$MgCl_2$ + 0·2 mM-$CaCl_2$; D, 5 mM-$CaCl_2$. Vertical bars represent standard errors of three replicates. All ATPase activities were measured in the presence of 0·1 mM-ouabain and 0·1 mM-EGTA.

providing most stimulation. However, ATPase activity was consistently 20–30% higher ($P < 0.005$) than the appropriate control values after incubation with phospholipase $A_2$, irrespective of the ionic content of the medium (Fig. 3).

*Fluorescence polarization measurements*

Fluorescence polarization readings, obtained at 25°C, for the sperm plasma membrane fraction and for a liposome preparation containing cholesterol:dioleoyl-phosphatidylcholine (molar ratio 1:1) were plotted as a function of optical density (450 nm), to correct for light scattering effects. Linear extrapolations of these plots back to zero optical density gave P values of 0·332 for the membrane fraction and 0·335 for the liposome preparation.

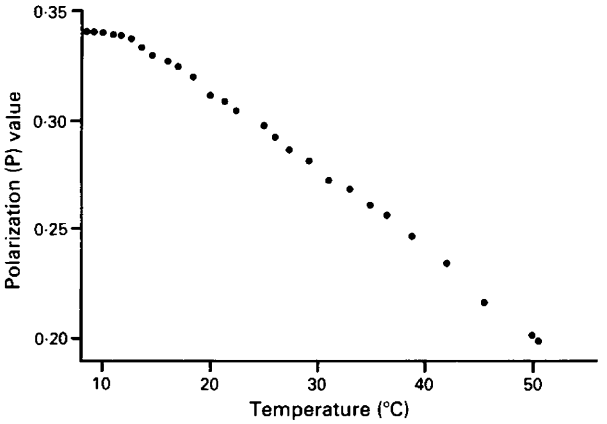


**Fig. 4.** A representative plot of the polarization value (P) for DPH in ram sperm plasma membrane vesicles, as a function of temperature.

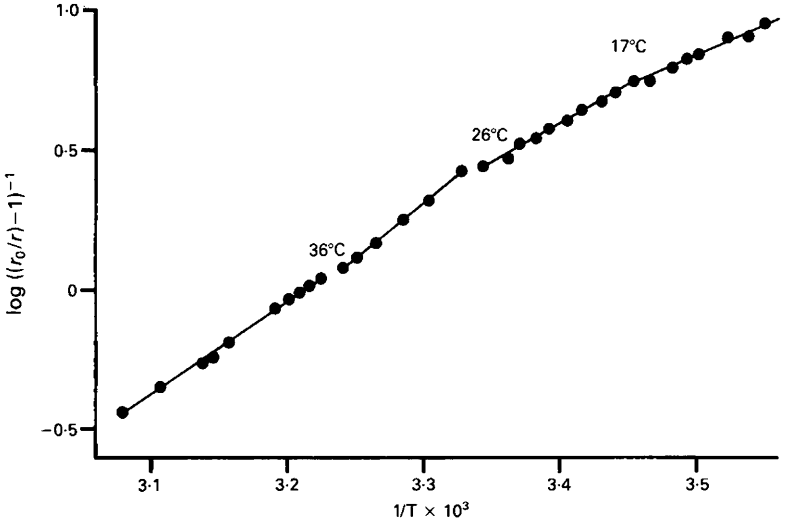


**Fig. 5.** A representative Arrhenius plot of the anisotropy parameter (see text for definition) for DPH in ram sperm plasma membrane vesicles. Four distinct linear regions of the graph are readily distinguished, with discontinuities at 17°C, 26°C and 36°C. Results obtained with different membrane preparations yielded equivalent plots.

When the P values for DPH in the membrane fraction were plotted as a function of temperature a smooth curvilinear response was obtained (Fig. 4). The effects of temperature upon the anisotropy parameter of DPH in the sperm plasma membrane fraction are illustrated by a representative Arrhenius plot in Fig. 5. The plot appears to be a complex sequence of four linear regions with departures from linearity at about 17°C, 24–26°C and 35–38°C. Data from two separate experiments, performed with different membrane preparations, gave mean (±s.e.m.) values of 16·4 ± 1·3°C, 26·4 ± 0·9°C and 35·6 ± 0·9°C for the three discontinuities. These results appear to indicate three separate thermotropic phase transitions in the membrane lipids.

## Discussion

Upon ejaculation into the female reproductive tract, spermatozoa experience an increase in environmental temperature of a few degrees, depending upon species, having been produced and stored in the testis and epididymis, respectively; both of these organs have developed sophisticated mechanisms for remaining below body temperature. Spermatozoa also experience drastic changes in environmental temperature during the procedures involved in cryostorage when the temperature is lowered to about 0–5°C, then either maintained for storage without freezing, or lowered further to that of liquid nitrogen. The reverse procedure involves a relatively rapid increase in temperature, typically reaching 30–37°C in a few seconds.

Temperaure changes of this nature characteristically modify cell membrane structure by altering the physical state of membrane lipids (for review, see Karnovsky *et al.*, 1982). Changes in lipid phase from a liquid–crystalline to a gel state, or *vice versa*, have been recognized as having profound effects upon membrane properties, including their permeability and ability to undergo membrane fusion reactions. It is therefore probable that membrane phase transitions are functionally important during the events which immediately precede fertilization, whilst those induced artificially during cooling and storage may contribute to the loss of sperm viability associated with these procedures.

Although this study was designed primarily to investigate the occurrence of membrane-lipid phase transitions in spermatozoa, one of the techniques chosen relied upon the detection of temperature-related changes in an integral membrane enzyme activity ($Ca^{2+}$–$Mg^{2+}$ ATPase); in contrast to the use of fluorescence polarization, this method provided information on both the occurrence of lipid-phase transitions and the way in which a membrane function may be affected by them.

The stimulatory effects of phospholipase $A_2$ upon the membrane ATPase examined in this study support the view that the activity of this particular enzyme is modulated by the physical state of the lipid environment. In a detailed study of this effect in erythrocyte and platelet membranes, Mostafa *et al.* (1984) concluded that the activation process is caused by small amounts of phospholipid hydrolysis, with the consequent release of lysolipids and free fatty acids. Membrane structure is known to be disrupted by these products of phospholipid hydrolysis (Poole *et al.*, 1970). Lysolecithin is known to promote plasma membrane and acrosomal membrane disruption in mammalian spermatozoa (Jones, 1976; Fleming & Yanagimachi, 1981). The phospholipase-induced stimulation of ATPase activity seen in this study is therefore interpreted as being a consequence of membrane-lipid disordering in the vicinity of the enzyme molecules; further studies of this effect may establish that the rate of calcium transport out of the spermatozoon is also lipid dependent.

Membrane ATPase measurements at various temperatures have been widely used for the study of membrane phase transitions (for review, see Kimelberg, 1977), and there is good reason to believe that integral membrane enzymes of this type are kinetically modified by changes in their lipid environment.

It was not possible to study the effects of temperature upon cytosolic enzymes to strengthen the interpretations made from Arrhenius plots of membrane ATPase activity regarding phase transitions in membrane lipids, because there is considerable evidence that some cytosolic enzymes also

produce discontinuous Arrhenius plots (see, for example, Massey *et al.*, 1966). These are widely regarded as reflecting changes in protein conformation or phase changes in the environment of the enzyme. Kimelberg (1977) reviewed these arguments in some detail, and concluded that some intramembranous enzymes are fundamentally affected by the physical state of their lipid environment. It is therefore neither possible to support nor refute the validity of the data derived from the ATPase experiments by recourse to cytosolic enzymes. It was, however, this precise point of debate which stimulated the use of DPH as a second, and independent, method of assessing phase transitions in the sperm plasma membrane preparations used here.

The use of fluorescence polarization has been discussed at length by previous authors who generally agree that steady-state polarization determinations using DPH give a good indication of membrane microviscosity. This applies especially to studies of isolated membrane fractions, since the use of whole cells can lead to significant errors through the interaction of the probe with intracellular proteins (Mély-Goubert & Freedman, 1980). Membrane-lipid phase transitions have been detected by fluorescence polarization in a diversity of cell types, including pig intestinal brush border cells (Ohyashiki, Takeuchi, Kodera & Mohri, 1982), rat colonic epithelial cell basolateral membranes (Brasitus, 1983) and plant cell membrane lipids (Raison & Wright, 1983). To our knowledge, however, there has only been one previous application of this technique to the study of sperm plasma membranes (Vijayasarathy *et al.*, 1982) but in that instance all measurements were carried out at a constant temperature.

In view of morphological findings that the sperm plasma membrane is organized as a number of distinct domains, each with its own characteristic structure (for reviews, see Friend, 1982; Holt, 1984), it is not surprising that the techniques used in this study should reveal at least three significant temperatures associated with changes in membrane conformation. From the fluorescence polarization study it would appear that lipid-phase transitions occur in the regions of 15–17°C, 24–26°C and 35–38°C; the second of these transitions was also apparent from the studies of membrane ATPase activity.

A study of this nature cannot establish the topographical location of the lipids involved in the phase transitions detected; however, in view of the complex heterogeneity of the sperm membrane it is likely that each transition point represents events in different membrane regions. Recent findings that membrane fractions from tails of bull spermatozoa are more than ten times richer in $Ca^{2+}$–$Mg^{2+}$ ATPase than the corresponding head membranes (Vijayasarathy & Balaram, 1982) would suggest that the enzymically detected phase transitions are probably associated with the sperm tail.

The apparent phase transition detected in the region of 35–38°C is potentially very important, because it is slightly below the normal body temperature in sheep, 39–40°C, but above the normal testicular and epididymal temperature of 32–33°C (Waites & Moule, 1961). It therefore appears that ejaculation of ram spermatozoa is accompanied by a gel–sol transition in at least part of the plasma membrane. The potential effects of such a change could be far reaching, with increased protein mobility and membrane fusogenicity as two of the more obvious consequences. Effects of this type have been correlated with the preliminary stages of fertilization (Friend, 1980), and it is attractive to consider that a strategy as simple as raising the environmental temperature of the spermatozoa might act to precipitate these later stages of sperm development. Fleming & Kuehl (1985) have shown that the time course of acquisition of acrosome reacting ability in guinea-pig spermatozoa was considerably faster at 44 than at 37°C; at 15 or 25°C none of the spermatozoa gained the ability to undergo the acrosome reaction, even after prolonged incubation. Whilst these findings are open to several interpretations they are certainly consistent with the idea that changes in membrane-lipid phase might promote the events which precede the acrosome reaction.

The phase transition detected by fluorescence polarization at 25–26°C probably corresponds to that observed at about 24°C by monitoring $Ca^{2+}$–$Mg^{2+}$ ATPase activity. Breitbart & Rubinstein (1983), who also studied ram sperm plasma membrane vesicles, found a discontinuity in their Arrhenius plot for calcium transport at 28°C, perhaps a reflection of the same membrane event.

The rationale for using liposomes with a high cholesterol content in these experiments was to test the possibility that the cholesterol content of the sperm plasma membrane could thereby be increased by transfer. Since the cholesterol:phospholipid ratio of ram spermatozoa has been reported as 0·38 (Darin-Bennett & White, 1977) and 0·59 (Holt & North, 1985), increasing this value towards the comparable estimate of 0·99 for human spermatozoa (Darin-Bennett & White, 1977) might be expected to help protect against cold-shock. A simple explanation for the apparent increase in phase transition temperature induced by cholesterol-containing liposomes in this study would therefore be that transfer of cholesterol or phosphatidylcholine occurred between the liposomes and the membrane vesicles. The lipid environment immediately surrounding the ATPase molecules thus becomes modified and more viscous. As these transfers are normally rather slow, with half-times measured in hours, it is difficult to reconcile this with the apparently immediate effects found in this study but there are two possible explanations: (1) the membrane lipids in the immediate vicinity of the enzyme are unusually receptive to exogenous lipids or (2) the exogenous lipids are able to interact with the membrane ATPase from outside the lipid bilayer. Clearly these possibilities need further investigation, especially in view of other findings that the same exogenous lipids exert a protective action upon intact spermatozoa during cooling and storage (W. V. Holt & R. D. North, unpublished observation).

Three types of phosphatidylcholine, of different fatty acid content, were used in this investigation, to see whether chain length or degree of saturation might influence the resultant enzyme kinetics. It is clear from the results that the apparent upwards shift in phase transition from 23–24°C to about 30°C was consistently obtained for all three types of fatty acid used; however, a second discontinuity appears to have been introduced at 19–20°C with dipalmitoylphosphatidylcholine. However, little significance can be attached to this finding since a second discontinuity in the other sets of data could have been overlooked. The identification of linear regions in these plots was achieved by visual assessment of the plotted points, in combination with linear regression analysis of the Arrhenius plots; obvious discontinuities were readily identified, but when there was some doubt as to the existence of discontinuities they were disregarded. These studies are therefore insufficiently detailed to allow conclusions to be drawn with respect to these subsidiary phase transitions.

A thermotropic phase transition at about 17°C was detected by fluorescence polarization. Although its significance is unclear it may be involved in the deleterious changes which accompany the cooling of spermatozoa for storage. Robertson & Watson (1986) have demonstrated that ram spermatozoa lose their ability to control intracellular calcium content below 18–20°C but this ability is restored upon rewarming, provided the initial cooling was carried out slowly. These findings may indicate that rapid temperature changes through this critical region might be particularly damaging by causing uncontrolled phase separations. There is some parallel here with findings that boar spermatozoa, which are highly susceptible to cooling damage, apparently acquire resistance if held above 15°C for several hours (Pursel *et al.*, 1972a, b). Watson (1979) proposed that this effect was a membrane 'stabilization', brought about by allowing time for phase changes to occur.

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