External ionic conditions, internal pH and motility of ram and boar spermatozoa

J-L. Gatti¹, C. Chevrier^{1,2}, M. Paquignon¹ and J-L. Dacheux¹

¹URA 1291 INRA–CNRS, Laboratoire de Physiologie de la Reproduction, Institut National de la Recherche Agronomique, 37380 Monnaie, France; and ²Laboratoire de Biologie de la Reproduction, Faculté des Sciences et Techniques, 37200 Tours, France

Internal pH and motility of testicular, epididymal and ejaculated ram and boar spermatozoa were studied as a function of external ionic composition. Internal pH was estimated by the amine distribution method and motility was characterized by percentage of cells that were motile and flagellar beat frequency. Upon dilution in media at different external pH values, internal pH of boar and ram spermatozoa changed rapidly towards the external pH. High external concentrations of Na⁺ or K⁺ had no effect on the rate of equilibration and only a slight effect on the final internal pH value, ruling out a role of Na⁺–H⁺ or K⁺–H⁺ exchange mechanisms in this process. In both species, a linear relationship was observed between internal and external pH but equilibration was incomplete suggesting that there is a complex regulatory mechanism. This result was unaffected by epididymal maturation and ejaculation. Ram and boar testicular spermatozoa showed no increase in movement after dilution, suggesting that simple changes in internal pH are not a sufficient trigger for motility. At high external pH, internal pH increased and motility of epididymal boar spermatozoa was initiated. Motility of ejaculated boar spermatozoa, and epididymal and ejaculated ram spermatozoa was less dependent upon external pH and affected only very slightly by the internal pH changes. K⁺ or Na⁺ had almost no effect on motility just after dilution. After 1 h of incubation, movement decreased. Maintenance of motility in sodium or potassium showed a sharp external pH optimum. Media without Na⁺ and K⁺ allowed a better conservation of motility at external pH > 8 for ram epididymal and ejaculated spermatozoa and at external pH > 6 for boar ejaculated spermatozoa.

Introduction

Mammalian spermatozoa obtained from the testis are immotile or show only weak axonemal movements without forward motility. To become motile, and also fertile, they must undergo a post-gonadal maturation during their epididymal transit (reviewed by Yanagimachi, 1981; Cooper, 1986).

In spite of numerous studies, the biochemical basis for the acquisition and the control of motility during epididymal transit and after ejaculation are still poorly understood. Internal Ca²⁺ and cAMP have been implicated as second messengers that initiate motility during epididymal maturation, but the external trigger remains unknown, mainly because both the composition of the fluid and the spermatozoa change at the same time during this process (for reviews Garbers and Kopf, 1980; Yanagimachi, 1981; Cooper, 1986). As they pass through the epididymis and during ejaculation, the plasma membrane surface proteins, lipids and enzyme activities of spermatozoa are strongly modified (for review see Cooper, 1986). These modifications also induce changes in the ionic permeabilities of plasma membranes (Chulavatnol and Yindepit, 1976; Hiiipakka and Hammerstedt, 1978; Zimmerman *et al.*, 1979; Hammerstedt *et al.*, 1979;

Lardy and San Agustin, 1989). Along the epididymis and during ejaculation, the composition of the media surrounding the spermatozoa is greatly modified (Mann, 1964; Yanagimachi, 1981; Cooper, 1986), and some of these modifications have been shown to control acquisition of motility by spermatozoa (for reviews see Garbers and Kopf, 1980; Yanagimachi, 1981; Cooper, 1986).

Changes in the properties of the plasma membrane, and particularly in its ionic conductances, in response to environmental factors have been shown to be powerful regulators of metabolism and motility activation in spermatozoa of different species; internal pH plays the role of a second messenger (Busa and Nuccitelli, 1984). The best example of this pH effect is sea urchin spermatozoa which are immotile and without respiration when their intracellular pH is under 7.5 and become highly motile and consume oxygen in response to an intracellular alkalization (Shapiro *et al.*, 1985). Several reports suggest a role for intracellular pH during the activation of motility in mammalian spermatozoa. Rat spermatozoa are immotile in epididymal cauda fluid and initiation of their motility is accompanied by an intracellular alkalization mediated by a sodium–proton exchange mechanism (Wong *et al.*, 1981; Wong and Lee, 1983). It is also proposed that inhibition of motility of spermatozoa of several species in the cauda epididymis is attributable to an

acidic intracellular pH due to the presence of lactate (Babcock, 1983; Acott and Carr, 1984; Turner and Reich, 1985; Carr *et al.*, 1985). Babcock *et al.* (1983) showed that a potassium-dependent intracellular pH alkalization stimulated both metabolism and motility of epididymal bull spermatozoa. Protein phosphorylations that were dependent upon changes in internal pH of bull spermatozoa were also reported (Carr and Acott, 1989).

Because little information on the mechanism controlling mammalian internal pH (pHi) of spermatozoa is available, we investigated the effect of external cations (H^+ , Na^+ and K^+) on the regulation of internal pH and the initiation and preservation of motility of spermatozoa obtained at different stages of maturation (testis, cauda epididymis and upon ejaculation). Ram and boar spermatozoa were used because they present opposing extremes of motility during epididymal maturation: ram spermatozoa are fully motile in the cauda epididymidis fluid, whereas boar spermatozoa are almost immotile (Dacheux and Paquignon, 1980a, b).

Materials and Methods

Collection of boar and ram spermatozoa

Ejaculated spermatozoa were collected with an artificial vagina for the ram and manually with a dummy sow for the boar. Epididymal spermatozoa were collected by retro-perfusion of the posterior region of the epididymis with paraffin oil within 30 min after surgical castration or slaughter of the animals. Spermatozoa were kept in the epididymal fluid without further dilution ($2-4 \times 10^9$ spermatozoa ml^{-1}). Ram and boar testicular spermatozoa were collected from conscious animals with a cannula inserted in the rete testis as described by Volgmayr *et al.* (1967) and Edwards *et al.* (1976). In general, samples of spermatozoa were divided for use in both pH and motility measurements. For pHi measurement, ram and boar testicular spermatozoa and ejaculated boar spermatozoa were first concentrated to about 10^9 cells ml^{-1} by centrifugation (5 min, 850–900 g); epididymal spermatozoa were used directly.

Media

For this study we used three different media with ionic composition in which the major cation was either sodium (sodium media: NaM), potassium (potassium media: KM) or cholinium (choline media: ChM). The sodium containing medium had the following composition (mmol l^{-1}): NaCl (100), NaOH (50), $CaCl_2$ (2), $MgCl_2$ (2), KCl (15), Tris-base (10), Hepes (10), Mes (10), glucose (5), pH adjusted with HCl or NaOH. For the choline media, NaCl, NaOH, Mes and KCl were omitted and substituted by (mmol l^{-1}): choline chloride (150), KOH (15), and the pH was adjusted with HCl. In the potassium media, NaCl and NaOH were substituted by KCl (100 mmol l^{-1}) and KOH (50 mmol l^{-1}). These media had the same osmolarity (310–320 mOsm) and were buffered to different pH values at 37°C just before use. Spermatozoa were added to this media containing the radioactive [^{14}C]methylamine and kinetics of uptake were followed as described below. No significant change in the external pH (pHe) value was observed 1 h after dilution owing to the sperm activity.

Chemicals were from Sigma (St Quentin, France), Merck (Nogent-sur-Marne, France) and Prolabo (Paris, France) in the best available grade.

Estimation of motility of spermatozoa

The sperm samples were diluted in the different media maintained at 37°C to a final concentration of 10^8 spermatozoa ml^{-1} . Percentage motility was estimated either subjectively (by visual observation under a phase contrast microscope) or objectively by laser Doppler velocimetry (Paquignon *et al.*, 1983); both methods gave results in agreement within a few per cent (Bork *et al.*, 1988). Sperm flagellar beat frequencies were measured under stroboscopic illumination (Strobex system 236, Shadwick-Helmuth, El Monte, CA); an average value was obtained by varying the frequency of the flash until the maximum proportion of the spermatozoa within the field of the microscope showed one flagellum. For motility observation, 1% BSA was added to the media to avoid sticking of spermatozoa to the slide. BSA had no effect on the pHi measurement.

Measurement of internal pH of the spermatozoa

Internal pH (pHi) of spermatozoa has previously been estimated by several methods: the spectral shift of the ^{31}P peak in NMR (Christen *et al.*, 1983a; Smith *et al.*, 1985; Robitaille *et al.*, 1987), the distribution of radioactive or fluorescent amines (Christen *et al.*, 1982; Lee *et al.*, 1983; Working and Meizel, 1983; Lee and Storey, 1985), and by fluorescence of pH-sensitive probes (Babcock, 1983; Babcock *et al.*, 1983; Vijayaraghavan *et al.*, 1985; Babcock and Pfeiffer, 1987; Tajima *et al.*, 1987; Carr and Acott, 1989; Florman *et al.*, 1989). Here, we used the distribution of radioactive amine because it presents several advantages over the other methods: (1) no preincubation and no washes of the cells were necessary, (2) this method has been demonstrated to be very reliable and accurate, and allows measurement of the internal pH over a large range of external pH values, and (3) measurement required only a few cells.

The theory of the amine distribution has been reviewed by Roos and Boron (1981) and Kotic and Slavic (1989), and a particular analysis in the case of spermatozoa is described by Christen *et al.* (1982). We calculated an internal pH value after measurement of the accumulation ratio of the amine using the following equation:

$$pHi = pHe - \log (Ar) \quad (1)$$

(Ar: ratio between internal and external amine concentration).

Because an amine equilibrates in all acidic internal compartments, the pHi measured for mammalian spermatozoa represents an average of both cytoplasmic and acrosomal pH. (The mitochondria, which are more alkaline, do not interfere (Christen *et al.*, 1982). The same problem of compartmental averaging might arise with molecules such as carboxyfluorescein derivatives, if an esterase activity is present in the acrosomal vesicle or in the mitochondria (Florman *et al.*, 1989; Parrich *et al.*, 1989) and also with ^{31}P -NMR if a low concentration of phosphate is present in one of the compartments (Robitaille *et al.*, 1987; Kotic and Slavic, 1989). Although this problem needs a more detailed

exploration, if any change occurs in one of the spermatozoa compartments the ratio of amine accumulation will be changed. Thus, even if an absolute cytoplasmic pH value cannot be directly derived, amine accumulation can detect relative pH changes and is very useful for comparing and studying the regulation of internal pH of spermatozoa.

Measurement of accumulation of radioactive methylamine

Spermatozoa were diluted 1:40 in the different medium (37°C) that contained the radioactive [^{14}C]methylamine (30 mCi mmol^{-1} from CEA, Saclay; final concentration, $1\text{--}4\text{ }\mu\text{mol l}^{-1}$). The first sampling was done immediately after dilution (about 20–30 s); thereafter aliquots were taken at intervals. Spermatozoa solution, 200 μl , was layered on 500 μl of *n*-hexane-silicone oil solution (1:10, w/v) in an Eppendorff microtube. Spermatozoa were separated from the reaction media by centrifugation for 30 s at 12 000 g; the supernatant was carefully removed and the spermatozoa pellet dissolved in scintillant (toluene scintillator, Packard, Croningen) before measurement of radioactivity. The internal cell volume was estimated by the difference between the total volume (obtained by the distribution of tritiated water) and the extracellular space of the pellet (measured with radioactive inulin, sucrose, or mannitol). With inulin and sucrose, variable results were obtained possibly because of some binding to the spermatozoa surface; mannitol seemed to be a better choice to estimate the external space (Ford and Harrison, 1983). From uptake of amine at low external pH and experiments using radioactive thiocyanate (not shown), we also estimated an external trapped volume: all data were in general agreement with a trapped volume equal to about 40% of the total water space of the pellet, and gave a volume of $4.5 \pm 2.5\text{ }\mu\text{l } 10^{-8}$ spermatozoa (mean $\pm$ SD for 14 separate experiments). Although higher than the $2.2\text{ }\mu\text{l } 10^{-8}$ spermatozoa obtained for human spermatozoa by Ford and Harrison (1983) and the $2\text{--}2.4\text{ }\mu\text{l } 10^{-8}$ spermatozoa measured for ram spermatozoa using electron spin resonance spectroscopy (Hammerstedt *et al.*, 1979), this value is close to the volume calculated from the measured size of spermatozoa. It is also in the same range as the $2.5\text{--}4.5\text{ }\mu\text{l } 10^{-8}$ spermatozoa obtained by electronic counting for both boar and ram spermatozoa (O'Donnell, 1969). The total pellet volume was estimated for each batch of spermatozoa and the internal pH calculated using equation (1).

Results

Measurement of internal pH of spermatozoa

Internal pH of boar spermatozoa. Typical curves of [^{14}C]methylamine uptake by ejaculated boar spermatozoa upon dilution in sodium media buffered at different pH values are shown (Fig. 1a). The rate of uptake of the amine is proportional to the concentration of its neutral form, whereas its total accumulation at equilibrium depends solely upon the transmembrane pH gradient (Chisten *et al.*, 1982). Thus, when the external pH was 9.0 an equilibrium was achieved less than 30 s after dilution, and an accumulation ratio (*Ar*) of about 13 was measured; this *Ar* value indicated that the cytoplasm of the

Fig. 1. Uptake of [^{14}C]methylamine and changes in internal pH of ejaculated boar spermatozoa after dilution in sodium media at different pH values. (a) Concentrated ejaculated boar spermatozoa were diluted in sodium media at different pH values (pH 9: $\blacktriangle$ — $\blacktriangle$; pH 8, $\triangle$ — $\triangle$; pH 7, $\bullet$ — $\bullet$; pH 6, $\circ$ — $\circ$) containing tracer amounts of radioactive methylamine. At the indicated time, aliquots of the solution were treated as described and radioactivity was measured. The accumulation is expressed both in c.p.m. of radioactive amine in sperm pellets and in accumulation ratio (*Ar*), which is the ratio between internal and external amine concentration. (b) Internal pH values resulting from the transformation of the *Ar* values by the equation $\text{pHi} = \text{pHe} - \log Ar$.

spermatozoa was much more acidic than the external media. An outflux of amine was observed during the following minutes, suggesting a change in the pH gradient (an alkalization of the cytoplasm). After 10–15 min, the amount of radioactivity reached a plateau (final *Ar* about 2.5) and was stable for at least 1 h (data not shown).

When the external pH was 8.0, the initial rate of methylamine uptake was lower than at pH 9, but two phases of accumulation were also observed: accumulation first reached an *Ar* value of 2.4, and thereafter a small release of amine occurred for 10 min (final *Ar* = 1.4). This result suggested that upon dilution the pH difference between external and internal media was smaller than that at pH 9 (internal pH still lower than external pH) and that a slight equilibration between internal and external pH took place thereafter.

At external pH values of 7.0 and 6.0, because of the very low concentration of neutral amine, the initial rate of uptake was small and the amounts of methylamine taken up by spermatozoa during the period of observation were very low, making it

Fig. 2. pHi as function of pHe in different ionic conditions and stages of spermatozoa maturation. (a,c): Internal pH values (reported from Tables 1 and 2) obtained 30 min after dilution of ejaculated boar (a) and ram (c) spermatozoa in choline (∇ — ∇), sodium ($\bullet$ — $\bullet$) and potassium (∇ — ∇) media buffered at different pH values. There is a linear relationship between the internal pH value reached and the external pH. (b,d): This result obtained in (a and c) was not changed by the stage of maturation: pHi values obtained in sodium media at different pH values for epididymal ($\circ$ — $\circ$), ejaculated ($\square$ — $\square$) and testicular ($\blacksquare$ — $\blacksquare$) spermatozoa of (b) boar and (d) ram spermatozoa.

difficult to obtain very precise A_r values. The fact that the accumulations slightly increased during 30 min indicated either that the equilibrium was not reached or that internal pH changed slowly.

The accumulation ratios were transformed into internal pH values of the spermatozoa (Fig. 1b). All the curves pointed toward a common origin which should correspond to the value of the pHi of the spermatozoa before dilution: in this case, the seminal plasma. From this particular set of curves, the initial internal pH appeared to be about 7.7. After dilution, at pHe 9.0 and 8.0, the internal pH increased in less than 10 min to reach a value of 8.6 and 7.8, respectively. When pHe was 7.0, internal pH decreased to a value of about 7.3; when pHe was 6.0, pHi dropped to a value of 6.6. For the reasons cited above these last pHi values should be viewed cautiously since they could be even lower if the probe equilibrium was not reached. The curves of methylamine uptake in choline and potassium media presented similar patterns (not shown).

Thirty minutes after dilution, in the three different media, the same linear relationship was observed between internal and external pH (Fig. 2a). These results indicated that the final pHi value of ejaculated spermatozoa did not depend on the presence of sodium or potassium ions in the external medium.

Internal pH of epididymal and testicular boar spermatozoa was also analysed following dilution in the same three media at

the different pHe. The presence of sodium and potassium did not affect the initial internal pH variation that occurred at dilution (not shown). The internal pH values of spermatozoa 30 min after dilution are summarized (Table 1). For the different maturation stages there was the same linear relationship between pHi and pHe and this was not influenced by sodium or potassium (Fig. 2b).

Internal pH of ram spermatozoa. Uptake of radioactive methylamine by ram spermatozoa was analysed in the same conditions used for boar spermatozoa. Methylamine accumulations by ejaculated ram spermatozoa followed kinetics similar to those obtained for boar spermatozoa (not shown). As for boar spermatozoa, the external pH but not the ionic composition of the media determined the pHi change at dilution.

The values obtained for internal pH after 30 min in the different media for ejaculated, epididymal and testicular spermatozoa are summarized (Table 2). The same linear relationship existed between internal and external pH regardless of the media (Fig. 2c) or the collection stage of the spermatozoa (Fig. 2d).

Measurement of motility and flagellar beat frequency

Percentage of motile cells and average flagellar beat frequency of the spermatozoa were measured 5 and 60 min after dilution in the different media in which internal pH was measured.

Motility of boar spermatozoa. Boar testicular spermatozoa were immotile in all our conditions of incubation (except for some flagellar twitching without propagating waves).

After collection, boar cauda epididymal spermatozoa were almost immotile in their fluid. Five minutes after dilution, spermatozoa movement was obtained in alkaline media (pHe 9), independently of the medium composition (Fig. 3a). Forward motility of spermatozoa was not induced and flagellar beating was always very irregular and asynchronous, so it was not possible to measure the average beat frequency. After 60 min large changes occurred (Fig. 3b); movements were observed in choline from pH 6 to 9, with an important increase at pH 7 and 8. In sodium media, spermatozoa were motile at pH 8, and an important decrease occurred at pH 9. In potassium, only a few spermatozoa still displayed a slow flagellar movement at pH 8.0, and no cell remained motile at pH 9. No synchronized flagellar beating and no progressive spermatozoa were observed after 1 h of incubation and spermatozoa trajectories were always highly erratic.

Ejaculated boar spermatozoa were highly motile in their seminal plasma. Five minutes after dilution, both motility and beat frequency depended on the pH of the medium and was almost unaffected by sodium or potassium (Fig. 4a, b). The percentage of spermatozoa that remained motile was only 5% at pH 5, whereas 70% were still moving at pH 7 and above. From pH 7 to 9, almost all motile spermatozoa displayed a forward motility with a flagellum beat frequency averaging 15–20 Hz. As the pH was lowered, spermatozoa presented more and more irregular flagellar movements with a decreased or non-measurable beat frequency (rated zero).

Table 1. Internal pH of boar spermatozoa in various ionic conditions

Media	pHe	Spermatozoa								
		Ejaculated				Epididymal			Testicular	
		pHi	SD	n	pHi	SD	n	pHi	SD	n
Choline	6	6.5	0.3	2	6.1	0.1	2	6.1	0.2	2
	7	7.3	0.1	4	6.4	0.1	2	6.9	0.1	2
	8	7.7	0.1	4	7.1	0.1	2	7.3	0.1	2
	9	8.4	0.2	4	8.1	0.3	2	7.7	0.1	2
Sodium	6	6.6	0.6	5	6.2	0.1	2	6.3	0.1	2
	7	7.0	0.4	5	6.5	0.1	2	6.9	0.1	2
	8	7.7	0.3	5	7.2	0.2	2	7.3	0.1	2
	9	8.3	0.3	5	8.1	0.1	2	7.8	0.1	2
Potassium	6	6.8	0.6	3	5.9	0.2	2	6.3	0.1	2
	7	7.0	0.3	3	6.3	0.1	2	6.9	0.1	2
	8	7.8	0.3	3	6.9	0.1	2	7.4	0.1	2
	9	8.3	0.4	3	7.8	0.1	2	8.4	0.2	2

Internal pH was measured 25–30 min after dilution of testicular, epididymal and ejaculated boar spermatozoa in choline, sodium or potassium media at various external pH values.

n: Number of different animals; SD: standard deviation. pHe: external pH; pHi: internal pH.

Table 2. Internal pH of ram spermatozoa in various ionic conditions

Media	pHe	Spermatozoa								
		Ejaculated				Epididymal			Testicular	
		pHi	SD	n	pHi	SD	n	pHi	SD	n
Choline	6	5.8	0.1	3	6.1	0.5	3	6.2	0.1	2
	7	6.7	0.1	3	6.5	0.3	3	6.7	0	2
	8	7.6	0.2	3	7.2	0.1	3	7.3	0.1	2
	9	8.3	0	3	8.0	0.1	3	8.1	0.1	2
Sodium	6	5.8	0.1	4	6.4	0.4	3	6.2	0.1	2
	7	6.6	0.2	4	6.6	0.3	3	6.7	0	2
	8	7.4	0.2	4	7.1	0.1	3	7.1	0	2
	9	8.3	0.3	4	7.9	0.2	3	8.2	0.1	2
Potassium	6	5.9	0.2	3	6.0	0.4	3	6.2	0.1	2
	7	6.7	0.2	3	6.6	0.1	3	6.7	0	2
	8	7.6	0.1	3	7.4	0.2	3	7.6	0.1	2
	9	8.4	0.2	3	8.1	0.1	3	8.5	0.2	2

Internal pH values obtained 25–30 min after dilution of testicular, epididymal and ejaculated ram spermatozoa in choline, sodium or potassium media at various external pH values.

n: Number of different experiments; SD: standard deviation. For ejaculated spermatozoa three experiments were done with pooled spermatozoa from two to three different animals.

After incubation for 60 min, the percentage of motile spermatozoa and the beat frequency (Fig. 4c, d) were largely reduced in media including sodium or potassium chloride, except in sodium at pH 8.0 in which 50% of spermatozoa retained their forward motility. In contrast, in choline chloride at pH 7, 8 and 9, the beat frequency and the percentage of

motile spermatozoa remained almost the same and spermatozoa exhibited progressive motility with high flagellar beat frequency.

Motility of ram spermatozoa. Like testicular boar spermatozoa, testicular ram spermatozoa remained almost immotile in

Fig. 3. Influence of the external ionic conditions on the motility of boar epididymal spermatozoa. Percentage of motility of epididymal spermatozoa was measured (a) 5 and (b) 60 min after dilution in choline ($\blacktriangledown$ — $\blacktriangledown$), sodium ($\bullet$ — $\bullet$) and potassium (∇ — ∇) media at different pH values. Two different animals were used; vertical bars indicate the standard errors.

Fig. 4. Influence of the external ionic conditions on the motility and flagellar beat frequency of boar ejaculated spermatozoa. Concentrated ejaculated spermatozoa were diluted to 10^8 spermatozoa ml^{-1} in choline ($\blacktriangledown$ — $\blacktriangledown$), sodium ($\bullet$ — $\bullet$) and potassium (∇ — ∇) media buffered at different pH values. The percentage of motile spermatozoa (a and c) and their average flagellar beat frequency (b and d) were measured (a and b) 5 and (c and d) 60 min after dilution. Spermatozoa from four different animals were used; vertical bars indicate the standard error deviation.

all conditions of incubation: only weak movements of the flagellum of some spermatozoa (5–10%) could be observed.

Epididymal ram spermatozoa showed motility in the cauda fluid after collection. Upon dilution, spermatozoa remained

Fig. 5. Motility and flagellar beat frequency of ram epididymal spermatozoa as a function of the external ionic conditions. (a and c) Percentage of motile spermatozoa and (b and d) average flagellar beat frequency were measured (a and b) 5 and (c and d) 60 min after dilution in choline ($\blacktriangledown$ — $\blacktriangledown$), sodium ($\bullet$ — $\bullet$) and potassium (∇ — ∇) media at different pH values. Means and SEM are for three different animals.

highly motile in a large range of external pH, although maximum motility was observed at pH 7 and 8 in sodium and potassium (Fig. 5a). In choline media a high percentage of motile spermatozoa was preserved at alkaline pH. Flagellar beat showed the same dependence upon pH, with an optimum at pH 7 in potassium and at about pH 8 in choline and sodium media (Fig. 5b).

Fig. 6. Influence of the external ionic conditions on the motility and flagellar beat frequency of ejaculated ram spermatozoa. (a and c) Percentage of motile spermatozoa and (b and d) average flagellar beat frequency were measured (a and b) 5 and (c and d) 60 min after dilution with choline ($\blacktriangledown$ — $\blacktriangledown$), sodium ($\bullet$ — $\bullet$) and potassium (∇ — ∇) media at different pH values. Means and SEM are for four different animals.

After incubation for 60 min, motility of epididymal spermatozoa remained the same in choline media (Fig. 5c) and increased at pH 5 in sodium and potassium media, but the beat frequency of the flagellae was still too low to produce a spermatozoa displacement (Fig. 5d). A decrease in motility occurred in potassium and sodium at pH over 7. After 1 h, in all media, the flagellar beat frequency decreased but was dependent on pH in a similar way as at 5 min.

Ejaculated spermatozoa were slightly less motile than epididymal spermatozoa but responded in a similar way. Motile spermatozoa with high beat frequency were observed upon dilution from pH 5.0 to 8.0 in all media (Fig. 6a, b). At more alkaline pH values, both the percentage of motile spermatozoa and the beat frequency were reduced in sodium and potassium media, but not in choline. In all media motility and flagellar beat decreased after incubation for 60 min. Maximum retention of motility in sodium and potassium media was at about pH 7.0 and in choline media at about 8.0 (Fig. 6c, d). The decrease was more pronounced at acidic pH. In choline media, a high percentage of spermatozoa was still motile at pH 9.0 and 10.0 after incubation for 1 h, although at pH 10 velocity of spermatozoa was strongly reduced with a speed of forward displacement related to the low beat frequency of the flagella.

Table 3. Initial internal pH of boar and ram spermatozoa

Species	Spermatozoa		
	Ejaculated	Epididymal	Testicular
Boar	7.4 ± 0.2 (3)	6.5 ± 0.2 (2)	$7.15 \pm 0.05^*$
Ram	7.1 ± 0 (2)	6.05 ± 0.15 (2)	6.8 ± 0 (2)

Internal pH values extrapolated at zero time (see Fig. 1) for boar and ram spermatozoa from testis, cauda epididymis and after ejaculation. Mean was obtained from the initial values extrapolated in the three different media. n: Numbers of different animals except * obtained on the same animal in two different experiments.

Discussion

Internal pH of spermatozoa was estimated by the radioactive methylamine distribution, which is based on the theory that cells are only permeable to the neutral form of the amine. However, certain cells were shown to be permeable to the ionized form of methylamine, and the internal pH value obtained by

methylamine was lower than that obtained either by microelectrode measurements or 5,5 dimethyl-2,4-oxazolidine-dione-2 (DMO) distribution (Boron and Roos, 1976; Roos and Boron, 1981; Monroe and Kimmich, 1986). Methylammonium is positively charged, and if the plasma membrane is permeable to this ion its flux will depend upon the value of plasma membrane potential (Boron and Roos, 1976; Monroe and Kimmich, 1986): it increases as the plasma membrane potential becomes more negative. It was also shown that, in this case, the error on pHi value increases with the size of the pH gradient, and becomes critical over a pH gradient of 2 (Boron and Roos, 1976; Monroe and Kimmich, 1986). With mammalian spermatozoa, several results indicate that methylammonium permeability is very low: (1) at low external pH when a large part of methylamine is in the ionized form (more than 99.999% of total methylamine at pH 6.0) the rate of methylamine uptake is very low; (2) this rate remains the same in media where the plasma membrane potential is very different (ChM versus KM); (3) no change in the rate of accumulation was noticed between pHe 7.0 and 6.0, although methylammonium concentration increased by a factor 10. Moreover, methylamine was previously used to measure the internal pH of the sea urchin spermatozoa upon dilution and the pHi values obtained were very similar when measured by methylamine, ethylamine, 9-aminoacridine and DMO distributions (Christen *et al.*, 1982, 1983b). We also used both methylamine and 9-aminoacridine distributions to measure the pHi of trout spermatozoa after dilution and again both amines gave similar results (Gatti, 1987). These results support the view that the internal pH values of ram and boar spermatozoa measured with methylamine were not underestimated and were meaningful for studying and comparing internal pH under different conditions.

The *in situ* pH of the fluid changes along the mammalian genital tract (Mann 1964; Cooper, 1986); for example, the pH values for the ram testicular, epididymal and ejaculated fluids are 7.1, 6.8 and 6.9, respectively (Setchell, 1970; White, 1980; Mann and Lutwak-Mann, 1981), and for the boar 7.1, 6.5 and 7.6 (Rodriguez-Martinez *et al.*, 1990). Methylamine accumulation curves were used in this study to extrapolate initial internal pH values for the spermatozoa at the time of dilution (Table 3). For both boar and ram an acidification of the cytosol of spermatozoa occurred during epididymal transit, and a rapid alkalization took place during the ejaculation process. For boar spermatozoa, the initial pHi values reported here agree with the *in situ* pH of the fluid measured *in vivo* with microelectrodes (Rodriguez-Martinez *et al.*, 1990). A study using P-NMR (Robitaille *et al.*, 1987) reported values for intracellular pH of about 7.0 ± 0.2 for ejaculated boar and ram spermatozoa which fit quite well with the value of 7.1–7.4 found here. Moreover, the variation of initial internal pH observed between cauda epididymis and ejaculated spermatozoa (from 6.5 to 7.2, respectively) that occurs in response to an increase in the surrounding pH during the ejaculation process agrees with our observations that pHi of spermatozoa can change rapidly in response to change in external pH.

Boar and ram spermatozoa at each stage of maturation altered their internal pH immediately after dilution to equilibrate with the pH value of the external media. In eukaryotic cells, intracellular pH is controlled by sodium–proton exchange or bicarbonate transport or both systems, the proton

permeability of the plasma membrane, the buffering power of the cell and the plasma membrane potential (Roos and Boron, 1981; Frelin *et al.*, 1988). The change upon dilution and the final value reached by the pHi of boar and ram spermatozoa were not highly affected by the external presence of sodium or potassium. These results rule out a role of simple mechanisms such as sodium–proton or potassium–proton exchange as pHi regulatory mechanisms. Moreover, since the plasma membrane potential of mammalian spermatozoa depends upon the potassium conductance (Rink, 1977; J.-L. Gatti, C. Chevrier, M. Paquignon and J.-L. Dacheux, unpublished results for boar and ram spermatozoa) protons are not distributed passively since in this case pHi should be very different in potassium, choline and sodium media.

After dilution, when internal pH is stabilized, we observed that for both ram and boar spermatozoa from the different parts of the genital tract, the same linear relationship existed between pHi and pHe. However, no complete equilibration between internal and external pH took place. This suggests that there must be control mechanisms for pHi, and that they are perhaps similar for the two species. Our results are different from reports that pHi of rat spermatozoa from cauda epididymis is controlled by a sodium–proton exchange similar to that found in the sea urchin spermatozoa (Wong *et al.*, 1981; Wong and Lee, 1983). The pHi of bull and ram spermatozoa can be alkalinized by changes in pHe only if external potassium is present (Babcock *et al.*, 1983); the way in which potassium acts on pHi is not clear but internal pH and calcium fluxes may be related either through Ca^{2+} – H^{+} exchange or voltage-dependent calcium channels (Babcock and Pfeiffer, 1987; Rigoni *et al.*, 1987). It is important to note that in these previous studies, the authors washed the spermatozoa and incubated them under acidic conditions before measuring the internal pH; our data indicated that this treatment induced important changes in the cytoplasmic pH of the spermatozoa. We did not attempt to detail the effect of calcium (calcium was always present in our media) on pHi regulation of boar and ram spermatozoa. We observed a small difference of about 0.2 pH unit in ram spermatozoa pHi when high or low concentration of potassium was used at alkaline pHe; but a further study will be necessary to analyse the reason for this difference.

It was demonstrated that there is an anion (HCO_3^- and SO_4^{2-}) transporter across the plasma membrane of boar spermatozoa (Okamura *et al.*, 1988). Several types of bicarbonate-transport system have been observed in eukaryotic cells; the activity of these mechanisms depends upon external and internal pH values or on the presence of sodium or on both (Frelin *et al.*, 1988). We did not include any bicarbonate in our media; the only bicarbonate present came from the dissolution of CO_2 from air and the CO_2 produced by the spermatozoa; this amount was perhaps enough to allow the bicarbonate exchange system to work. However, the addition of external bicarbonate (up to 40 mmol l^{-1}) to boar spermatozoa does not affect the internal pH value and the activity of this transport mechanism is strongly dependent upon the value of the external pH (Tajima *et al.*, 1987). It, therefore, seems unlikely that internal pH of ram and boar spermatozoa can be mainly regulated by a bicarbonate-transport system.

Lactate has been implicated as an important regulatory system for pHi of mammalian spermatozoa (Acott and Carr, 1984;

Carr *et al.*, 1985; Turner and Reich, 1985; Carr and Acott, 1989). Lactate is present at different concentrations along the genital tract: 0.6 and 0.7 mmol l⁻¹ in boar and ram testicular fluid (White, 1973) and 6.6 and 2.0 mmol l⁻¹ in ram and boar cauda epididymal fluid, respectively (Mann, 1964). The same concentration of lactate was found in the ejaculate as in the cauda epididymis (Mann, 1964). Hence, lactate may play a role in control of spermatozoa internal pH *in situ* and this could explain some of the pHi differences observed between testicular, cauda and ejaculated spermatozoa. However, in all our media the 40 times dilution of semen should produce a rapid efflux of lactate from spermatozoa (lactate pKa is 3.7), and then result in an increase of the internal pH; lactate diffusion cannot explain the acidification observed in some media at dilution. Because only small amounts of lactate are carried over into the incubation media, its contribution to the regulation of internal pH after dilution is negligible. This notion is also supported by the fact that washing the spermatozoa with a phosphate saline buffer just before the pHi measurement did not change the final pHi values found 30 min after dilution (results not shown).

We looked for a relation between pHi changes, ionic composition of the dilution media and motility of spermatozoa. For both ram and boar, under the experimental conditions used, it was not possible to activate motility of testicular spermatozoa by simple dilution. Because the different media used produced changes in the internal pH and in the plasma membrane potential, any of these factors (and even a combined effect of pHi and plasma membrane change) can be ruled out as 'simple' triggers for acquisition of motility by spermatozoa during epididymal maturation. Several studies involved an increase in internal cAMP in the acquisition of motility by caput epididymal spermatozoa and suggested that the cAMP content is controlled by the value of internal pH during the maturation process (Dacheux and Paquignon, 1980a, b; Garbers and Kopf, 1981; Hoskins *et al.*, 1983; Vijayaraghavan *et al.*, 1985). Indirectly our results suggest that the cAMP production cannot be induced by short-term changes in pHi (see also, Babcock *et al.*, 1983).

Cauda epididymal spermatozoa quiescence, depending upon the species, is believed to result in an effect of a proteinaceous immobilizing factor or a low internal pH value due to lactate accumulation or to both factors; motility is subsequently initiated by dilution of the immobilizing factor or an alkalinization due to the diffusion of lactate (Acott and Carr, 1984; Carr *et al.*, 1985; Turner and Reich, 1985; Carr and Acott, 1989). We observed that motility of epididymal boar spermatozoa (which is inhibited in the epididymal fluid) is induced upon dilution only in alkaline media independently of the presence of sodium or potassium. Since in alkaline media an internal pH increase occurred, change in pHi might play a role in this activation. This is also supported by the fact that the *in situ* pHi of spermatozoa in cauda epididymidis is about 6.4 and after dilution this internal pH value was inhibitory for motility even for ejaculated spermatozoa. At ejaculation, an increase in pHi was observed suggesting that activation of motility that takes place at ejaculation in boar may involve a pHi change, although some other factors (such as the presence of bicarbonate) are also necessary for a full activation (Tajima *et al.*, 1987). This situation looked similar to that found for spermatozoa of bovine and some other species, in which lactate could act as a reversible inhibitor of motility by lowering internal pH (Babcock *et al.*,

1983; Carr and Acott, 1989). In contrast, ram epididymal spermatozoa that were already motile in the epididymal fluid remained highly motile (with a forward motility) in a large range of external pH, although their pHi increased or decreased. This shows that there are some important functional differences between the spermatozoa of these two species.

External pH could affect motility of spermatozoa directly by its effect on the pHi value. This contention is suggested by the variations in flagellar beat frequency. It was demonstrated that the flagellar apparatus is sensitive to pH and that there are complex relations between axoneme, cAMP and pH (Ishijima and Witman, 1987; Gotz *et al.*, 1988). The activity of several key enzymes of metabolism and of some of the Ca²⁺-binding proteins depends upon pH. Moreover, increasing or decreasing the external pH can act on the other ionic permeabilities of the plasma membrane and also on the availability of metabolizable substrates for the cell via the inhibition of metabolite membrane transporters (Ballesteros *et al.*, 1983).

We observed that a large extracellular sodium or potassium concentration is not absolutely required for the initiation of motility of spermatozoa, and can even have a negative effect on motility preservation; this is especially true at alkaline pHe. The positive effect of choline on the conservation of motility is striking and no clear explanation can be found. Choline is present in the male genital tract where it can serve as a precursor for phosphorylcholine, phosphatidylcholine and glycerophosphorylcholine (Mann, 1964; Cooper, 1986) and may play the role of an osmolytic protecting compound.

In conclusion, this study showed that pHi of boar and ram spermatozoa varied *in situ* during epididymal maturation. No short-term effect of this pHi change on the acquisition of motility can be seen, although an inhibitory action on the acquired motility of boar spermatozoa was suggested. Internal pH of boar and ram spermatozoa changed immediately upon dilution by a similar mechanism which was unaffected by epididymal maturation and did not depend upon sodium or potassium. This or these mechanisms are unknown. Large concentrations of sodium and potassium are not necessary for maintaining acquired motility in boar and ram spermatozoa and further study will be necessary to detail the regulatory mechanism of pHi.

This work was initiated during the Thesis of J-L. Gatti under the direction of R. Christen, URA 671, CNRS, Villefranche-sur-mer, France. This work was supported by the Centre National de la Recherche Scientifique, and by grants from the Institut National de la Recherche Agronomique, the Institut National de la Santé et de la Recherche Médicale (contrat de recherches externes 834005 and 894004). Part of these results was presented at the VI International Congress on Spermatology held in Siena, Italy, 1990 and published as an abstract (Gatti *et al.*, 1992).

References

- Acott TS and Carr DW (1984) Inhibition of bovine spermatozoa by cauda epididymal fluid: II. Interaction of pH and a quiescence factor *Biology of Reproduction* **30** 926-935
- Babcock DF (1983) Examination of the intracellular ionic environment and of ionophore action by null point measurement employing the fluoresceine chromophore *Journal of Biological Chemistry* **258** 6380-6389

- Babcock DF and Pfeiffer DR (1987) Independent elevation of cytosolic $[Ca^{++}]$ and pH of mammalian sperm by voltage-dependent and pH-sensitive mechanisms *Journal of Biological Chemistry* **262**, 15041–15047
- Babcock DF, Rufo GA and Lardy HA (1983) Potassium-dependent increases in cytosolic pH stimulate metabolism and motility of mammalian sperm *Proceedings of the National Academy of Sciences USA* **50** 1327–1331
- Ballesteros LM, Delgado NM, Rosado A and Hernandez O (1983) Effect of steroid hormones on membrane sugar transport in human spermatozoa *Archives of Andrology* **11** 95–100
- Bork K, Chevrier C, Paquignon M, Jouannet P and Dacheux J-L (1988) Analyse de la motilité et du mouvement flagellaire des spermatozoïdes de verrat au cours du transit épididymaire *Reproduction, Nutrition, Développement* **28**, 1307–1315
- Boron WF and Roos A (1976) Comparison of microelectrode, DMO, and methylamine methods for measuring intracellular pH *American Journal of Physiology* **231** 799–809
- Busa WB and Nuccitelli R (1984) Metabolic regulation via intracellular pH *American Journal of Physiology* **246** R409–R438
- Carr DW and Acott TS (1989) Intracellular pH regulates bovine sperm motility and protein phosphorylation *Biology of Reproduction* **41** 907–920
- Carr DW, Usselman MC and Acott TS (1985) Effect of pH, lactate, and viscoelastic drag on sperm motility: a species comparison *Biology of Reproduction* **30** 913–925
- Christen R, Shackmann RW and Shapiro BM (1982) Elevation of the intracellular pH activates respiration and motility of sperm of the sea urchin, *Strongylocentrotus purpuratus* *Journal of Biological Chemistry* **257** 14 881–14 890
- Christen R., Shackmann RW, Dalquist FW and Shapiro BM (1983a) ^{31}P -NMR analysis of sea urchin sperm activation *Experimental Cell Research* **149** 289–294
- Christen R, Shackmann RW and Shapiro BM (1983b) Metabolism of sea urchin sperm: interrelationships between intracellular pH, ATPase activity and mitochondrial respiration *Journal of Biological Chemistry* **258** 5392–5399
- Chulavatnol M and Yindepit S (1976) Change in surface ATPase of rat spermatozoa in transit from the caput to the cauda epididymis *Journal of Reproduction and Fertility* **48** 91–97
- Cooper TG (1986) *The Epididymis Sperm Maturation and Fertilization*. Springer-Verlag, Berlin.
- Dacheux J-L and Paquignon M (1980a) Relations between the fertilizing ability, motility and metabolism of epididymal spermatozoa *Reproduction, Nutrition, Development* **20** 1085–1099
- Dacheux J-L and Paquignon M (1980b) Effect of caffeine on ram and boar spermatozoa. Influence of their stage of maturation and the medium; initiation of progressive motility of testicular spermatozoa. In *Testicular Development, Structure and Function* pp 513–522 Eds E Steinberger and A Steinberger. Raven Press, New York
- Edwards E, Dacheux J-L and Waites GMH (1976) Effect of α -chlorhydrin on the metabolism of testicular and epididymal spermatozoa of the ram *Journal of Reproduction and Fertility* **48** 265–270
- Florman HM, Tombes RM, First NL and Babcock DF (1989) An adhesion-associated agonist from the zona pellucida activates G protein-promoted elevation of internal Ca^{2+} and pH that mediates mammalian sperm acrosomal exocytosis *Developmental Biology* **135** 133–146
- Ford WCL and Harrison A (1983) D-(1- C^{14})-mannitol and (U- C^{14})-sucrose as extracellular space markers for human spermatozoa and the uptake of 2-deoxyglucose *Journal of Reproduction and Fertility* **69** 479–487
- Frelin C, Vigne P, Ladoux A and Lazdunski M (1988) The regulation of the internal pH in cells from vertebrates *European Journal of Biochemistry* **174** 3–14
- Garbers DL and Kopf GS (1980) The regulation of spermatozoa by calcium and cyclic nucleotides *Advances in Cyclic Nucleotide Research* **13** 251–306
- Gatti J-L (1987) Rôle du pH Interne et du Potentiel de Membrane dans l'Activation de la Mobilité et du Métabolisme des Spermatozoïdes. Thèse de doctorat en Science de la Vie, Université de Nice
- Gatti J-L, Chevrier C, Paquignon M, Dacheux J-L and Christen R (1992) Relation between internal pH (pHi) and motility of ram and boar spermatozoa. In *Comparative Spermatology 20 years after* pp 369–372 Ed. B Baccetti. Raven Press, New York
- Gotz JS, Gardner TK, Kanous KS and Lindemann CB (1988) The interaction of pH and cyclic adenosine 3',5'-monophosphate on activation of motility in Triton X-100 extracted bull sperm *Biology of Reproduction* **39** 1129–1136
- Hammerstedt RH, Keith AD, Hay S, Deluca N and Amann R (1979) Change in ram sperm membranes during epididymal transit *Archives of Biochemistry and Biophysics* **196**, 7–12
- Hiiipakka RA and Hammerstedt RH (1978) Changes in 2-deoxyglucose transport during epididymal maturation of ram sperm *Biology of Reproduction* **19** 1030–1035
- Hoskins DD, Acott TS, Critchlow L and Vijayaraghavan S (1983) Studies on the role of cyclic AMP and calcium in the development of bovine sperm motility *Journal of Submicroscopy and Cytology* **15** 21–27
- Ishijima S and Witman GB (1987) Flagellar movement of intact and demembrated, reactivated ram spermatozoa *Cell Motility and Cytoskeleton* **8** 375–391
- Kotic A and Slavic J (1989) *Intracellular pH and its Measurement*. CRC Press Inc, Boca Raton
- Lardy H and San Agustin J (1989) Caltrin and calcium regulation of sperm activity. In *The Cell Biology of Fertilization* pp 29–39 Eds H Schatten and G Schatten. Academic Press, New York
- Lee MA and Storey BT (1985) Evidence of the plasma membrane impermeability to small ions in acrosome-intact mouse spermatozoa bound to mouse zonae pellucidae, using an aminoacridine fluorescent probe: time course of the zona-induced acrosome reaction monitored by both chlortetracycline and pH probe fluorescence *Biology of Reproduction* **33** 235–246
- Lee HC, Johnson CH and Epel D (1983) Changes in internal pH associated with initiation of fertility and acrosome reaction of sea urchin sperm *Developmental Biology* **95** 31–45
- Mann T (1964) *The Biochemistry of Semen and of the Male Reproductive Tract*. John Wiley and Sons, New York
- Mann T and Lutwak-Mann C (1981) *Male Reproductive Function and Semen*. Springer-Verlag, Berlin
- Monrose MH and Kimmich GA (1986) Quantitative use of weak bases for estimation of cellular pH gradients *American Journal of Physiology* **250** C418–C422
- O'Donnell JM (1969) Electronic counting and sizing of mammalian spermatozoa and cytoplasmic droplets *Journal of Reproduction and Fertility* **19** 263–272
- Okamura N, Tajima Y and Sugita Y (1988) Decrease in bicarbonate transport activities during epididymal maturation of porcine sperm *Biochemistry and Biophysical Research Communications* **3** 1280–1287
- Paquignon M, Dacheux JL, Jeulin C and Fauquenot A (1983) Laser light scattering study of spermatozoa motility of domestic animals. In *The Sperm Cell* pp 332–335 Ed. J Andre. Martinus Nijhoff, The Hague
- Parrish JJ, Susko-Parrish JL and First NL (1989) Capacitation of bovine sperm by heparin: inhibitory effect of glucose and role of internal pH *Biology of Reproduction* **41** 683–689
- Rigoni F, Dell'antone P and Deana R (1987) Evidence for a dpH-driven Ca^{2+} uptake in EGTA-treated bovine spermatozoa *European Journal of Biochemistry* **169** 417–422
- Rink TJ (1977) Membrane potential of guinea pig spermatozoa *Journal of Reproduction and Fertility* **51** 155–157
- Robitaille PM, Robitaille PA, Martin PA and Brown GG (1987) Phosphorus-31 nuclear magnetic resonance studies of spermatozoa from the boar, ram, goat and bull *Comparative Biochemistry and Physiology* **87B** 285–296
- Rodriguez-Martinez H, Ekstedt E and Einarsson S (1990) Acidification of epididymal fluid in the boar *International Journal of Andrology* **13** 238–243
- Roos A and Boron WF (1981) Intracellular pH *Physiological Reviews* **61** 296–434
- Setchell BP (1970) Testicular blood supply, lymphatic drainage and secretion of fluid. In *The Testis* pp 101–221 Eds AD Johnson, WR Gomes and NL Vandemark. Academic Press, New York
- Shapiro BM, Schackmann RW, Tombes RM and Kazazoglou T (1985) Coupled ionic and enzymatic regulation of sperm behavior *Current Topics in Cell Regulation* **26** 97–113
- Smith MB, Babcock DF and Lardy HA (1985) A ^{31}P -NMR study of the epididymis and the epididymal sperm of the bull and hamster *Biology of Reproduction* **33** 1029–1040
- Tajima Y, Okamura N and Sugita Y (1987) The activating effect of bicarbonate on sperm motility and respiration at ejaculation *Biochimica et Biophysica Acta* **924** 519–529
- Turner TT and Reich GW (1985) Cauda epididymal sperm motility: comparison among five species *Biology of Reproduction* **32** 120–128
- Vijayaraghavan S, Critchlow LM and Hoskins DD (1985) Evidence for a role for cellular alkalization in the cyclic adenosine 3',5'-monophosphate-mediated initiation of motility in bovine caput spermatozoa *Biology of Reproduction* **32** 489–500

- Volgmayr JK, Scott TW and Setchell BP** (1967) Metabolism of testicular fluid collected from conscious rams *Journal of Reproduction and Fertility* **14** 87–99
- White IG** (1973) Biochemical aspects of spermatozoa and their environment in the male reproductive tract *Journal of Reproduction and Fertility Supplement* **18** 225–235
- White IG** (1980) Secretion of the male reproductive tract and seminal plasma. In *Reproduction in Farm Animals* pp 189–202 Ed. ESE Hafez. Lea and Febiger, Philadelphia
- Wong PYD and Lee WM** (1983) Potassium movement during sodium-induced motility initiation in the rat caudal epididymal spermatozoa *Biology of Reproduction* **28** 206–212
- Wong PYD, Lee WM and Tsang AYF** (1981) The effects of extracellular sodium on acid release and motility initiation in rat caudal epididymal spermatozoa *in vitro* *Experimental Cell Research* **131** 97–104
- Working PK and Meizel S** (1983) Correlation of increased, intra-acrosomal pH with the hamster sperm acrosome reaction *Journal of Experimental Zoology* **227** 97–107
- Yanagimachi R** (1981) Mechanism of fertilization in mammals. In *Fertilization and Embryonic Development in-vitro* pp 81–182 Eds L Mastroianni Jr and JD Biggers. Plenum Press, New York
- Zimmerman KJ, Crabo BG, Moore R, Weisberg S, Deibel FC and Graham EF** (1979) Movement of sodium and potassium into epididymal boar spermatozoa *Biology of Reproduction* **21** 173–179