The internal pH (pHi) of sperm of the sea urchin, *Strongylocentrotus purpuratus*, was estimated by measuring the accumulation of the weak bases [14C]methylamine, [14C]diethylamine, or 9-aminoacridine under conditions where cellular respiration was activated or inhibited. When 9-aminoacridine fluorescence measurements are corrected for binding to intracellular components, the pHi estimates agree quantitatively with those obtained from [14C]amine distributions. The pHi of sperm decreased when the intracellular [K⁺] was elevated above the physiological value of 10 mM, or when the external pH was substantially decreased below the physiological 8.0, or when Na⁺ was absent from the seawater. At the decreased pHi values, sperm respiration and motility were inhibited; conversely, both respiration and motility increased when the pHi was elevated. Increased respiration occurred whether the pHi was increased by altering the external pH, [K⁺] or [Na⁺], or by the addition of NH₄Cl to the medium. In all cases, the activation of respiration and motility were linked, suggesting a unitary control mechanism, some possibilities for which are presented.

Sperm are cells that face markedly different demands at distinct portions of their lives. For example, sea urchin sperm remain quiescent in the testes for months; immediately upon spawning (and dilution) into seawater, they respire and swim at a maximal rate (30). Upon encountering a sea urchin egg, the sperm undergo the acrosome reaction (exocytosis from an apical vesicle) that allows them to bind and fuse with the homologous egg (reviewed in Ref. 1). These rapid alterations in activity that follow their dilution in the external medium require a coordination of metabolism with external demands. The mechanism of this coordination is not well understood. Sea urchin sperm provide several advantages in studies of the biochemical basis of such metabolic coordination. They can be obtained in large quantities and induced to undergo activation (increased respiration and motility) and the acrosome reaction synchronously and within seconds. Additionally, sperm are relatively simple cells, devoid of the complex machinery to replicate DNA or synthesize proteins.

The activation of respiration and motility of sperm that occurs upon dilution is sensitive to the ionic composition of seawater. For example, sperm that are diluted into seawater of low pH or elevated K⁺ are immotile (3-8). Na⁺ is required for initiation of motility (2), and the acrosome reaction is associated with 22Na⁺ uptake and H⁺ efflux (9). At a seawater pH of 6.0 (in contrast to the normal 8.0) the respiration of sea urchin sperm is inhibited (10-12) as are the acrosome reaction (e.g. see Ref. 1) and motility. At low external pH, sperm motility can be initiated in the presence of an egg peptide (12) if Na⁺ is present.

Na⁺-dependent H⁺ efflux could lead to an increased pHi, which might act as an intracellular messenger to regulate the activation response of sperm. In a previous study (17), we found that the intracellular pH of sperm was acidic with respect to seawater. In the present study, medium estimations of the pH of sperm diluted into media of ionic compositions that either permit or inhibit sperm activation. These studies utilize the ability of weak acids or bases to traverse membranes in their uncharged forms and to accumulate intracellularly according to pH gradients (13-16). We show in this study that increased respiration and motility are associated with an increase in pH, and suggest how both could be coordinately regulated.

**MATERIALS AND METHODS**

**Collection of Gametes**—Spermatozoa from the sea urchin, *Strongylocentrotus purpuratus*, were obtained by intracoelomic injection of 0.5 M KCl; they were collected as "dry" (undiluted) sperm (2-6 x 10^10 sperm/ml) and kept on ice. For experiments at low Na⁺ concentrations, unless otherwise indicated, sperm were washed by 200-500-fold dilution into Na⁻-free medium (ChSW) and centrifugation (Sorvall, SS34, 10 min, 1000 x g); the pellet was resuspended in ChSW. Dry sperm from the starfish, *Pisaster ochraceus*, were obtained by dissection of the gonads. All experiments were performed between 10 and 12 °C except where noted.

**Media**—ASW was of the following composition: 360 mM NaCl, 50 mM MgCl₂, 10 mM CaCl₂, 10 mM KCl, 30 mM 4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid, pH 8.0. In some experiments the buffer was 12.5 mM Tris and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. For ChSW, three times recrystallized choline chloride was used instead of NaCl; fresh solutions were used. The pH was adjusted with KOH and Tris base so that K⁺ was 10 mM. When K⁺ was varied, K⁺ was substituted for Na⁺ in ASW, so that [K⁺] + [Na⁺] = 370 mM; Ca⁺-free medium was made by omitting Ca²⁺ from ASW; 1 mM EGTA was added as noted in the figures and the pH was readjusted.

**Sperm Respiration**—Respiration rates were determined by continuous recording with a Clark oxygen electrode. A 100-fold dilution of dry sperm was made into 5-mL medium, unless indicated otherwise. The respiratory rates in ASW were constant until about 80% of the O₂ was depleted from the medium and measurements were made only up to this point. In long term experiments, the medium was periodically reoxygenated by bubbling air through it. A 100% reading in the figures is for medium in equilibrium with air at the indicated temperature.

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*The abbreviations used are: pHi, intracellular pH; Et,NH, diethylamine; 9-AA, 9-aminoacridine; DMO, dimethyloxalondine-2,4-dione; pHₑ, extracellular pH; Ar, accumulation ratio; ASW, artificial seawater; ChSW, choline-substituted for Na⁺ in artificial seawater; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid KSW, K⁺ substituted for Na⁺ in artificial seawater.*
Intracellular pH and Respiration

Sperm Motility—Estimates of motility were obtained by microscopic observation of a thick droplet of sperm by darkfield microscopy. Only sperm in liquid suspension (i.e. those not close to the surface of the slide) were examined, since swimming artifacts occur in the vicinity of glass surfaces. Sperm motility was scored qualitatively in five categories from completely immotility (0) to full activity (4).

Results of Quantitative Assays—Dry sperm were diluted 100-fold into media containing radioactive EtNH₃ or DMO (17). [¹⁴C] Diethylamine (65.5 mCi/mmol) from ICN was used at a final concentration of 2.0-10⁻⁴ μM and DMO at 2-4 μM. Separation of the sperm from the reaction medium was by centrifugation through silicone oil (General Electric Versamid F-50) in an Eppendorf microfuge for 30-45 s at full speed. Extracellular and total medium-water spaces were determined with tritiated water and [¹⁴C]ulin in sucrose, as previously described (45). The average value for sperm intracellular water space in 25 determinations was 0.71 ± 0.04 (S.E.) μl/10⁵ sperm and was nearly the same for either Ca²⁺-free ASW or for ChSW; pH, = pH, - log₂₀₂(X), where X is the ratio of internal to external amine concentration (17). EtNH₃ was used because it reached an equilibrium level more rapidly than methyamine did. For example, when [¹⁴C]EtNH₃ was added 30 min after dilution of sperm into Ca⁺⁺-free ASW, a constant Ar was achieved with a t₁/₂ of 45 s; the final Ar was identical with that found when sperm were incubated in [¹⁴C]EtNH₃ from the time of dilution and sampled at 35 min. Thus, changes in the Ar for [¹⁴C]EtNH₃ that occurred during the first hour of sperm dilution at pH 8.0 (e.g. Fig. 3) reflect changes in pH, and not a slow diffusion of the amine.

The uptake of [¹⁴C]DMO was performed analogously to that for EtNH₃ (17).

Accumulation of Fluorescent Amines—For continuous monitoring of amine accumulation in a sample, we followed the quenching of fluorescence of 9-AA as it is taken up by cells (e.g. Ref. 13). Measurements were performed with a Perkin Elmer MFP 44A fluorescence spectrophotometer, with excitation of 382 nm and emission at 454 nm. With starfish sperm, measurements were made with an Aminco Bowman Fluorometer (380 nm excitation, 440 nm emission). In order to test whether any of the 9-AA fluorescence that remains after quenching in the suspension is from sperm which have accumulated the dye, the fluorescence was measured before and after removal of the sperm by centrifugation. Under all conditions, the fluorescence of the supernatant was within 5% of that for the sperm suspension. This demonstrates that uptake of 9-AA almost completely quenches its fluorescence. It follows that the fluorescence serves as a measure of the extracellular 9-AA remaining in solution. Over the range of concentrations used in these experiments, the fluorescence of the solution was directly proportional to the 9-AA concentration and inversely proportional to the sperm concentration.

Binding of 9-AA to intracellular components can complicate quantitative pH measurements (13). However, in sperm there is an excess of 9-AA binding sites, and thus the ratio of bound to free intracellular 9-AA is nearly constant over the range of 9-AA concentrations used (see Appendix 3). The binding can therefore enhance the quenching due to accumulation in response to the pH gradient. Calculation of the intracellular pH with 9-AA is given by Equation 12 of Appendix 3:

\[ pH = pH_e - \log\left(\frac{Q}{1 - Q'} \times \frac{1 - Q'}{1 - Q}\right) \]

where Q is the fraction of fluorescence quenched in intact sperm and Q' is the fraction quenched when the ΔpH is collapsed by either Trion X-100 or the iophanes nigerin or monensin. For example, for the curve in Fig. 1B at pH, = 7.0, Q' is 0.25 and Q is 0.73. These values give pH, = 7.0 (Table II). Q' is not dependent upon the pH or the ionic compositions of seawaters used. With corrections for binding, quantitative equivalent pH, values were obtained with 9-AA and EtNH₃, but the final equilibrium was reached much more slowly: with 9-AA the processes took 20-30 min at pH, = 8.0 and about 4 h at pH, = 6.9 (see Fig. 1B). This slow uptake precluded use of 9-AA for kinetic analyses, but did permit estimates of equilibrium values of pH, to compare with data using [¹⁴C]EtNH₃.

RESULTS

Sea urchin sperm accumulate radioactive and fluorescent amines (17). The extent of accumulation is a function of the external and internal pH. The Ar may be used to calculate the pH, when the size of the internal acidic compartment is estimated, provided that the accumulation is due only to the transmembrane pH gradient and not to binding (see “Discussion”). Amines are more useful for estimates of pH, than weak acids like DMO, since DMO concentrates within the basic mitochondrion. We may treat the sperm as a two-compartment system, with the mitochondrion as one compartment and the remainder of the cell as the second, in order to estimate the mitochondrial uptake of each probe. Using the previously obtained (17) Ar for DMO (0.45) and EtNH₃ (5.0), it can be calculated that 80% of the DMO is within the mitochondrion, whereas only 5% of the EtNH₃ is intramitochondrial (see Appendix 1). These data suggest that amines are reasonable indicators of the average cytoplasmic pH, as long as there is no significant accumulation in acidic organelles. The acrosomal granule may be such an acidic organelle (17, 44), but because of its extremely small size (<0.06% of the cell volume, as calculated from electron micrographs and water volume measurements) EtNH₃ accumulation there would be only a small fraction of the total.

Regulation of the pH, of Sperm by Seawater Ionic Composition—The pH, of sperm was affected by the ionic composition of the seawater. As shown in Tables I and II, the pH, and Na⁺ concentration both influenced the pH, in the normal 360 mM Na⁺, a decrease in pH, from 8 to 7 caused a change of only a few tenths in pH, (Tables I and II); whereas lower pH, led to further decreases in pH, (Tables I and II); whereas lower pH, led to further decreases in pH, (Tables I and II). The greater stability of pH, in the presence of Na⁺ at pH, = 7.8 was also observed by measurement of 9-AA accumulation as described in Fig. 1A. Notice that the quenching (uptake) of the amine was greatest at pH, = 8.3 and least at pH, = 6.9, indicating a decrease in trans-

| Seawater composition | pH, | Ar, pH, | Ar, pH, |
|----------------------|-----|---------|---------|
| ASW                  | 5.0 | 0.22    | 0.22    |
| ASW                  | 6.0 | 0.31    | 0.38    |
| ASW                  | 7.0 | 0.95    | 0.95    |
| ASW                  | 8.0 | 6.3     | 4.3     |

TABLE I

Influence of external ionic composition on internal pH

Dry sperm were diluted 100-fold into the indicated seawater at the specified pH, and incubated with [¹⁴C]EtNH₃ for 25 min; for Experiment 3, sperm were first washed in the same medium. Separation of the sperm from the reaction media and determination of the Ar and pH, were performed as under “Materials and Methods.” Both Ar and pH, values are illustrated to indicate the size of change in the measured value (Ar) that can occur with relatively minor effect on the calculated pH, since the Ar reflects the ΔpH across the membrane.
membrane ΔpH as the pH, was decreased. The calculated pH, values corresponding to these data are shown in Table II, under "ASW."

In the absence of Na+ (<0.5 mM) lower values of pH, were found at each pH, even at the normal pH of seawater, 7.9–8.0 (Tables I and II). This is shown in Fig. 1B, where approximately the same amount of uptake of 9-AA occurred at all pH, from 6.9–8.3, indicating that a relatively stable transmembrane ΔpH existed over this range. The calculated pH, values that correspond to each pH, are shown in Table II, under "ChSW."

Fig. 1B shows that in the absence of Na+, the kinetics of amine penetration are slower at a more acidic pH. This is because the penetration of an amine is a passive diffusion phenomenon, so that the unidirectional influx of the amine (equal to the initial rate of influx) is proportional to the external concentration of the diffusing species, that is, the nonprotonated form of the amine (see Fig. 4 of the Miniprint, Appendix 3). Since this concentration decreases at more acidic external pH, the initial rate of uptake is lower when the external pH is more acidic. In the presence of Na+, the total amount of amine taken up by the sperm is reduced at more acidic pH, so that although the rate of penetration is slower, the plateau is reached in a short period of time. Without sodium, the same amount of amine is accumulated at equilibrium, whatever the pH, is. Since the unidirectional flux of amine entering the cells is decreased at a more acidic pH, it takes a much longer time to achieve equilibrium (see Fig. 1 and Appendix 2 in the Miniprint). In the absence of Na+, [14C]Et2NH uptake is also slower at pH 7 than at pH 8 (not shown), but reaches an apparent equilibrium in approximately 1 h; this level is maintained for at least 3 h (<0.1 pH change) which indicates the slow accumulation of 9-AA is not due to gradual alterations of pH. The requirement for extracellular Na+ in order to maintain a relatively constant pH, has been seen in other cells (19–23). In sea urchin sperm at pH, = 8, the pH, increased with increasing [Na+] in the seawater up to approximately 100 mM Na+ (Fig. 2), after which a relatively constant pH, was maintained. Unlike Na+, Ca2+ had only a small influence upon pH, (Table I).

The K+ concentration in seawater had a significant effect on the final value of pH, as well as on the kinetics of the intracellular pH change upon sperm dilution (Fig. 3). Sperm that were diluted into ASW containing normal K+ (10 mM) maintained a pH, of approximately 7.5. When they were diluted into seawater with an increased K+ concentration (50 mM), a transient internal acidification was seen, followed by an adjustment toward pH 7.5. The alterations in the accumulation ratio for Et2NH indicated a time-dependent alteration in pH, that occurred following sperm dilution, for they occurred on a longer time scale than that needed for Et2NH diffusion (see "Materials and Methods"). Because it binds within the sperm, 9-AA takes a much longer time to reach equilibrium (see Fig. 1 and Appendix 2 in the Miniprint) and cannot be used for rapid determination of internal pH. At high K+ concentrations (200 and 370 mM, Fig. 3) the sperm reached an even more acidic pH, which did not revert to an alkaline value.

**TABLE II**

**Effect of Na+ on the regulation of pH, as determined by 9-AA accumulation**

Sperm were incubated as described in Fig. 1 until an apparent equilibrium was reached. The pH, was calculated after correction for binding as described in the Miniprint. No Ca2+ was present in these seawaters, which also included 1 mM EGTA.

| pH, | ASW* | ChSW* |
|-----|------|-------|
| 8.3 | 7.5  | 7.0   |
| 7.9 | 7.4  | 7.0   |
| 7.4 | 7.3  | 6.5   |
| 6.9 | 7.3  | 6.1   |

* Na+ = 360 mM.  
* Na+ < 0.3 mM.

**Fig. 1. Dependence of pH, on extracellular [Na+].** Dry sperm were diluted 500-fold into seawater containing 360 mM sodium (A), or Na+ free ChSW (B), each with 2.5 μM 9-AA. Ca2+ was absent from both media which also contained 1 mM EGTA. The decrease in fluorescence (due to the uptake and quenching of the dye, see "Materials and Methods") was followed with time at each external pH.

**Fig. 2. Effect of [Na+] on pH, and respiratory rate.** Dry sperm were diluted 100-fold into artificial seawater with increasing concentrations of Na+ at pH 8.2. The pH, (C) estimated by diethylamine uptake and the respiratory rates (B) were both determined at 8 min after dilution. Inset, a replot of the data to show the respiratory rate at each value of pH.
at acidic pH, was not due to sperm death. Rather, the inhibition of respiration correlated with a decrease in pHi (e.g., Figs. 1 and 2; Tables I and II).

When sperm were diluted into Na⁺-free seawater, their respiration was suppressed, and addition of increasing concentrations of Na⁺ led to increased respiration (Figs. 2 and 4B). The increased respiratory rate correlated with an internal alkalinization (Fig. 2). As previously reported (2), sperm are immotile in Na⁺-free seawater; motility was activated by concentrations of Na⁺ similar to those that activate respiration. In our experiments both processes were activated together, usually at some pH between 7.0 and 7.5, although the estimated pH value for activation varied from one batch of sperm to another.

When sperm were diluted into high concentrations of K⁺, respiration was inhibited even if Na⁺ was present (Fig. 4C). At intermediate concentrations of K⁺ (25 and 50 mM), respiration began, but only after a delay which increased with increasing K⁺ concentration (Fig. 4C). When the results of Fig. 4C were compared with those of Fig. 3, an interesting correlation was found. Respiration was inhibited whenever the pH was acidic and activation of respiration correlated with intracellular alkalinization. Thus, during the transient phase of acidic pH, found in 25–50 mM K⁺, respiration was transiently suppressed.

Extracellular Na⁺ and pH appear to act synergistically to

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**Fig. 3. Effects of potassium on pH.** Dry sperm were diluted 100-fold into ASW containing K⁺ as indicated and [¹⁴C]Et₂NH, and the accumulation ratio of the amine (Ar) was determined as under "Materials and Methods" as a function of time. ▲ 10 mM K⁺; ▼ 50 mM K⁺; ▲ 200 mM K⁺; ▲ 370 mM K⁺. After 120 min of incubation, NH₄Cl was added (final concentration 25 mM) and the accumulation ratio was measured 5 min later. The internal pH as calculated from the Ar values is shown on the right scale of the figure.

**Fig. 4. Sperm respiration in media of differing composition.** A, effect of pH on respiration. Dry sperm were diluted 100-fold into ASW adjusted to different pH values and the concentration of oxygen was measured with an oxygen electrode as described under "Materials and Methods." The pH of each sample is shown above the curve. B, effect of Na⁺ and NH₄⁺ on sperm respiration. Dry sperm were diluted 200-fold into ChSW at pH 8.0, conditions where respiration was not initiated, or into the media of defined Na⁺ or NH₄⁺ concentrations as shown above each curve. C, effect of extracellular [K⁺] on respiration. Dry sperm were diluted 200-fold into seawater at pH 8.2 of varying [K⁺]. K⁺ was substituted 1:1 for Na⁺ so that [K⁺] + [Na⁺] = 370 mM. All abscissas are given as time in minutes.
activate respiration as shown in Table III, where the effect of changing the external pH at various extracellular [Na'] was examined. Sperm respiration was triggered if the external pH was raised to a sufficient value, and the external pH required to trigger respiration was lower at higher external concentrations of Na'. For example, similar respiratory rates were observed at pH 9 and <0.3 mM Na', pH 8 and 10 mM Na', and pH 7 and 30 mM Na'.

**TABLE III**

**O2 consumption as a function of pH, and Na' concentration**

Dry sperm (30 µl) were diluted into 5 ml of Ca²⁺ and Na⁺ free seawater (1 mM EGTA), with different concentrations of NaCl and adjusted to different pH with Tris base or HCl. Respiratory rates were measured at 15 min.

| External Na' (mM) | pH 6 | pH 7 | pH 8 | pH 9 |
|-------------------|-----|-----|-----|-----|
| 0                 | 0.5 | 0.5 | 0.5 | 10.9 |
| 1                 | 0.5 | 6.1 |     |     |
| 10                | 1.4 | 9.2 |     |     |
| 50                | 2.7 | 10.0| 10.8|     |

**DISCUSSION**

In this study, we found that the sperm pH was more acidic when the extracellular K⁺ was increased, or when no Na⁺ was present in the dilution medium, or when the external pH was decreased. A decreased pH has been found in another species of sea urchin sperm by 31P-NMR when the pH, is decreased or external K⁺ is increased (28). Changes in the internal pH of the sperm might affect the acrosomal reaction (17, 39), their viability (27), and also their respiration and motility (2, 26, 38, 46). In this paper, we found a good correlation between increased pH and activation of sperm respiration and motility, whether the pH was altered by changing the extracellular K⁺, Na⁺, or pH, or by addition of NH₄Cl. Similar results on the correlation between motility and pH have been found by Lee et al. (26, 46) when sea urchin sperm were diluted in a sodium free medium, whereas other invertebrate sperm may control motility independently of pH (37).

The inhibition of both sperm respiration and motility by low pH could be caused by independent effects on the two processes, or by inhibition of one component of a tightly linked reaction. Motility of the sea urchin sperm is effected by the dynein ATPase of the flagellar axoneme (31, 32). Thus, motility and respiration have the potential for being linked, since the ATP generated by respiration is used for motility.
and the ADP so produced is required for respiration of tightly coupled mitochondria. If motility and respiration were tightly linked and axonemal movement was inhibited by low pH, both motility and respiration would cease. In fact, both in situ and in a macromolecular aggregate, the dynein ATPase has an alkaline pH optimum, and its activity increases sharply around pH 7.5 (33), although the purified enzyme has a broader optimum (34). Axonemal motion in permeabilized sperm preparations is activated over a very narrow pH range (4), from pH 7.3 to 7.8. This is the pH range in which the respiration and motility are increased in vivo. Changes in cAMP and cGMP levels are also associated with stimulation of motility and respiration at low pH by the peptide "speract" (12), so these nucleotides, as well as protein phosphorylation may play some role in the regulation (24, 35, 36).

The apparent pH, is expressed as a net value for the whole cell. This is a useful but unsophisticated measurement, since multiple intracellular compartments contribute to the net pH, that is obtained, and the size of the relevant compartment can not be determined with precision (15). Additional and less valid assumptions would be needed to determine the pH of any individual intracellular compartment. Still, the reported average pH values are useful in comparing one set of experimental conditions with another, allowing estimates of changes in pH associated with cellular activity, even if the absolute values of the cytoplasmic pH cannot be determined with certainty.

Since the pH of the sperm is affected by both the pH and Na" concentration, part of the regulation of pH might involve Na"-dependent H" movements (2, 26, 46). Such movements have been proposed to account for pH regulation in other cells (19-23). The synergistic effect of extracellular Na" and pH on activation of respiration is consistent with this idea. The same respiratory rate can be obtained at lower pH, if the extracellular Na" is elevated (Table III). Since we have not examined the fluxes of all other ions under the conditions of these experiments, we cannot tell whether other Na" dependant ionic fluxes, such as chloride-bicarbonate exchanges (19-21, 29) are involved in the regulation of pH. The possibility that protons are equilibrated across the membrane according to a Donnan equilibrium seems unlikely, since depolarization of the plasma membrane with elevated K" (17) leads to a strong acidification instead of an alkalnization. This membrane depolarization might affect the Na"-dependent H" efflux mechanism or other ionic permeabilities needed to regulate the internal pH.

A question raised by these studies is whether the weak bases used to estimate the intracellular pH can also distribute across the membrane in their protonated form with a rate similar to the nonprotonated form. To the extent that this occurred, their utility as pH indicators would be decreased, since the mechanism of accumulation would also depend upon the membrane potential of the cell. The following observations suggest that this is not the case. Measurements of the sperm membrane potential with lipid-soluble ions (tetr phenylphosphonium+, triphenylmethylphosphonium+, thiocyanate-) provide results which cannot explain the accumulation ratios obtained with the pH probes. For example, depolarization of the membrane potential with increased external K" (17) would lead to a reduced accumulation of permeable cations such as Et,NH". The opposite effect was seen in this study; more amine accumulated in 200 mM K" than in 10 mM K". Also, reducing the external pH of sea water from 8 to 7 has little effect on the membrane potential. However, this decrease in pH leads to a reduction in the initial rate of influx of the amine, as expected if the nonprotonated form is the diffusing species, since its concentration is decreased 10 times by a decrease of 1 unit in the external pH. Finally, similar results are obtained with any amine used to measure the changes in the internal pH (diethylamine, methylamine, and 9-ami noacridine).

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Supplementary Material to: Elevation of the Intracellular pH Activates Respiration and Motility of Sperm of the Sea Urchin Strongylocentrotus purpuratus.

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Appendix 1:

Two-compartment Distribution of an Amine and a Weak Acid.

If we assume that there contain only two compartments, the mitochondria and the rest of the cell, then the following set of equations can be derived. We define:

- $V_m$: fractional volume of the mitochondria
- $V_o$: fractional volume of the cytosol
- $A_m^{\text{H+}}$: concentration in the mitochondria/cytosol/external medium
- $A_o^{\text{H+}}$: concentration in the mitochondria/cytosol/external medium
- $A_{\text{m}}$: the amine accumulation ratio
- $A_{\text{o}}$: the acid accumulation ratio

and $b = 0.45$ (reference 17). The following equation (11) allows to calculate the time constant of the uptake:

$$t = \frac{1}{b}$$

This shows that the initial rate of uptake is decreased at more acidic external pH, that the level of amine accumulation is proportional to the transmembrane $pH$, and that the amine accumulation is slower when the internal pH is more acidic.

Similar calculations, although much more tedious, can be performed even if the amine is bound intracellularly, provided the binding sites are not saturated (see Appendix 2). Equations are then more difficult to simplify, but $pH_{\text{m}}$ and $pH_{\text{o}}$ have the same influence on the parameters of the scheme, although the calculations are not detailed here, this result can easily be demonstrated by taking the derivatives of these expressions with respect to $pH_{\text{m}}$, $pK_a$, and $pK_b$.

These calculations do not imply that the $pH_{\text{m}}$ or $pH_{\text{o}}$ are changing during the experiment, that is, if the cells change their internal $pH$, if the $pH_{\text{m}}$ is not well buffered, or if the respiration of cells alters the $pH_{\text{m}}$.

Appendix 2: Quantitative Determination of Intracellular pH in Sea Urchin sperm with 9-aminoacridine.

INTRODUCTION

Although the weak amines have been used to estimate intracellular $pH$ (pHi) in sperm (17, 42, 43) as well as in other cells, organelles and membrane vesicles (13, 14, 55), the determination of the $pH$ in intact cells is more tedious. For example, in sea urchin sperm and hamster (44, 47) sperm, as in other systems (55-58), the uptake of 9-A is up to 2 orders of magnitude greater than the uptake of the nonmetabolized amine. This difference is attributed to cellular binding of the amine system, consequently, the 9-A molecule is more acidic than the free amine; changes that occur with the membrane reaction and the onset of motility and respiration. If the binding of 9-A could be estimated quantitatively, then this reaction would be much more useful for estimation of intracellular $pH$.

Calculation of $pH_i$ using 9-Am with correction for binding: We define:

$$[A_m], [A_o]$$: intra- and extracellular unbound free (9-A)

$$[A_m^+]$$: intra- and extracellular protonated free (9-A)

$$[A_m^{\text{H+}}]$$: total concentration of bound 9-A which is assumed here to be intracellular. This assumption will be justified in the Results. The total intracellular concentration of 9-A is:

$$[A_m] = [A_m^+] + [A_m^{\text{H+}}]$$

Accumulation of amine is response to a pH gradient with subsequent intracellular binding can be treated as follow. (If the assumption of Schmidlin and colleagues (44, 47) that the unprotonated amine rapidly equilibrates to $A_m$, $A_o$, and $A_{\text{m}}$ (48) the protonated amine is essentially impermeable and does not accumulate in response to the membrane potential, $A_{\text{m}}$, where $K_b$ is the acid dissociation constant for the amine (for 9-A, $K_b = 10^{-11}$) when the pH of the medium is $< 7.0$ (and the free amine is protonated), that the concentration of protonated amine approximates the total free amine concentration.

From reference (42):

$$[A_m] = [A_m]_{\text{eq}}$$

Combining the equations (11) and (21):

$$[A_m]_{\text{eq}} = [A_m]_{\text{eq}}$$

Equation (21) indicates that in order to determine the intracellular $pH$ in cells when the amines are used, one must be able to measure the amount of free and bound amine, that will be a function of the free intracellular amine concentration, the total concentration of intracellular binding sites (eqs. (11), (12), and (13) for a single heterogeneous system in the apparent dissociation constant, $K_b$. At or above pH 9, where $K_b$ is the acid dissociation constant for the amine (for 9-A, $K_b = 10^{-11}$), when the pH of the medium is $< 7.0$ (and the free amine is protonated), that the concentration of protonated amine approximates the total free amine concentration.
Intracellular pH and Respiration

The observed parameters for measurements with 9-AA are δ, the fraction of the fluorescence that is quenched, and the ratio of the free amino to the intracellular volume. These are related to the free concentration by

\[ [A_9] = [A_9]_0 \frac{1 - \delta}{\delta} \left( [A_9] - f \right) \]  

and

\[ [A_9]_0 = (1 - \delta) [A_9] \]  

where [A_9] is the initial (9-AM) prior to sperm addition (47).

RESULTS

At 10°C intact sea urchin sperm accumulated 0.04% relatively slowly (Figure 1A, dotted line). Near 0.6% of the 9-AM was taken up by sperm and released by subsequent addition of 0.04% (or higher) Triton X-100, under conditions previously shown to disrupt the sperm plasma membrane (25). When Triton X-100 was added to the 9-AM solution before sperm addition (Figure 1A, dotted line), uptake (quenching) of 9-AM to the same final level occurred immediately upon sperm addition. Treatment of sea urchin sperm with 0.04% Triton X-100 causes the immediate release of K+ (Schubert, unpublished data) and renders sperm permeable to ATP (33), but leaves the nucleus relatively intact (33). Gabel, Opperwier and Edmonds, unpublished data).

Figure 1. Accumulation of 9-AM into sperm and the effect of Triton X-100 on uptake.

Figure 2. Accumulation of 9-AM into sperm in the presence of 10 mM CaCl₂ and 0.6% Triton X-100 (open circles), 10 mM CaCl₂ and 0.04% Triton X-100 (open squares), and 10 mM CaCl₂ (open triangles). The fluorescence before and after sperm addition was 100 (data not shown). The percentage of 9-AM released was calculated using 9-AM uptake at 30 min after Triton X-100 addition as 100%. The solution was made 0.04% Triton X-100. Final sperm concentration was 2.5 x 10⁶ sperm/ml.

Intracellular pH and Respiration

When Triton X-100 was added to sperm, the immediate release of K+ (Schubert, unpublished data) and renders sperm permeable to ATP (33). Gabel, Opperwier and Edmonds, unpublished data).

In experiments on intact sperm at 0.6 - 2.0 x 10⁶ sperm/ml, with the binding parameters obtained from Figure 1 and (34), 0.04% or 0.08% of the binding sites were occupied at pH 6.9 - 7.4. The percentage of 9-AM bound was calculated with the intact sperm as 100%. The solution was made 0.04% Triton X-100. The percentage of 9-AM released was calculated using 9-AM uptake at 30 min after Triton X-100 addition as 100%. The solution was made 0.04% Triton X-100. Final sperm concentration was 2.5 x 10⁶ sperm/ml.

The binding parameters for 9-AM are comparable to those for binding of 2,6-diaminopurine (adenosine) to bacteriophage DNA (37, 41). The dissociation constant compares with values of 25-30 nM for 9-AM interaction with isolated DNA (48, 49). These values vary as a function of temperature, [Mg²⁺], and other parameters such as salt concentration (50-52). The total number of high affinity binding sites for 9-AM per sperm is calculated with the data that 0.04% Triton X-100, 2.0 x 10⁶ sperm/ml, and 9-AM was added to sperm. The ratio of bound to free bound (1:1) was calculated with the intact sperm as 100%. The percentage of 9-AM released was calculated using 9-AM uptake at 30 min after Triton X-100 addition as 100%. The solution was made 0.04% Triton X-100. Final sperm concentration was 2.5 x 10⁶ sperm/ml.

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The intracellular pH and respiration

The intracellular pH and respiration are important factors in cellular function. The pH can affect enzyme activity, protein stability, and the proton motive force, which drives ATP synthesis. The respiration rate can provide insights into metabolic activity and energy production. Studies have shown that changes in intracellular pH can significantly impact cellular processes, including pH-dependent enzyme activities and the regulation of ion channels.

One method to determine the intracellular pH is through the use of pH-sensitive fluorescent probes. These probes can be used to measure the pH difference across the plasma membrane, providing valuable information about the pH gradient and its regulation within the cell.

The measurement of intracellular pH is crucial for understanding various cellular processes, including pH-dependent signaling pathways, ion homeostasis, and the response to environmental stress. Accurate measurements require the development of sensitive and specific probes, as well as robust experimental techniques to interpret the data accurately.

In summary, studying intracellular pH and respiration is essential for gaining a deeper understanding of cellular function and the mechanisms that govern these processes. 

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**Table 1**

Comparison of average intracellular pH values determined using 9-AM or [14C]-diethylamine.

| Condition            | pH 7.4 | pH 7.7 |
|----------------------|--------|--------|
| 9-AM                 | 7.43   | 7.43   |
| + jelly (1 min later)| 7.70   | 7.72   |
| + jelly (3 min later)| 7.16   | 7.23   |

There were dilutions of 0.1% dry powder into ASW. To test the sample 2.5 μM 9-AM was added to another part of the [14C]-diethylamine was added. Both samples were included for 20 min until equilibrium was reached. At 1 min, which was prepared previously described [9], was then added to both samples at a final concentration of 24 μM Methylene blue. Fluorescence measurements were performed using a fluorescence spectrophotometer. The pH values for [14C]-diethylamine were determined as described in Materials and Methods.

The use of a pH-sensitive fluorescent probe allows us to test the underlying assumptions on which the weakly-permeating technique rests, that the protoclated form of the amine (NAP) does not accumulate with respect to the intracellular membrane potential. For 9-AM, the concentration of A varies 10-fold, over a range of 6.8 to 7.9, but of NAP changes by only 10% over this range (Fig. 1). The validity of the [14C]-diethylamine technique rests on the assumption that the protoclated form of the amine is the difficulty species.

Another assumption in these studies is that 9-AM does not alter the pH, as it accumulates. In fact, because it accumulates to a great extent, μM 9-AM is as effective as 5 μM methylene blue in increasing pH when measured with greater quantities of [14C]-diethylamine. Additionally, in order to validate the intraluminal free [9-AM] in a cell, a correction factor for the amine concentration is required. The level of free 9-AM should reach below 5 μM (Fig. 1). Hence the intracellular [9-AM] present after the cells have reached equilibrium should be less than 5 μM for sper in ASW and should be even lower at lower values of pH.

**Figure 4**

The initial rate of 9-AM uptake as a function of pH. *One amine was diluted 1250 μM (0.5 1000) molar solution of 9-AM. The pH was adjusted by mixing of 9-AM was adjusted (42). The fluorescence decay was monitored by adding one percent of the amines is the difference between the pH, using a model bounded with the assumption that the decaying emission at a given pH, time, is followed by the fluorescence. The amine concentration of 9-AM is the difference between the quenching of 9-AM fluorescence, with a change in the intracellular pH of the cell. Other cell classes than those described in Table 1, demonstrate the nuclear uptake of 9-AM, so that the nuclear uptake is due to the nuclear pH. The intracellular pH of 9-AM uptake system to obtain quantitatively accurate pH data.