Intracellular pH of Sea Urchin Eggs
Measured by the Dimethyloxazolidinedione (DMO) Method

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ABSTRACT   Intracellular pH (pH_i) of sea urchin eggs and embryos was determined using DMO (5,5-dimethyl-2,4-oxazolidinedione). By this method, the pH_i of *Lytechinus pictus* eggs increased after fertilization from 6.86 to 7.27, and this higher pH_i was maintained thereafter, as has been previously observed with pH microelectrodes. The same general result was obtained with the eggs of *Strongylocentrotus purpuratus*, in contrast to previous estimates of the pH of egg homogenates from this species, which had indicated a rise and then fall of pH_i after fertilization. pH_i did not significantly change during early cell divisions. Studies of treatments that alter pH_i confirmed that ammonia alkalizes and acetate acidifies the cells. The regulation of pH_i by embryos in acidic seawater is impaired if sodium is absent, whereas unfertilized eggs can regulate pH_i in acidic, sodium-free seawater.

A major event regulating biosynthesis and transport after the fertilization of sea urchin eggs is an increase of intracellular pH (pH_i) (1-3). This pH_i change was first suggested by (*a*) the Na^+-dependent release of acid by eggs to the surrounding seawater during the first 10 min after fertilization, implying an increase in pH_i, and (*b*) the initiation of some of the changes of fertilization by weak bases, which would be expected to increase pH_i. An additional observation was that Na^+ is required for the initiation of development during the same time period in which Na^+-dependent acid release is occurring, and this Na^+ requirement could be bypassed by incubation in weak bases. The initial measurements of this pH_i change, made by homogenizing eggs in isotonic KCl (4-6), indicated that the pH_i of unfertilized *Strongylocentrotus purpuratus* eggs averaged 6.4 and rose to nearly 6.8 within 4 min after fertilization. 10 min after fertilization, pH_i began to slowly decline back to unfertilized levels, which it reached ~60 min later (6).

Measurements of pH_i of a different species, *Lytechinus pictus*, with pH microelectrodes confirmed that these eggs alkalize by ~0.4 pH unit after sperm activation, but the pH_i was greater (6.84 in unfertilized eggs), and never reacidified as in the homogenate studies with *S. purpuratus* embryos (7). Stable pH-microelectrode records have not been obtained in *S. purpuratus*, so that direct comparisons on the same species have not been possible (S. Shen, personal communication).

A question therefore arises as to whether the pH_i change after fertilization is transient or permanent. Are these species differences? Or do the different values reflect types of damage done to the cells by electrode impalement or the breakage of compartments during homogenization? Resolution of this issue is important to understanding the causal relationships among the various fertilization-initiated changes within the embryo. For example, if the pH_i change is permanent, it could directly account for the permanent increases of cell activity, without the necessity of evoking another regulating factor that is "locked in" by a transient pH_i rise (1).

We have undertaken the present investigation to resolve this question by using an alternate technique of measuring pH_i—the DMO method (8-10). DMO (5,5-dimethyl-2,4-oxazolidinedione) is a weak acid that, when acting ideally (see Materials and Methods), distributes at equilibrium between intracellular and extracellular compartments on the basis of their respective pHs. Thus, by determining the intracellular and extracellular concentration of [^{14}C]DMO and the extracellular pH, pH_i can be calculated. Intracellular pH as determined by DMO ($DMO-pH_i$) has been found to agree well with pH_i determined by other methods, especially pH microelectrodes. In squid giant axons and barnacle muscle fibers, $DMO-pH_i$ is 0.05 pH unit more acidic than microelectrode-pH_i (11), but in the plant *Nitella*, the agreement is not as good ($DMO-pH_i$ is 0.2 pH unit more acidic than microelectrode-pH_i; see reference 12). The DMO method will not suffer from the same artifacts that can arise from the other two methods, such as damage to compartments of differing pH during homogenization or ionic leakage during microelectrode recording (for example, the resting membrane potential of *Lytechinus* is 55 mV less when

impaled by a pH microelectrode than when impaled by a single standard microelectrode, implying an ionic leak around the pH microelectrode; see reference 13). In collaboration with Dr. Robert Gillies (14), we carried out preliminary DMO measurements of *S. purpuratus* egg pH_i and in this report present a more extensive analysis of these results, comparing these to *L. pictus* and also describing pH_i during the cell cycle. In addition, we have studied the effects of ammonia and external sodium on pH_i in eggs and embryos.

Our results show that pH_i measured by DMO (*a*) behaves in both species very much as does that measured by microelectrodes, and unlike that estimated by the cell lysis method; (*b*) does not change significantly during or after the first and second cell divisions; and (*c*) is regulated by a Na^+-independent mechanism before fertilization and by a Na^+-dependent mechanism after fertilization.

MATERIALS AND METHODS

Handling of Gametes

Shedding of eggs and sperm of *S. purpuratus* and *L. pictus* was induced by injection of 0.5 M KCl into the coelomic cavity. The semen was collected dry and stored at 4°C. The eggs, dejellied by mechanical swirling and several washes in Millipore-filtered (Millipore Corp., Bedford, Mass.) seawater (MFSW; 0.45 μm), were resuspended in MFSW (except for the experiments of Fig. 8; see Materials below) at a concentration of 2.5% by volume as determined by sedimenting the unfertilized eggs in Bauer-Schenck tubes. The seawater was buffered with 10 mM HEPES, pH 7.70, except where noted. (The external pH was adjusted to 7.7 rather than 8.0 to increase the amount of permeant DMO.) To fertilize, a few microliters of semen was diluted and added to the egg suspension. In all experiments, the eggs were continuously stirred gently and the temperature maintained at 16–18°C.

Materials

In the experiments of Fig. 8, eggs were resuspended in artificial seawater of the following composition: NaCl, 460 mM; KCl, 10 mM; CaCl₂, 10 mM; MgCl₂, 27 mM; MgSO₄, 28 mM; NaHCO₃, 2.5 mM; Tris, 5 mM; PIPES, 5 mM. For sodium-free seawater (ONaSW), NaCl was replaced by choline Cl and NaHCO₃ by KHCO₃. Tritiated sorbitol, [³H]inulin, [¹⁴C]DMO and [¹⁴C]methylamine were purchased from New England Nuclear (Boston, Mass.). Unlabeled sorbitol and DMO were obtained from Sigma Chemical Co. (St. Louis, Mo.), and polyvinyl "microfuge" centrifuge tubes (1.5 ml) from VWR Scientific Inc. (subsid. of UNIVAR, San Francisco, Calif.).

Experimental Protocol

In a routine experiment, 1.0 μCi of ¹⁴C-DMO in ethyl acetate was dried with nitrogen, redissolved in seawater, and added to 40 ml of an egg suspension (final DMO concentration, 5×10^{-7} M). This concentration of DMO allowed normal plutei to develop. After permitting sufficient time for equilibration (usually 30 min), 1-ml samples were placed in microfuge tubes, and 50 μl of a seawater solution containing 0.25 μCi [³H]sorbitol and 0.1 mg unlabeled sorbitol was added to each tube (final sorbitol concentration was ~0.01% wt/vol). The tubes were gently mixed and spun an Eppendorf Micro Centrifuge 5412 (Brinkmann Instruments, Inc., Westbury, N. Y.) for 5 s, then turned off and allowed to spin down. The total spinning time was 20–25 s, which did not sediment unattached sperm. Two supernatant samples were taken, one for the immediate measurement of external pH and the other for counting the radioactivity in the supernate. The remaining supernate was aspirated and the pellet frozen in dry ice/isopropanol. When all samples had been taken, these frozen pellets were squeezed out of the microfuge tubes into predessicated, preweighed scintillation vials (8 ml) and the wet weight measured. Aliquots of the corresponding supernates were also placed in vials and weighed. After all samples had been weighed, they were dried at 70°C for 30 min, then dessicated overnight with P₂O₅ under a slight vacuum. Then all samples were reweighed to determine the dry weight, completely digested with 1 ml of NCS (New England Nuclear) or BTS-450 (Beckman Instrument Co.) at room temperature, and counted in a Beckman LS-8000 scintillation counter with "automatic quench correction" (an external standard channels ratio method) after addition of 5 ml of scintillation solution (5 g PPO and 100 mg POPOP in 1 l of toluene). The ³H and ¹⁴C counts were always corrected for spillover and efficiencies as in reference 15,[1] using quenched standards made up like experimental samples, except with known activities of [³H]toluene or [¹⁴C]toluene, and the pH_i calculated as below on a DEC PDP-11 computer (Digital Equipment Corp., Marlboro, Mass.) or a TI59 programmable calculator (Texas Instruments Inc., Digital Systems Div., Houston, Tex.).

Methylamine pH_i was determined in the same way, except: (*a*) 0.5 μl methylamine stock (New England Nuclear; 50 μCi/ml EtOH) was added to each milliliter of egg suspension instead of DMO, and (*b*) methylamine-pH_i was calculated by the equation found in reference 11 (methylamine pK_a was taken to be 11.8).

Calculation of pH_i

First, it must be pointed out that DMO-pH_i is an average pH_i, so that compartments having pHs different from that of the cytoplasm will influence DMO-pH_i. Because DMO is an acid, it will be excluded from acidic compartments and DMO-pH_i will not be significantly different from the true cytoplasmic pH as long as the relative volume of the acidic compartments (e.g., lysosomes) is small. On the other hand, DMO will accumulate in basic compartments (e.g., mitochondria), and it may therefore indicate an erroneously alkaline pH if these compartments are very basic or constitute a large proportion of intracellular volume (11, 14).

To calculate pH_i from DMO distribution, one must assume: (*a*) that the plasma membrane is not permeable to ionized DMO, and that the concentration of un-ionized DMO inside and outside the cell is equal after equilibration (when the plasma membrane potential is very large, permeability of ionic DMO can become a major problem, as in reference 16), and (*b*) that DMO is completely inert, i.e., not metabolized or bound. In two plant systems (17, 18) it apparently is not inert, but in animal systems, DMO has behaved as an inert substance (8, 9).

Use of the following equation requires that the intracellular and extracellular concentration of DMO be known, so corrections for intracellular and extracellular space in the pellet are made. The equation for pH_i calculation, given the above assumptions, is:

$$pH_i = pK_a + \log_{10}\left(\left[\frac{D_t}{D_o}\left(\frac{S_o}{S_o - S_t}\right) - \frac{S_t}{S_o - S_t}\right]\left[10^{pH_o - pK_a} + 1\right] - 1\right),$$

where: pK_a = pK_a of DMO (here taken as 6.32; see reference 11), D_t = dpm of DMO in pellet sample/mg water, D_o = dpm of DMO in supernate/mg water, S_t = dpm of sorbitol in pellet sample/mg water, S_o = dpm of sorbitol supernatant sample/mg water, and pH_o = extracellular pH. (The reference for this form of the equation is 19; see reference 20 for a partial derivation and reference 21 for a complete derivation.)

Extracellular Markers

As stated above, the intracellular and extracellular space in the pellet must be known. Some previous workers (14, 19) have preferred to estimate internal spaces by comparing ³H₂O and [¹⁴C]sorbitol (or other extracellular marker) spaces in samples parallel to those with [¹⁴C]DMO. We have chosen a method that allows the intracellular space to be determined on the same samples that are incubated in DMO (11). The pellet space that is accessible to tritiated sorbitol is taken as extracellular water (W_o) and the difference between the wet and dry weights of the pellet is the total water (W_t). Intracellular water is therefore $W_i = W_t - W_o$. The values obtained for W_i by either method are not statistically different at the 0.05 level: in one experiment on the same batch of unfertilized *S. purpuratus* eggs, the W_i in 1-ml samples of the egg suspension was 11.51 ± 0.435 μl (mean ± SEM, $n = 5$) as determined by the [³H]sorbitol-weighing protocol and 10.98 ± 0.264 μl ($n = 4$) by the ³H₂O-[¹⁴C]sorbitol method.

To make sure that the sorbitol space was a reliable indicator of W_o, unfertilized eggs were incubated in sorbitol for various times. The results are shown in Fig. 1, where the W_i is normalized for the quantity of cells by dividing by the dry weight (dw) of the pellets. W_i/dw remains relatively constant, with a slight decline, over at least 30 min. The difference between the values in *L. pictus* and *S. purpuratus* may be attributable to differences in egg density. Because intracellular water, not volume, is the measured parameter, an egg packed with yolk proteins will have a smaller W_i/dw than the corresponding intracellular volume/dw calculated on the basis of the egg diameter (this value is 4–5 by our determination and also by Harvey [22]). Unlike values for other types of cells, W_i/dw values are probably <4 in eggs because they are packed with yolk proteins. This discrepancy is acceptable, however, because it is the DMO concentration in the intracellular water, not the volume, which is the desired quantity as far as the pH_i calculation is concerned.

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[1] The reader should beware of a misprint in the denominator of the upper equation in reference 15.

The slow decline of W_i/dw with time, as seen in Fig. 1, is probably the result of sorbitol uptake or binding, and not a result of equilibration with extracellular spaces, because (a) long incubations (>1 h) yield W_i/dw values that can be negative; (b) the unfertilized egg surface is relatively smooth and does not have major surface irregularities (as in other cells [11]) that might retard complete equalization of sorbitol concentration into extracellular spaces; and (c) fertilized eggs of L. pictus, which do have vitelline envelopes and hyalin layers that might slow sorbitol diffusion, in fact exhibit just as rapid an equilibration of sorbitol (as indicated by equal values of W_i/dw for unfertilized and fertilized eggs; see Table I). Therefore, because sorbitol equilibrates rapidly with eggs and then is slowly taken up, the cells in routine assays were incubated with [³H]sorbitol for 40–60 s before processing.

We also tried [³H]inulin as an extracellular marker with S. purpuratus eggs. Whereas inulin gave W_i/dw values for unfertilized eggs that were comparable to those obtained by the use of sorbitol, the W_i/dw for fertilized eggs was almost always negative. Negative values were calculated for samples incubated with inulin within the first 5–10 min after fertilization, which suggests that inulin may become incorporated into the newly forming vitelline envelope.

Table I summarizes the W_i/dw data for unfertilized, fertilized, and ammonia-treated eggs incubated in [³H]sorbitol for 40–60 s. In L. pictus, there is no significant difference of W_i/dw at the 0.05 level between unfertilized and fertilized or between unfertilized and ammonia-treated eggs. These data show that sorbitol is permeable to the vitelline space and probably to the hyalin space of L. pictus (if not, W_i/dw would have doubled, because the diameter of the vitelline envelope is ~1.3 times that of the unfertilized cell.)

In S. purpuratus, however, W_i/dws of unfertilized and fertilized eggs are significantly different. Again, sorbitol must be permeable to the vitelline envelope or the difference would be greater. However, the hyalin layer, which is thicker in S. purpuratus than in L. pictus, may exclude sorbitol, resulting in an increased W_i/dw for the zygote of the former species. The difference is not attributable to incomplete sorbitol equilibration, because incubation of fertilized eggs in sorbitol for up to 20 min did not decrease W_i/dw further. This difference may cause DMO-pH_i of fertilized S. purpuratus as reported in this paper to be ~0.05 pH unit lower than the "actual" fertilized pH_i.

RESULTS

DMO Equilibration and Control Experiments

The uptake of DMO into the eggs of L. pictus and S. purpuratus is plotted in Fig. 2A and B as a function of time. It is obvious that 10 min is sufficient for equilibration. Because there is no further DMO uptake after 10 min, DMO is probably not converted into other compounds within these cells, as has been found in some plant cells (17). Zygotes of both species exhibit a pH_i that is 0.4 pH unit more basic than that of eggs (Table II). Table II shows further that the DMO-pH_i of L. pictus eggs and zygotes are quite similar to the previously published values obtained with pH microelectrodes. pH_i of S. purpuratus eggs (7.08) and zygotes (7.47) are consistently higher than their L. pictus counterparts (6.86 and 7.27, respectively).
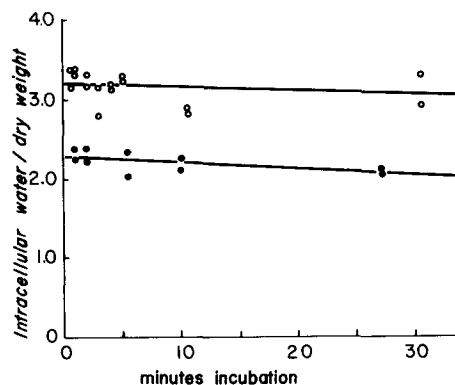
Three control experiments were done to determine if DMO

FIGURE 1 Determination of intracellular water/dry weight of unfertilized eggs of L. pictus (O) and S. purpuratus (●) with [³H]-sorbitol. Incubation time in this and all other figures includes the centrifugation time. Least-squares regression lines are also plotted.

TABLE I

Comparisons of W_i/dw

	Average W_i/dw (±SEM)	n
L. pictus		
Unfertilized	3.04 ± 0.040	47
Fertilized	3.11 ± 0.021	137
NH_3 (5 and 10 mM)	2.99 ± 0.029	71
S. purpuratus		
Unfertilized	2.17 ± 0.020	74
Fertilized	2.56 ± 0.032	117
NH_3 (5 and 10 mM)	2.10 ± 0.018	75

binds within sea urchin eggs. If DMO is binding, the ratio of intracellular to extracellular DMO will change if all binding sites become occupied as the total DMO concentration is increased. If DMO acts as an ideal pH probe, on the other hand, this ratio will be equal at all concentrations of DMO (23). Fig. 3 illustrates that the ratio is constant from 5×10^{-7} M to 10^{-4} M DMO. Only at millimolar concentrations does the ratio noticeably change in L. pictus, and this is a concentration that would be expected to acidify pH_i directly (DMO at these high concentrations can act like another weak acid, dinitrophenol, which is known to acidify sea urchin eggs; see references 3, 10, and 24).

Another control for DMO binding is to equilibrate intracellular and extracellular pH and see if DMO distributes accordingly. Unfertilized S. purpuratus eggs were treated with 0.1% Triton X-100, which causes the cells to swell, but not to lyse. We observe that pH_i was within 0.05 pH unit of pH_o (7.7) under these conditions, whereas if significant DMO binding had occurred, the calculated pH_i would have differed from pH_o.

A final control is to vary the extracellular pH and see if the DMO distribution changes accordingly. If DMO is binding inside the cell, its distribution would not be markedly different at different external pH (this would lead to very different calculated pH_is at different pH_os); if, however, DMO is distributing according to the pH gradient, more DMO should enter at lower pH_o and less DMO at high pH_o (this assumes the cells can maintain a constant pH_i at different pH_os, which these cells can only partly accomplish; see below). Therefore, if DMO is acting ideally, it will indicate a relatively constant pH_i over a range of pH_o; this is in accordance with the data of Fig. 8 for cells in sodium seawater at pH_os between 6.8 and 7.8 (within the ability of these cells to regulate pH_i in acidic or basic seawater).

pH_i and Fertilization

Fig. 4 shows the time-course of pH_i changes after fertilization. The pH_i obtained with the DMO method for L. pictus is compared with pH_i obtained using pH microelectrodes (2). This microelectrode record, which was shortened for comparison with the DMO data, was considered most representative by Dr. S. Shen (personal communication), and its later time-points (not shown) confirm that pH_i stabilizes at 7.19. The agreement between the two methods is good, and the variability of L. pictus pH_i as measured by DMO and microelectrodes is similar (Table II).

The data of Fig. 4 confirms that the pH_i of both L. pictus and S. purpuratus rises after fertilization and remains high, unlike the homogenate data. Fig. 5 depicts the same result under the conditions (6) used to determine pH_i by homogeni-
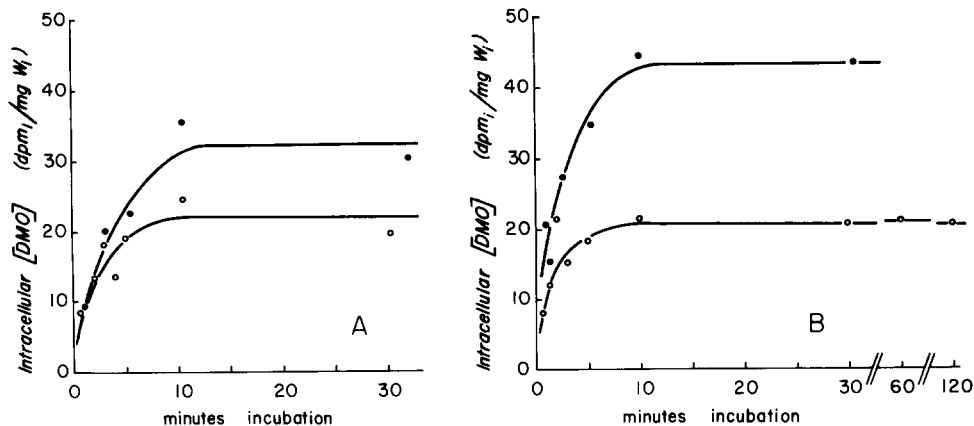
FIGURE 2 (A) Equilibration of DMO with *L. pictus* unfertilized eggs (○) and zygotes (●). Each point is the average of duplicate determinations. Zygotes were fertilized in batches at different times to create an asynchronously fertilized population so that any changes in pH$_i$ as development proceeded would not perturb the equilibration curve. (B) *S. purpuratus* eggs and zygotes, as in A. W$_i$, intracellular water.

TABLE II

pH$_i$ of *L. pictus* and *S. purpuratus*

	Average pH$_i$ ± SEM	n
L. pictus: microelectrode*		
Unfertilized	6.84 ± 0.02	44
Fertilized	7.26 ± 0.06	8
L. pictus: DMO		
Unfertilized	6.86 ± 0.019	27
Fertilized‡	7.27 ± 0.015	38
S. purpuratus: DMO		
Unfertilized	7.08 ± 0.011	36
Fertilized‡	7.47 ± 0.015	32

* From reference 7.
‡ Between 10 and 30 min after fertilization.

zation: namely, *S. purpuratus* eggs in MFSW without added buffer. The slight drop in pH$_i$ at 18 min is probably not significant when compared with the general variability of the data. In this experiment, acid release was observed (extracellular pH dropped from 7.85 to 7.55), but no later decrease in pH$_i$ after fertilization occurred.

pH$_i$ and Ammonia

Ammonia is known to increase pH$_i$ and activate some of the developmental changes that are normally triggered by sperm (1, 7). Ammonia probably raises pH$_i$ by binding protons within the cell after moving through the cell membrane in the uncharged form (25). Figs. 6 and 7 show how egg pH$_i$ is affected by incubation in 5 and 10 mM NH$_4$Cl. For *S. purpuratus*, the effect of either concentration of NH$_4$Cl is the same: pH$_i$ increases 0.4 pH unit within the first 15 min and then remains stable. In *L. pictus*, total equilibration is not achieved by 15 min after the addition of NH$_4$Cl, but other experiments show that pH$_i$ does stabilize at 60 min after NH$_4$Cl addition (data not shown). (Note that it is the microelectrode data in Fig. 6 A that show unusual acidity, whereas the DMO data are within the usual range of pH$_i$. Also note that the DMO-pH$_i$ for untreated *L. pictus* eggs in Fig. 7 A is about 0.15 pH unit higher than average.)

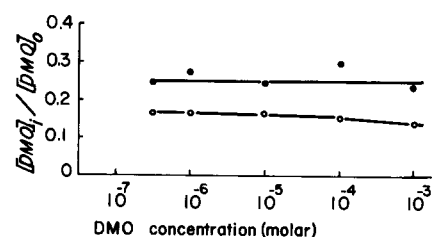A difference in pH$_i$s as measured by DMO or pH microelec-

trodes is the rise time after NH$_4$Cl addition. In all cases, DMO-pH$_i$ responds more rapidly. Differences of pH$_i$ before and after treatment are noticeable within 2 min, whereas microelectrode-pH$_i$ is only just beginning to change after 2 min. An explanation for this discrepancy is proffered in the Discussion.

pH$_i$ and Acetate

Sodium acetate has been reported to lower the pH$_i$ of *L. pictus* embryos (2). We have performed a few experiments of this kind on *S. purpuratus* with DMO. In one experiment, for example, the pH$_i$ of zygotes suspended in pH$_o$ 7.75 seawater was 7.73 ± 0.013 (± SEM, n = 3), and pH$_o$ 6.93 seawater lowered the pH$_i$ to 7.24 ± 0.012 (n = 3); but after a 40-min incubation in pH$_o$ 6.93 seawater plus 10 mM sodium acetate, the pH$_i$ dropped 0.19 pH unit further to 7.05 ± 0.009 (n = 3).

Acidic Compartments

Previously, pH$_i$ as measured by the weak base methylamine had been reported to increase from 6.45 to 7.0 after fertilization (14). We could not confirm such a change: methylamine-pH$_i$ of *S. purpuratus* eggs was 6.41 ± 0.038 (n = 3) and of 35-min zygotes was 6.30 ± 0.020 (n = 3). Lee and Schuldiner[2] have found that the distribution of the fluorescent weak base 9-aminoacridine (9AA, also a pH$_i$ indicator; see reference 14) also does not change after fertilization. Interestingly, 9AA is

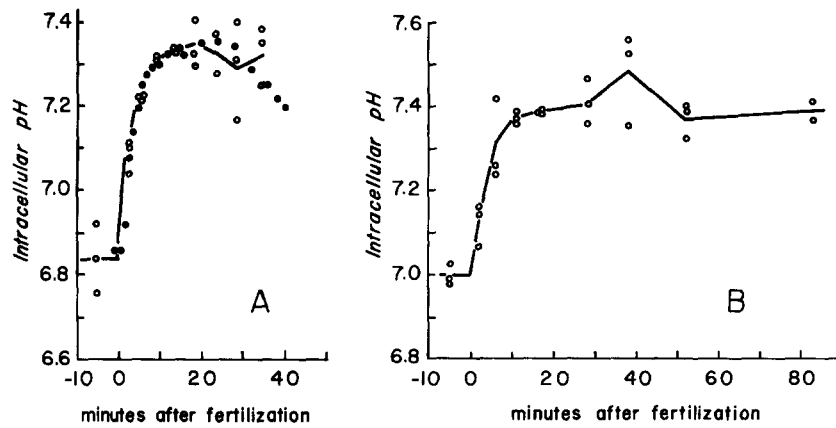[2] H. C. Lee and S. Schuldiner. Unpublished observations.

FIGURE 4 (A) pHᵢ before and after fertilization in L. pictus eggs as determined by DMO (O) and pH microelectrodes (●). The lines connect the average of each DMO triplicate. The pH-microelectrode data comes from reference 2, by permission of Dr. S. Shen. (B) S. purpuratus egg pHᵢ determined by DMO, as in A.
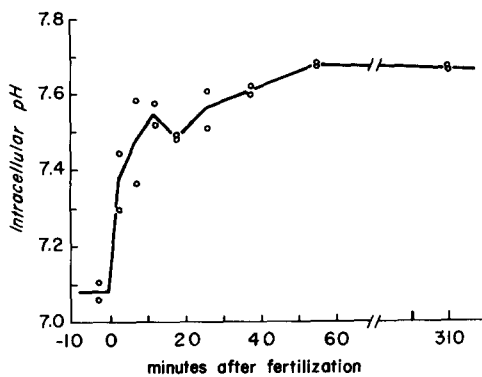

FIGURE 5   pHᵢ before and after fertilization of S. purpuratus eggs in unbuffered seawater (only 2.5 mM NaHCO₃). The pH₀ had dropped from 7.85 to 7.55 by 20 min after fertilization. The line connects the averages of each DMO duplicate. At 310 min, the embryos were at the 32-cell stage.

not distributed uniformly within the cell—in the fluorescence microscope, the 9AA fluorescence is primarily in distinct "granules." These are likely to be acidic compartments, but they are not cortical granules because they are not discharged after fertilization. Ammonia or nigericin will discharge the 9AA fluorescence of these granules in vivo and in vitro.[2] We conclude that the pH of these "9AA-acidic compartments" does not change after sperm activation and that the pHᵢ measured by methylamine is probably influenced by a low pH within these same compartments.

pHᵢ Regulation and Na⁺

External Na⁺ is required for the acid release and pHᵢ increase after fertilization (13, 26). We wanted to know if external Na⁺ was required for pHᵢ regulation in the face of external pH "insults." Eggs or zygotes were suspended in artificial seawater or ONa seawater of various pH₀s and the pHᵢs were then determined. The results are plotted in Fig. 8. The ability of unfertilized eggs to regulate pHᵢ was not markedly different whether or not Na⁺ was present—i.e., the slopes of the pHᵢ vs. pH₀ plots are about the same, with the possible exception of very high pH₀, when eggs appear to regulate pHᵢ better in ONa seawater. (Note that pHᵢ of these unfertilized eggs at pH₀ 7.7

is higher than the typical values.) The tendency for pHᵢ of eggs in ONa seawater to be somewhat more acidic is reproducible.

In contrast to unfertilized eggs, however, the ability of already fertilized eggs to regulate pHᵢ is significantly impaired in ONa seawater (zygotes were transferred to ONaSW 10 min after fertilization). As seen in Fig. 8 B, embryos in acidic seawater can regulate pHᵢ much better if sodium is present. Samples of these embryos were fixed 120 min after fertilization. The embryos in ONaSW at pH 6.35 (pHᵢ = 6.3) and 6.55 (pHᵢ = 6.5) had only 20–30% chromosomal condensation, whereas condensation was >80% in all other samples (usually 95–100%).

pHᵢ during Cell Division

Fig. 9 illustrates representative experiments performed during the time of cell division. As observed with microelectrodes (2), L. pictus pHᵢ sometimes drops by 0.1–0.15 pH unit 30–60 min before the time of cell division, which coincides with a decrease in the incorporation of amino acids into protein (2). This drop is not, however, consistently observed—other DMO measurements of L. pictus pHᵢ show no differences at any time between 60 and 130 min after fertilization. Unlike pHᵢ fluctuations in Physarum (27) or Tetrahymena (14), fluctuations of pHᵢ during cell division are not significant in embryos of either L. pictus or S. purpuratus. The significance of these results is discussed below.

DISCUSSION

Comparison of the Methods of Determining pHᵢ

Clearly, DMO gives reproducible values for sea urchin egg pHᵢ that correspond with those obtained by pH microelectrodes. We, in fact, were surprised by how well the two methods agree, considering that DMO-pHᵢ can be affected by permeability of the plasma membrane to the ionic form of DMO and by cellular inhomogeneity of pH (in, for example, basic compartments such as mitochondria), whereas microelectrode-pHᵢ can be biased by ionic leaks resulting from damage from the electrodes. Because the potential artifacts of the two methods are different, it is likely that the measured pH values are representative of cytoplasmic pH, which by both methods is seen to increase substantially after fertilization and to remain high. Also, the responses of DMO-pHᵢ and microelectrode-pHᵢ to treatment with ammonia or acetate are generally similar.
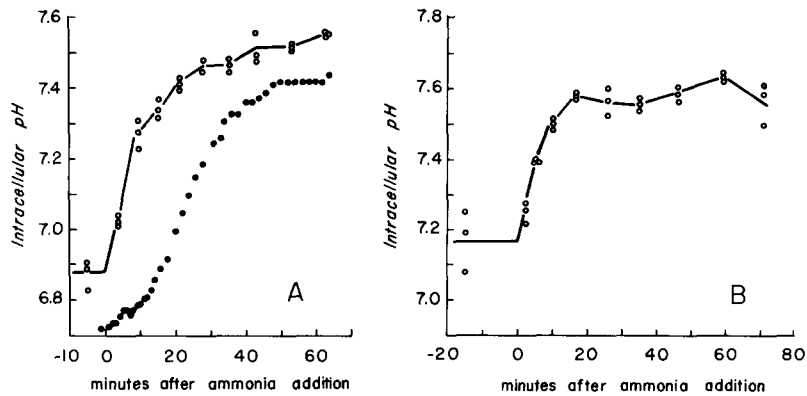
FIGURE 6 (A) pH_i before and after the addition of 5 mM NH₄Cl in *L. pictus* eggs as measured by DMO (O) and pH microelectrodes (●). The lines connect the average of each DMO triplicate. The pH-microelectrode data comes from reference 2, by permission of Dr. S. Shen. (B) *S. purpuratus* egg pH_i before and after treatment with 5 mM NH₄Cl determined by DMO, as in A.
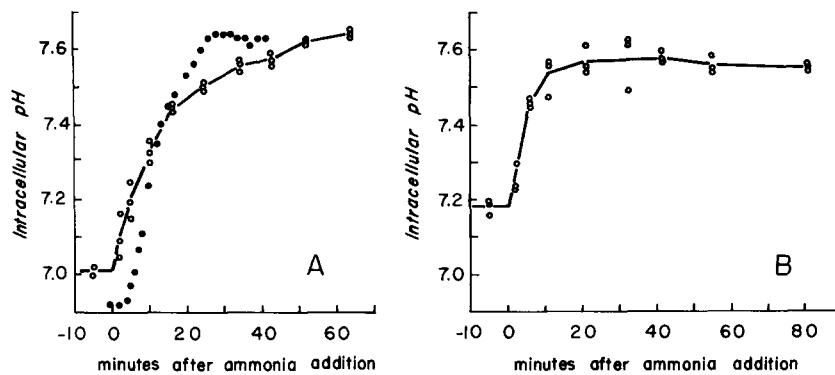

FIGURE 7 (A) pH_i before and after the addition of 10 mM NH₄Cl in *L. pictus* eggs as measured by DMO (O) and pH-microelectrodes (●). The lines connect the average of each DMO triplicate. The pH-microelectrode data comes from reference 7, by permission of Dr. S. Shen. (B) *S. purpuratus* egg pH_i before and after treatment with 10 mM NH₄Cl determined by DMO, as in A.

Neither DMO-pH_i nor microelectrode-pH_i displays the low pH_i values (as much as 0.6 pH unit lower) or the reacidification of pH_i after fertilization that has been reported for homogenate-pH_i (6). Why does the pH of egg homogenates reacidify after fertilization? The answer is unknown, but perhaps acidic compartments, e.g., lysosomes, become more fragile in zygotes and are therefore ruptured during homogenization.

The Role of Na

It has been suggested (4, 26) that the alkalization immediately after fertilization is mediated by a Na^+-H^+ exchange, thereby accounting for the stoichiometry of Na^+ uptake to proton release and the Na^+ requirement for acid release. Experiments in which we varied external pH in Na-free seawater investigated whether eggs and zygotes can regulate their steady-state pH_i in the absence of Na^+. As shown, the unfertilized egg regulates pH_i just as well in the presence or absence of Na. However, Na is involved in pH_i regulation after the period of Na^+-dependent acid release is over. It would appear that in both cases—acid release at fertilization and pH_i regulation of embryos in acidic seawater—Na is necessary for "pumping" acid out of the cell. Therefore fertilization induces a Na^+-dependent pH_i increase and activates a Na^+-dependent "pH-stat."

Cell Cycle: pH_i and Protein Synthesis

The data of Fig. 9 demonstrate that significant modulation of pH_i during the early cleavages does not occur. It must be noted, however, that this method would not be able to detect very brief changes of pH_i. Also, if cell-cycle synchrony is not excellent, small changes might be glossed over.

The data does show that there is no correlation between pH_i and the rate of protein synthesis after the first cell division. In *L. pictus*, the protein synthesis rate increases substantially after the first and again after the second cell divisions (see Fig. 5 of reference 2), whereas no stable pH_i changes occur after these cleavages. Therefore, pH_i cannot be the major regulator of protein synthesis after the first cell division (2).

Acidic Compartments

Even though DMO-pH_i and microelectrode-pH_i compare favorably, they do differ during ammonia treatment—DMO-pH_i increases more rapidly (Figs. 6 and 7). Similarly, Lee and Schuldiner[2] have found that the distribution of 9AA within the egg begins to change almost immediately after NH₄Cl is added: the granular fluorescence rapidly disappears (Triton X-100 and the ionophores nigericin and monensin will also "discharge" this particulate fluorescence). This suggests that the fluorescent granules are acidic, lysosomal-like vesicles that can be rapidly
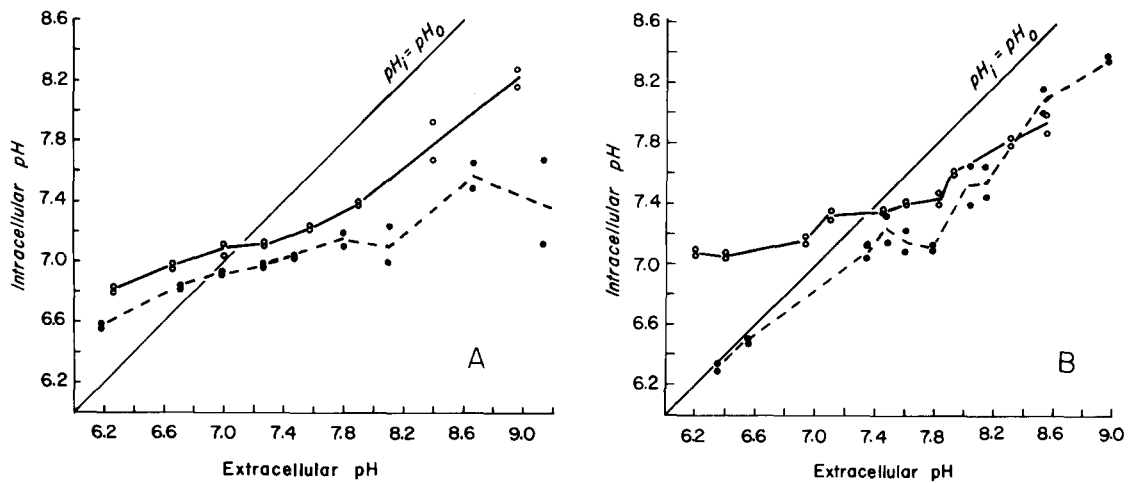
FIGURE 8 (A) pH_i of unfertilized S. purpuratus eggs suspended in seawater of various pH_os. Eggs are in artifical seawater (O) or sodium-free seawater (●). Eggs were incubated at these pH_os in DMO for 85 min before processing. Eggs in sodium-free seawater were washed three times and resuspended in choline seawater (see Materials and Methods). pH_o was adjusted with HCl or KOH. (B) pH_i of fertilized S. purpuratus eggs suspended in seawater of various pH_os. Zygotes are in artificial seawater (O) or sodium-free seawater (●) and were incubated at these pH_os in DMO for 60 min before processing, at which time they had been fertilized for 80 min. Zygotes in sodium-free seawater were washed three times and resuspended between 15 and 20 min after fertilization in choline seawater. pH_o was adjusted with HCl or KOH. A preliminary experiment indicated that a 60-min incubation was sufficient for stabilization of zygote pH_i at different pH_os in artificial or sodium-free seawater (there was no significant difference of pH_i between zygotes that had incubated 40 min and those that had incubated 70 min).
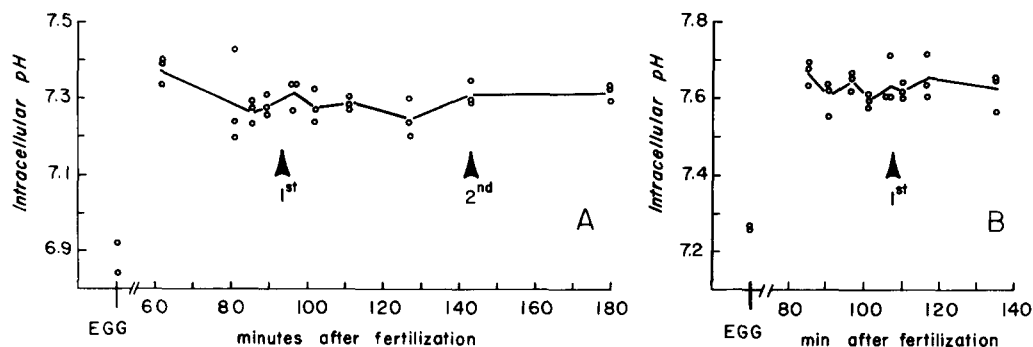

FIGURE 9 (A) pH_i of L. pictus embryos during the cell division cycle. Egg pH_i is also plotted for comparison. Arrowheads mark the time of 50% division for the first and second cleavages. The time between the completion of division in the "fastest" cells and the "slowest" cells was ~18 min. (B) pH_i of S. purpuratus embryos during the cell division cycle. The pH_i of eggs from this same batch of cells is plotted for comparison. The arrowhead marks the time of 50% division for the first cleavage. The time between the completion of division in the "fastest" cells and the "slowest" cells was ~10 min.

alkalized by the extracellular application of ammonia. In this regard, macrophage lysosomes react to extracellular ammonia in exactly this way (28). The rapid response of DMO-pH_i to ammonia is consistent with this interpretation. DMO will be excluded from acidic compartments, but as the latter are alkalized by ammonia, the DMO will redistribute into them. The intracellular concentration of DMO will therefore increase and an apparently higher DMO-pH_i will be calculated. This interpretation suggests the inflowing ammonia initially alkalizes acidic compartments and only afterwards increases the cytoplasmic pH (as measured by the pH microelectrode).

Interpretation of Experiments that Alter pH_i

Two other important conclusions arise from the above results. First, using ammonia or acetate as a reagent to change pH_i will have side-effects. In the former case, ammonia appears to discharge the pH of acidic compartments, whereas fertilization will not. Therefore, it does not specifically affect cytoplas-

mic pH. Moreover, it is well known that protein degradation is inhibited by treatments, including ammonia, that raise intralysosomal pH (29, 30). Consequently, just because ammonia treatment mimicks a fertilization-induced event (e.g., protein synthesis) does not mean that the event is necessarily regulated by the pH_i increase at fertilization—the ammonia may act by a totally different mechanism that is a side-effect of the drug. In the same way, acetate can have serious side-effects that limit its usefulness in diagnosing pH_i-regulated processes. Because it is a major intermediate of biochemical pathways, it will certainly disrupt metabolism. Also, like any weak acid at these concentrations, acetate should be capable of uncoupling oxidative phosphorylation (24).

As an example, recent experiments on another echinoderm system—maturation and fertilization of starfish oocytes (31)—reveal that even though 1-methyladenine (1-MA) treatment initiates meiosis and induces a fourfold to sevenfold increase of amino acid incorporation into protein, it does not trigger a

pH$_i$ change. Ammonia increases and acetate decreases both pH$_i$ and percent-incorporation, as in sea urchins. Yet pH$_i$ does not change throughout normal 1-MA activation, and therefore does not appear to be the regulatory factor in the activation of protein synthesis in these oocytes. These effects of ammonia and acetate are not surprising; one may merely be affecting cell activity via pH in a situation where pH$_i$ is not the normal regulator or by the side-effects discussed above.

The second major conclusion prompted by the ammonia data is that investigations using DMO must address the problem of pH compartmentation. Perhaps methylamine and/or 9AA should be routinely used in conjunction with DMO. A final but related issue is the possibility of local pH changes within the cytoplasm that are not pervasive enough to change the overall DMO distribution but regulate restricted events (such as actin polymerization [32]). Until recently, such local pH measurements were not possible. However, with the application of new techniques (33), these questions should be answerable.

NOTE ADDED IN PROOF: M. M. Winkler, G. B. Matson, J. W. B. Hershey, and E. M. Bradbury have measured pH$_i$ in *S. purpuratus* with ^{31}P-NMR and have obtained values very similar to those reported in this paper: 7.12 for unfertilized eggs and 7.55 for zygotes (personal communication and manuscript in preparation).

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