

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

CARL HIRSCHIE JOHNSON and DAVID EPEL

Department of Biological Sciences, Hopkins Marine Station, Stanford University, Pacific Grove, California 93950

**ABSTRACT** Intracellular pH ( $\text{pH}_i$ ) of sea urchin eggs and embryos was determined using DMO (5,5-dimethyl-2,4-oxazolidinedione). By this method, the  $\text{pH}_i$  of *Lytechinus pictus* eggs increased after fertilization from 6.86 to 7.27, and this higher  $\text{pH}_i$  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  $\text{pH}_i$  after fertilization.  $\text{pH}_i$  did not significantly change during early cell divisions. Studies of treatments that alter  $\text{pH}_i$  confirmed that ammonia alkalizes and acetate acidifies the cells. The regulation of  $\text{pH}_i$  by embryos in acidic seawater is impaired if sodium is absent, whereas unfertilized eggs can regulate  $\text{pH}_i$  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 ( $\text{pH}_i$ ) (1–3). This  $\text{pH}_i$  change was first suggested by (a) the  $\text{Na}^+$ -dependent release of acid by eggs to the surrounding seawater during the first 10 min after fertilization, implying an increase in  $\text{pH}_i$ , and (b) the initiation of some of the changes of fertilization by weak bases, which would be expected to increase  $\text{pH}_i$ . An additional observation was that  $\text{Na}^+$  is required for the initiation of development during the same time period in which  $\text{Na}^+$ -dependent acid release is occurring, and this  $\text{Na}^+$  requirement could be bypassed by incubation in weak bases. The initial measurements of this  $\text{pH}_i$  change, made by homogenizing eggs in isotonic KCl (4–6), indicated that the  $\text{pH}_i$  of unfertilized *Strongylocentrotus purpuratus* eggs averaged 6.4 and rose to nearly 6.8 within 4 min after fertilization. 10 min after fertilization,  $\text{pH}_i$  began to slowly decline back to unfertilized levels, which it reached ~60 min later (6).

Measurements of  $\text{pH}_i$  of a different species, *Lytechinus pictus*, with pH microelectrodes confirmed that these eggs alkalize by ~0.4 pH unit after sperm activation, but the  $\text{pH}_i$  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  $\text{pH}_i$  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  $\text{pH}_i$  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  $\text{pH}_i$  rise (1).

We have undertaken the present investigation to resolve this question by using an alternate technique of measuring  $\text{pH}_i$ —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 [ $^{14}\text{C}$ ]DMO and the extracellular pH,  $\text{pH}_i$  can be calculated. Intracellular pH as determined by DMO (DMO- $\text{pH}_i$ ) has been found to agree well with  $\text{pH}_i$  determined by other methods, especially pH microelectrodes. In squid giant axons and barnacle muscle fibers, DMO- $\text{pH}_i$  is 0.05 pH unit more acidic than microelectrode- $\text{pH}_i$  (11), but in the plant *Nitella*, the agreement is not as good (DMO- $\text{pH}_i$  is 0.2 pH unit more acidic than microelectrode- $\text{pH}_i$ ; 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  $\mu$ m), 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; CaCl<sub>2</sub>, 10 mM; MgCl<sub>2</sub>, 27 mM; MgSO<sub>4</sub>, 28 mM; NaHCO<sub>3</sub>, 2.5 mM; Tris, 5 mM; PIPES, 5 mM. For sodium-free seawater (ONaSW), NaCl was replaced by choline Cl and NaHCO<sub>3</sub> by KHCO<sub>3</sub>. Tritiated sorbitol, [<sup>3</sup>H]inulin, [<sup>14</sup>C]DMO and [<sup>14</sup>C]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. (subsidi. of UNIVAR, San Francisco, Calif.).

### Experimental Protocol

In a routine experiment, 1.0  $\mu$ Ci of [<sup>14</sup>C]DMO in ethyl acetate was dried with nitrogen, redissolved in seawater, and added to 40 ml of an egg suspension (final DMO concentration,  $5 \times 10^{-7}$  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  $\mu$ l of a seawater solution containing 0.25  $\mu$ Ci [<sup>3</sup>H]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 desiccated overnight with P<sub>2</sub>O<sub>5</sub> 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 <sup>3</sup>H and <sup>14</sup>C counts were always corrected for

spillover and efficiencies as in reference 15,<sup>1</sup> using quenched standards made up like experimental samples, except with known activities of [<sup>3</sup>H]toluene or [<sup>14</sup>C]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  $\mu$ l methylamine stock (New England Nuclear; 50  $\mu$ Ci/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  $pH_i$ s 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:

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

where:  $pK_a = pK_a$  of DMO (here taken as 6.32; see reference 11),  $D_i =$  dpm of DMO in pellet sample/mg water,  $D_o =$  dpm of DMO in supernate/mg water,  $S_i =$  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 <sup>3</sup>H<sub>2</sub>O and [<sup>14</sup>C]sorbitol (or other extracellular marker) spaces in samples parallel to those with [<sup>14</sup>C]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 ( $W_i$ ). Intracellular water is therefore  $W_i = W_i - W_o$ . 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  $11.51 \pm 0.435 \mu$ l (mean  $\pm$  SEM,  $n = 5$ ) as determined by the [<sup>3</sup>H]sorbitol-weighing protocol and  $10.98 \pm 0.264 \mu$ l ( $n = 4$ ) by the <sup>3</sup>H<sub>2</sub>O-[<sup>14</sup>C]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.

<sup>1</sup> 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

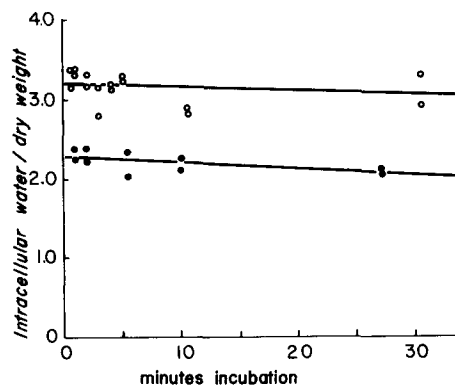


FIGURE 1 Determination of intracellular water/dry weight of unfertilized eggs of *L. pictus* (○) and *S. purpuratus* (●) with [ $^3H$ ]sorbitol. Incubation time in this and all other figures includes the centrifugation time. Least-squares regression lines are also plotted.

TABLE I  
Comparisons of  $W_i/dw$

	Average $W_i/dw$ ( $\pm$ SEM)	n
<i>L. pictus</i>		
Unfertilized	3.04 $\pm$ 0.040	47
Fertilized	3.11 $\pm$ 0.021	137
NH <sub>3</sub> (5 and 10 mM)	2.99 $\pm$ 0.029	71
<i>S. purpuratus</i>		
Unfertilized	2.17 $\pm$ 0.020	74
Fertilized	2.56 $\pm$ 0.032	117
NH <sub>3</sub> (5 and 10 mM)	2.10 $\pm$ 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  $5 \times 10^{-7}$  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  $pH_i$ s at different  $pH_o$ s); 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  $pH_o$ s, 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  $pH_o$ s 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-

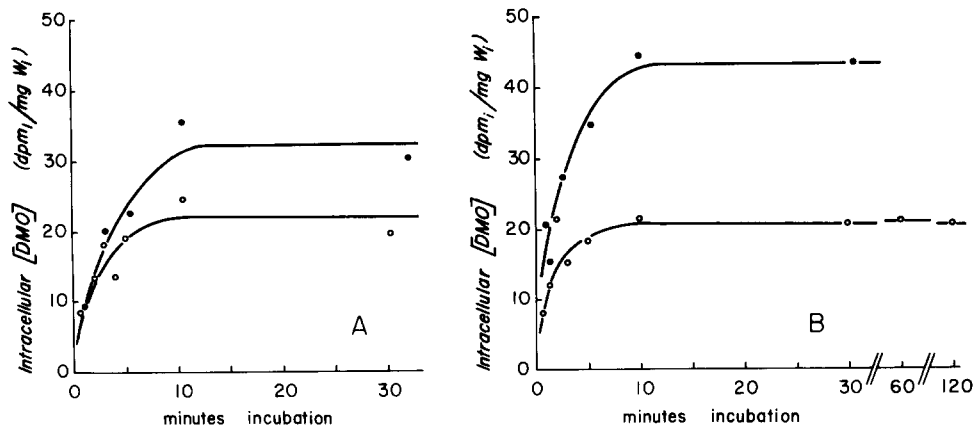


FIGURE 2 (A) Equilibration of DMO with *L. pictus* unfertilized eggs (○) and zygotes (●). Each point is the average of duplicate determinations. Zygotes were fertilized in batches at different times to create an asynchronously fertilized population so that any changes in  $pH_i$  as development proceeded 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 $pH_i \pm$ SEM	n
<i>L. pictus</i> : microelectrode*		
Unfertilized	$6.84 \pm 0.02$	44
Fertilized	$7.26 \pm 0.06$	8
<i>L. pictus</i> : DMO		
Unfertilized	$6.86 \pm 0.019$	27
Fertilized‡	$7.27 \pm 0.015$	38
<i>S. purpuratus</i> : DMO		
Unfertilized	$7.08 \pm 0.011$	36
Fertilized‡	$7.47 \pm 0.015$	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  $NH_4Cl$ . For *S. purpuratus*, the effect of either concentration of  $NH_4Cl$  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  $NH_4Cl$ , but other experiments show that  $pH_i$  does stabilize at 60 min after  $NH_4Cl$  addition (data not shown). (Note that it is the microelectrode data in Fig. 6A 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. 7A is about 0.15 pH unit higher than average.)

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

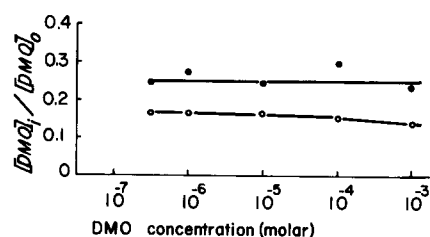


FIGURE 3 The ratio of intracellular DMO concentration ( $[DMO]_i$ ) to extracellular DMO concentration ( $[DMO]_o$ ) as the total DMO concentration is varied for *L. pictus* (○) and *S. purpuratus* (●) unfertilized eggs. Eggs were incubated for 70 min in  $pH_o$  7.7 seawater with the indicated DMO concentration. Each point is the average of duplicate determinations.

trodes is the rise time after  $NH_4Cl$  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  $7.73 \pm 0.013$  ( $\pm$  SEM,  $n = 3$ ), and  $pH_o$  6.93 seawater lowered the  $pH_i$  to  $7.24 \pm 0.012$  ( $n = 3$ ); 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  $7.05 \pm 0.009$  ( $n = 3$ ).

### 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  $6.41 \pm 0.038$  ( $n = 3$ ) and of 35-min zygotes was  $6.30 \pm 0.020$  ( $n = 3$ ). Lee and Schuldiner<sup>2</sup> 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

<sup>2</sup> H. C. Lee and S. Schuldiner. Unpublished observations.

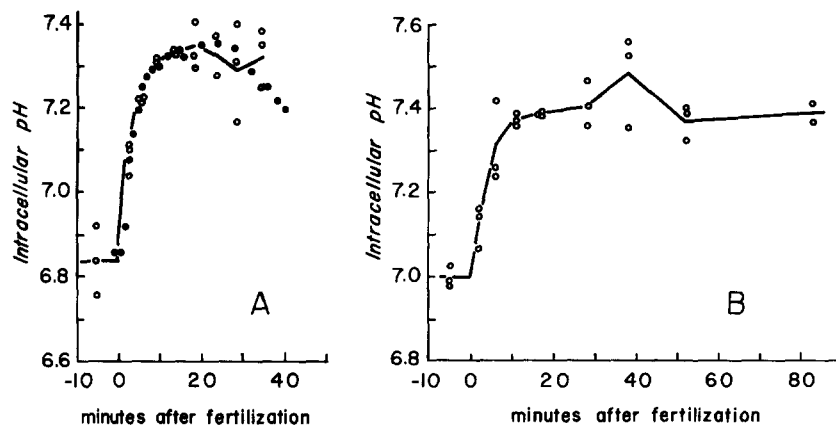


FIGURE 4 (A)  $pH_i$  before and after fertilization in *L. pictus* eggs as determined by DMO (○) and pH microelectrodes (●). The lines connect the average of each DMO triplicate. The pH-microelectrode data comes from reference 2, by permission of Dr. S. Shen. (B) *S. purpuratus* egg  $pH_i$  determined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

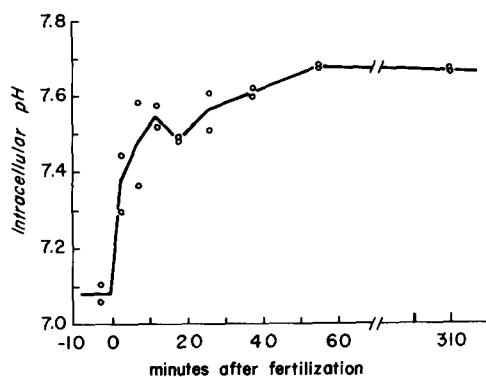


FIGURE 5  $pH_i$  before and after fertilization of *S. purpuratus* eggs in unbuffered seawater (only 2.5 mM  $\text{NaHCO}_3$ ). The  $pH_o$  had dropped from 7.85 to 7.55 by 20 min after fertilization. The line connects the averages of each DMO duplicate. At 310 min, the embryos were at the 32-cell 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*.<sup>2</sup> We conclude that the pH of these “9AA-acidic compartments” does not change after sperm activation and that the  $pH_i$  measured by methylamine is probably influenced by a low pH within these same compartments.

### $pH_i$ Regulation and $\text{Na}^+$

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

is higher than the typical values.) The tendency for  $pH_i$  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  $pH_i$  is significantly impaired in ONa seawater (zygotes were transferred to ONaSW 10 min after fertilization). As seen in Fig. 8B, embryos in acidic seawater can regulate  $pH_i$  much better if sodium is present. Samples of these embryos were fixed 120 min after fertilization. The embryos in ONaSW at pH 6.35 ( $pH_i = 6.3$ ) and 6.55 ( $pH_i = 6.5$ ) had only 20–30% chromosomal condensation, whereas condensation was >80% in all other samples (usually 95–100%).

### $pH_i$ during Cell Division

Fig. 9 illustrates representative experiments performed during the time of cell division. As observed with microelectrodes (2), *L. pictus*  $pH_i$  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*  $pH_i$  show no differences at any time between 60 and 130 min after fertilization. Unlike  $pH_i$  fluctuations in *Physarum* (27) or *Tetrahymena* (14), fluctuations of  $pH_i$  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 $pH_i$

Clearly, DMO gives reproducible values for sea urchin egg  $pH_i$  that correspond with those obtained by pH microelectrodes. We, in fact, were surprised by how well the two methods agree, considering that DMO- $pH_i$  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- $pH_i$  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- $pH_i$  and microelectrode- $pH_i$  to treatment with ammonia or acetate are generally similar.

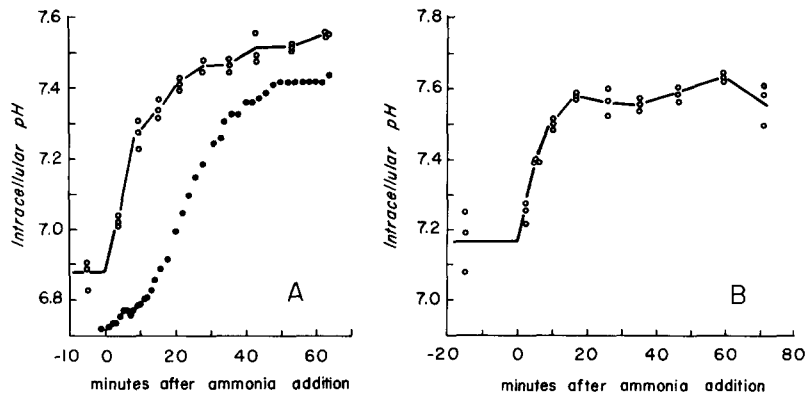


FIGURE 6 (A)  $pH_i$  before and after the addition of 5 mM  $NH_4Cl$  in *L. pictus* eggs as measured by DMO (○) and pH microelectrodes (●). The lines connect the average of each DMO triplicate. The pH-microelectrode data comes from reference 2, by permission of Dr. S. Shen. (B) *S. purpuratus* egg  $pH_i$  before and after treatment with 5 mM  $NH_4Cl$  determined by DMO, as in A.

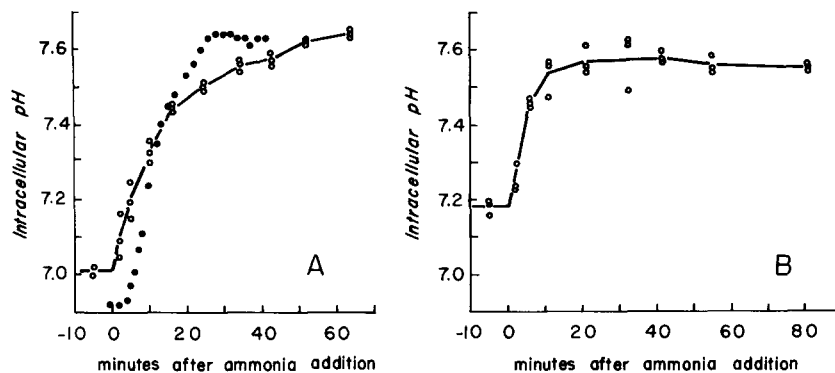


FIGURE 7 (A)  $pH_i$  before and after the addition of 10 mM  $NH_4Cl$  in *L. pictus* eggs as measured by DMO (○) and pH-microelectrodes (●). The lines connect the average of each DMO triplicate. The pH-microelectrode data comes from reference 7, by permission of Dr. S. Shen. (B) *S. purpuratus* egg  $pH_i$  before and after treatment with 10 mM  $NH_4Cl$  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<sup>2</sup> have found that the distribution of 9AA within the egg begins to change almost immediately after  $NH_4Cl$  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

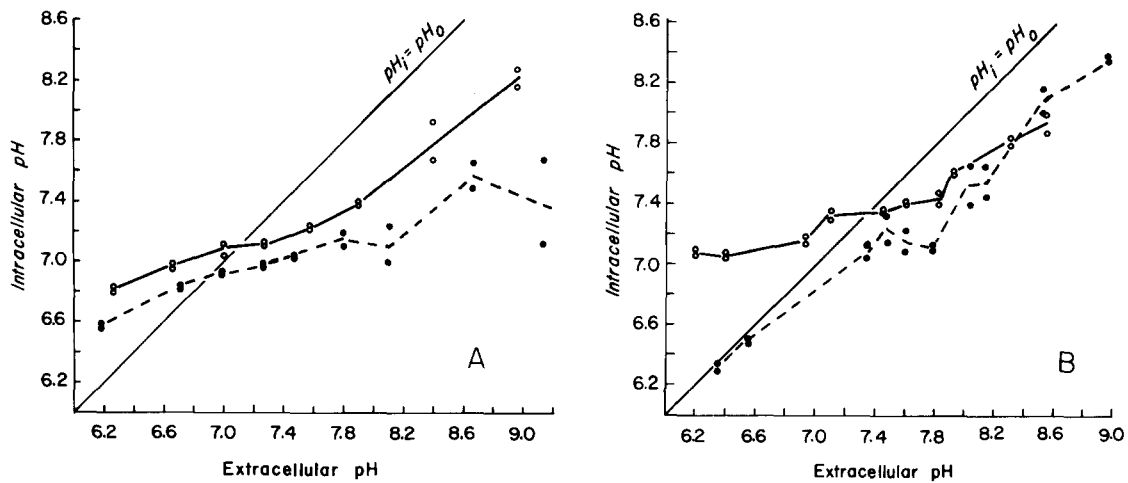


FIGURE 8 (A)  $pH_i$  of unfertilized *S. purpuratus* eggs suspended in seawater of various  $pH_o$ s. Eggs are in artificial seawater (○) or sodium-free seawater (●). Eggs were incubated at these  $pH_o$ s in DMO for 85 min before processing. Eggs in sodium-free seawater were washed three times and 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  $pH_o$ s. Zygotes are in artificial seawater (○) or sodium-free seawater (●) and were incubated at these  $pH_o$ s 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  $pH_o$ s 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).

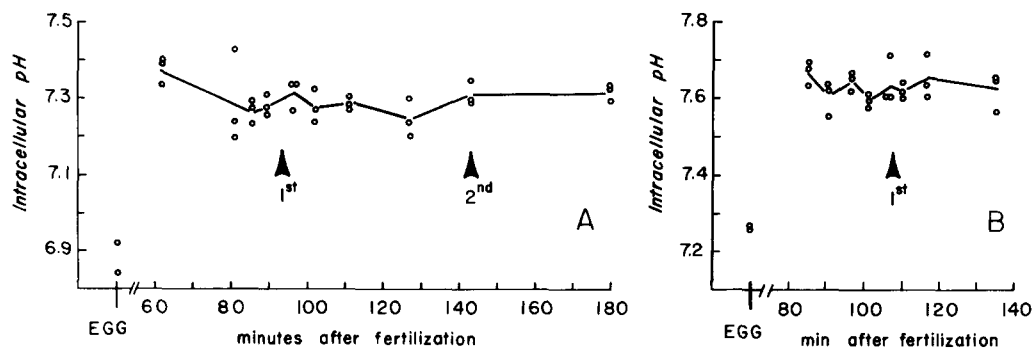


FIGURE 9 (A)  $pH_i$  of *L. pictus* embryos during the cell division cycle. Egg  $pH_i$  is also plotted for comparison. Arrowheads mark the time of 50% division for the first and second cleavages. The time between the completion of division in the "fastest" cells and the "slowest" cells was ~18 min. (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 alkalinized 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 alkalinizes 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 cytoplasmic

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<sub>i</sub> change. Ammonia increases and acetate decreases both pH<sub>i</sub> and percent-incorporation, as in sea urchins. Yet pH<sub>i</sub> 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<sub>i</sub> 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.

We thank Dr. Robert Gillies and Jennifer Price for their assistance in the early stages of this project, Dr. Albert Roos for his advice concerning these experiments and his critical reading of the manuscript, and Dr. Sheldon Shen for permission to use much of his pH-microelectrode data.

This work was supported by a grant from the National Science Foundation.

Received for publication 10 November 1980, and in revised form 19 January 1981.

NOTE ADDED IN PROOF: M. M. Winkler, G. B. Matson, J. W. B. Hershey, and E. M. Bradbury have measured pH<sub>i</sub> in *S. purpuratus* with <sup>31</sup>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).

## REFERENCES

1. Epel, D. 1978. Mechanisms of activation of sperm and egg during fertilization of sea urchin gametes. *Curr. Top. Dev. Biol.* 12:185-246.
2. Grainger, J. L., M. M. Winkler, S. S. Shen, and R. A. Steinhart. 1979. Intracellular pH controls protein synthesis rate in the sea urchin egg and early embryo. *Dev. Biol.* 68:396-406.
3. Shen, S. S., and R. A. Steinhart. 1980. Intracellular pH controls the development of new

- potassium conductance after fertilization of the sea urchin egg. *Exp. Cell Res.* 125:55-61.
4. Johnson, J. D., D. Epel, and M. Paul. 1976. Intracellular pH and activation of sea urchin eggs after fertilisation. *Nature (Lond.)*. 262:661-664.
5. Nishioka, D., and D. Epel. 1977. Intracellular pH and activation of sea urchin eggs at fertilization. *J. Cell Biol.* 75 (2, Pt. 2):40a (Abstr.).
6. Lopo, A., and V. D. Vacquier. 1977. The rise and fall of intracellular pH of sea urchin eggs after fertilisation. *Nature (Lond.)*. 269:590-592.
7. Shen, S. S., and R. A. Steinhart. 1978. Direct measurement of intracellular pH during metabolic derepression of the sea urchin egg. *Nature (Lond.)*. 272:253-254.
8. Waddell, W. J., and T. C. Butler. 1959. Calculation of intracellular pH from the distribution of 5,5-dimethyl-2,4-oxazolinedione (DMO). Application to skeletal muscle of the dog. *J. Clin. Invest.* 38:720-729.
9. Waddell, W. J., and R. G. Bates. 1969. Intracellular pH. *Physiol. Rev.* 49:285-329.
10. Roos, A. 1978. Weak acids, weak bases, and intracellular pH. *Respir. Physiol.* 33:27-30.
11. Boron, W. F., and A. Roos. 1976. Comparison of microelectrode, DMO, and methylamine methods for measuring intracellular pH. *Am. J. Physiol.* 231:799-809.
12. Spanswick, R. M., and A. G. Miller. 1977. Measurement of the cytoplasmic pH in *Nitella translucens*. *Plant Physiol. (Bethesda)*. 59:664-666.
13. Shen, S. S., and R. A. Steinhart. 1979. Intracellular pH and the sodium requirement at fertilisation. *Nature (Lond.)*. 282:87-89.
14. Gillies, R. J., and D. W. Deamer. 1979. Intracellular pH: methods and applications. *Curr. Top. Bioenerg.* 9:63-89.
15. Fox, B. W. 1976. Techniques of sample preparation for liquid scintillation counting. In *Laboratory Techniques in Biochemistry and Molecular Biology*. T. S. Work and E. Work, editors. American Elsevier Publishing Co., Inc., N. Y. 5:227.
16. Raven, J. A., and F. A. Smith. 1978. Effect of temperature and external pH on the cytoplasmic pH of *Chara corallina*. *J. Exp. Bot.* 29:853-866.
17. Leguay, J. J. 1977. The 5,5-dimethyl-2,4-oxazolinedione-2 [<sup>14</sup>C]-4-dione distribution technique and the measurement of intracellular pH in *Acer pseudoplatanus* cells. *Biochim. Biophys. Acta.* 497:329-333.
18. De Michelis, M. I., J. A. Raven, and H. D. Jayasuriya. 1979. Measurement of cytoplasmic pH by the DMO technique in *Hydrodictyon africanum*. *J. Exp. Bot.* 30:681-695.
19. Poole, D. T., T. C. Butler, and W. J. Waddell. 1964. Intracellular pH of the Ehrlich ascites tumor cell. *J. Nat. Cancer Inst.* 32:939-946.
20. Irvine, R. O. H., S. J. Saunders, M. D. Milne, and M. A. Crawford. 1960. Gradients of potassium and hydrogen ion in potassium-deficient voluntary muscle. *Clin. Sci. (Oxf.)*. 20: 1-18.
21. Johnson, C. H. 1981. Activation of echinoderm gametes: the role of intracellular pH. Appendix to PhD Thesis, Stanford University, Stanford, Calif.
22. Harvey, E. B. 1956. *The American Arbacia and Other Sea Urchins*. Princeton University Press, Princeton, N. J. 79.
23. Roos, A. 1975. Intracellular pH and distribution of weak acids across cell membranes: a study of D- and L-lactate and of DMO in rat diaphragm. *J. Physiol. (Lond.)*. 249:1-25.
24. McLaughlin, S. G. A., and J. P. Diiger. 1980. Transport of protons across membranes by weak acids. *Physiol. Rev.* 60:825-863.
25. Boron, W. F., A. Roos, and P. DeWeer. 1978. NH<sub>4</sub>Cl and other weak bases in the activation of sea urchin eggs. *Nature (Lond.)*. 274:190.
26. Epel, D. 1978. Intracellular pH and activation of the sea urchin egg at fertilization. *ICN-UCLA Symp. Mol. Cell. Biol.* 12:367-378.
27. Gerson, D. F., and A. C. Burton. 1977. The relation of cycling of intracellular pH to mitosis in the acellular slime mould *Physarum polycephalum*. *J. Cell. Physiol.* 91:297-303.
28. Ohkuma, S., and B. Poole. 1978. Fluorescence probe measurements of the intralysosomal pH in living cells. *Proc. Natl. Acad. Sci. U. S. A.* 75:3327-3331.
29. Wibo, M., and B. Poole. 1974. Protein degradation in cultured cells. *J. Cell Biol.* 63:430-440.
30. Amenta, J. S., and S. C. Brocher. 1980. Mechanism of protein turnover in cultured fibroblasts: differential inhibition of two lysosomal mechanisms with insulin and NH<sub>4</sub>Cl. *Exp. Cell Res.* 126:167-174.
31. Johnson, C. H., and D. Epel. 1980. Intracellular pH does not regulate protein synthesis of starfish oocytes. *J. Cell Biol.* 87 (2, Pt. 2): 142a (Abstr.).
32. Begg, D. A., and L. Rebhun. 1979. pH regulates the polymerization of actin in the sea urchin egg cortex. *J. Cell Biol.* 83:241-248.
33. Heiple, J. M., and D. L. Taylor. 1980. Intracellular pH in single motile cells. *J. Cell Biol.* 86:885-890.