Regulation of Intracellular pH in Sea Urchin Eggs by Medium Containing Both Weak Acid and Base

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Key words: intracellular pH/acetic acid/ammonia/pyranine/sea urchin egg/pH buffering power

ABSTRACT. To establish a method of pH regulation and to understand the pH regulation mechanism in the cell, we investigated the pH response of unfertilized or fertilized eggs of sea urchin, applying sea water containing both weak permeant acid, acetic acid and/or base, ammonia, to eggs. Pyranine was employed as a pH indicator to measure intracellular pH (pHi) by microfluorometry. The unfertilized/fertilized eggs had a pHi of 6.80/7.34 and 6.81/7.32 for Schaphechinus mirabilis and Hemicentrotus pulcherrimus, respectively. With the addition of both acetic acid and ammonia to the media, pHi changed linearly against extracellular pH (pH0) between 6–8 and was almost equal to pH0 at the concentration of 20 mM acetate and ammonia. This mixed application was proved to be available for regulating pHi at the desired value within a wide range involving the original pHi by a single solution system. pHi after the treatment was dependent on various factors, such as the concentration of the weak acid and base, the pH before the treatment, and pH buffering power in the cytoplasm. The latter was estimated to be 43 mM and 58 mM in unfertilized and fertilized eggs, respectively, from the measurement of pHi change induced by microinjecting a HEPES solution, assuming that the pH buffering power is caused by phosphate.

pH is one of the important factors that regulate the activity of the cell. For example, when the intracellular pH (pHi) in the unfertilized egg of sea urchins is suppressed at a lower level, the egg is placed in a quiescent state. With fertilization as a turning point, the pHi rises and the egg starts development (3, 8, 11, 20, 25). In Xenopus eggs, changes in pHi occur during cell cycle from interphase to mitosis (7).

For investigating the role of pHi, it is necessary to regulate pHi at an adequate level. There are three main methods for regulating pHi. The first is injection of pH buffer solutions into the cell. The second is treatment of the cell with an ionophore such as nigericin. The third is the application of weak acid or base in an extracellular medium. Because the third method is straightforward, it has been used widely (see 18 for a review).

The principle of this method using a weak acid and a weak base is briefly as follows. When the pH (pH0) of a medium containing acetic acid as the weak acid is lowered, molecules of acetic acid, but not acetic ions produced in the medium, can permeate the cell membrane. Inside the cell, they separate into H+ and acetic ions. This results in the decrease in pHi. In contrast, when the pH of a medium containing ammonia as the weak base is elevated, molecules of ammonia enter the cell, associate with H+, and become ammonium ions. As a result, the pHi is shifted higher (10).

In sea urchin, this method has been utilized in many investigations (2, 6, 17, 19, 21, 26). In these previous reports, either the weak acid or the weak base has been used separately. Although acetic acid is suitable only for lowering pHi and ammonia for raising pHi, the pHi is not predictable with pH0 in either case, and the measurement of pHi is necessary in both experiments. Moreover, it is difficult to attain pHi around the original pHi with these treatments. In order to regulate pHi within a wide range of pH involving the original pHi by a single solution system, we had an idea to apply sea water containing both weak acid and weak base to the eggs.

In this study, we investigated the pHi response of unfertilized or fertilized eggs when applying sea water con-
taining both weak acid and base in order to determine whether or not this method avails itself to the regulation of pH₄ at adequate values in sea urchin eggs. To measure pH₄ quantitatively, we employed pyranine as a fluorescent pH indicator. We also reported the pH₄ response of the cell against the separate application of either weak acid or weak base, for comparison. Moreover, we discussed the mechanism of pH₄ response of the cell in reference to a physicochemical model for the cell membrane which is passively permeated only by molecules of both acetic acid and ammonia as described in the appendices.

MATERIALS AND METHODS

Gametes. Sea urchins, Schaphechinus mirabilis and Hemicentrotus pulcherrimus were used. Gametes were obtained by injection of 1 mM acetylcholine sea water into the body cavity. Eggs were washed three times with artificial sea water (Jamarin U, Jamarin Laboratory, Osaka, Japan), kept at 15°C, and used within 4 hrs of shedding. Sperm were collected "dry", kept at 4°C in a refrigerator and diluted just before use. Fertilized eggs were demembranated by 1 M urea, washed in Ca-free sea water (Ca free Jamarin U, Jamarin Laboratory) shortly after insemination, and cultured in artificial sea water. They were used from 15 to 40 mins after fertilization. All experimental operations were carried out at 20–22°C.

Solutions. Pyranine (HPTS, l-hydroxy-pyrene-3,6,8-trisulphate, Tokyo Kasei, Tokyo, Japan) was dissolved at 10 mM in 50 mM MOPS (3-morpholinopropanesulfonic acid) adjusted to pH 7.0.

The composition of pH reference solutions was as follows: 40 mM MES (2-morpholinoethanesulfonic acid, monohydrate), 40 mM MOPS, 40 mM EPPS (3-[4-(2-hydroxyethyl)-1-piperazinyl] propanesulfonic acid), and 105.9–174.6 mM KCl. pH was adjusted by KOH to 6.3, 6.8, 7.3 or 8.0. Because the pKₐ of pyranine is dependent on ionic strength, appropriate amounts of KCl were added in pH reference solutions to adjust their ionic strength to 200 mM.

Four types of artificial sea water, whose composition and/or pH were changed (pHSW), were applied as perfusion media. The composition and pH of these media are shown in Table I. Normal-pHSW was artificial sea water containing neither sodium acetate nor ammonium chloride, but its pH was changed. Ac-pHSW and NH₃-pHSW contained sodium acetate and ammonium chloride, respectively. Ac⁺NH₃-pHSW was the sea water containing both sodium acetate and ammonium chloride. The concentration of acid or base was usually 20 mM. In the case of studying the relation between pH₄ change and concentration of acid and/or base, the concentrations of acid and base were used at 10, 20, 30 and 40 mM. The pH of perfusion media was adjusted by a pH meter (F-7, Hitachi-Horiba, Tokyo, Japan).

Measurement of the ratio of fluorescence. A Nikon photomicroscope equipped with epillumination (EFD, Nikon) was used. A 50 W halogen or 100 W mercury arc lamp was used as a light source for fluorescence. Light passed through one of two interchangeable narrow-band excitation filters (415 or 454 nm interference filter, Optical Cortings Japan, Shizuoka, Japan) was reflected by the dichroic mirror and illuminated the sample through an objective (Fluor 20 x /NA 0.75). Fluorescence was collected by the objective, passed through the dichroic mirror, and then passed through a barrier filter (>520 nm). It was then detected with a photomultiplier tube (1P21, Hamamatsu Photonics, Hamamatsu, Japan) mounted at the focal plane of the microscope camera. The signal was amplified and plotted directly on a sheet of chart paper with a chart recorder. The ratio of fluorescence excited at 454 nm to that excited at 415 nm was calculated.

pH calibration curve. To make a pH calibration curve, 10 mM pyranine was added to the pH reference solutions mentioned above, so that the final concentration of pyranine was 30 μM. The resultant solutions were loaded into microslides (Vitro Dynamics Inc. , Rockaway, N.J.), which are long glass tube with a rectangular orifice of 100 μm x 1000 μm. They were set on the microscopic stage so that light path length came to 100 μm. The ratio of fluorescence intensity at two

<table>
<thead>
<tr>
<th>Table I. Composition of pHSW (mM)</th>
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<tbody>
<tr>
<td>Composition</td>
</tr>
<tr>
<td>CH₃COONa</td>
</tr>
<tr>
<td>NH₄Cl</td>
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<tr>
<td>NaCl</td>
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<td>KCl</td>
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</tr>
<tr>
<td>HEPES</td>
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<td>pH range</td>
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</tbody>
</table>

Arrow shows the same figure as the left column. pH was adjusted by NaOH.
Regulation of Intracellular pH in Sea Urchin Eggs

![Calibration curve of pyranine.](image)

**Fig. 1.** Calibration curve of pyranine. The fluorescence ratio (454 nm/415 nm) of pyranine is plotted against pH. Each point of data is the mean of 3–6 measurements. The standard deviation of each point is below 0.01. The solid line represents a calibration curve. From the Henderson-Hasselbach equation of pyranine, the equation of the calibration curve that indicates the relation between pH and the ratio of fluorescence intensities (R) is obtained as

$$R = \frac{B(A-B)}{10^{-\mathrm{pH}}+A}$$

where A is constant, B is the maximum ratio of fluorescence, C is the minimum ratio of fluorescence, and R is the fluorescence ratio at two wavelengths of 454 nm and 415 nm at a certain pH. A, B, and C were calculated statistically from the values of R and pH. The pKₐ of pyranine was estimated to be 7.00±0.07 at the ionic strength of 200 mM from the curves such as this one.

**Microinjection and measurement of pH.** pH was measured by microinjecting the fluorescent pH indicator, pyranine, into the egg. Injection procedures were based on the method of Hiramoto and described elsewhere (8, 9). Eggs stuck firmly to the surface of a coverslip coated with 0.01% poly-L-lysine and the coverslip was set on the injection chamber. Pyranine solution was injected with 0.3–0.4% of the egg volume, such that the final concentration of pyranine in the egg cytoplasm was 30–40 μM. Following the injection, the coverslip was transferred to the perfusion chamber and mounted on the stage of a Nikon fluorescence microscope. First of all, pH was measured in normal sea water before application of PHSW. The PHSW was applied to one edge of the perfusion chamber with a pipette and was withdrawn by a piece of tissue paper (Kimwipe S200, Kimberly-Clark Corp.) from the other edge. In our experimental system, the extracellular medium was changed rapidly and so it could be replaced within 30 seconds after the onset of perfusion. After the onset of application of PHSW, pH was measured discontinuously during the 15–20 minutes period. In some experiments, pH was measured for more than 15–20 min even after returning to normal sea water from PHSW.

**Measurements of pH buffering power of eggs.** pH buffering power was calculated from the pH change when 1 M HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid) solution at pH 8.0 was microinjected into eggs. The volume of the HEPES solution microinjected was 3% of the egg volume and then the final concentration of HEPES in the egg cytoplasm was 30 mM. From the pH difference between pH before and after microinjection, pH buffering power was calculated according to the equations presented in appendix (1).

**RESULTS**

1. **pH of unfertilized and fertilized eggs of sea urchins**

To regulate pH, at appropriate values, the pH has to be accurately measured. We employed the microfluorometric measurement by microinjecting the fluorescent pH indicator, pyranine. Upon fertilization, the pH of the fertilized egg was measured at 30–40 s after insemination and reached a stable level within 3–4 min. Therefore, pH was measured at 15–40 min after insemination in this study. As shown in Table II, pH was measured at about 6.8 and 7.3 at a level of 10⁻² order in unfertilized and fertilized eggs, respectively, of both species, *S. mirabilis* and *H. pulcherrimus*. The pH values in unfertilized and fertilized eggs, and the pH change upon fertilization obtained in this study were similar to those in the previous reports (8, 12, 20, 22). The standard deviation of pH was, however, varied and was about 0.1 in *S. mirabilis*. This was possibly caused by the difference between individual eggs and batches, but not by any perturbation in pH caused by the measuring procedures, since fertilization and/or cleavage proceeded normally in these pH-measured eggs. The standard deviation was also similar to those obtained by means of microelectrode or microinjecting pH indicator (20, 22). Judging from these data, the accuracy of pH measured by pyranine was deemed sufficient to measure the pH change during the pH regulation.

<table>
<thead>
<tr>
<th>Species</th>
<th>pHₜ</th>
<th>pHₜ</th>
<th>pHₜ</th>
<th>pHₜ</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Scaphechinus mirabilis</em></td>
<td>6.80±0.08 (51)</td>
<td>7.34±0.09 (185)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Hemicentrotus pulcherrimus</em></td>
<td>6.81±0.05 (90)</td>
<td>7.32±0.06 (89)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

pH is represented as mean±SD., the figure in parentheses is the number of measured samples.

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Table II. INTRACELLULAR pH OF SEA URCHIN EGGS
2. Regulation of pHj by treatment with PHSW

In sea urchins, ammonia has been used for raising pHj and acetic acid has been used for lowering pHj in many reports. In this study, we applied to the eggs the medium containing both acetic acid and ammonia at equal concentration. Briefly, by treatment with this mixture (Ac+NH3-PHSW), pHj was found to be regulated in a wide pH range around the original pHj by a single solution system and pHj after treatment was found to nearly coincide with pHj. We report below the results of the mixed treatment in comparison with the results of the separate treatment with either acetic acid or ammonia.

2-1. pHj response during treatment with PHSW in fertilized eggs

The time course of pHj change in fertilized eggs during treatment with PHSW. Fig. 2a shows typical examples of the time course of pHj change in fertilized eggs during treatment with 20 mM Ac+NH3-PHSW at pH 6.80, 7.30 and 7.80. The time course of pHj change had two phases; pHj shifted rapidly in the first phase, especially within one minute at pH 7.80. Then it decreased slightly and became almost steady in the second phase.

Typical examples of the time course of pHj change during treatment with 20 mM Ac-PHSW are shown in Fig. 2b. pHj decreased in a similar manner to the case of Ac+NH3-PHSW at pH 6.8, though the steady level of pHj is different. Typical examples of the time course of pHj change during treatment with NH3-PHSW are also shown in Fig. 2b. In the case of treatment with NH3-PHSW at pH 7.8, the pHj changed in a similar manner to that in the case of Ac+NH3-PHSW at pH 7.8, whereas, in the case at pH 7.3, it shifted to about pH 7.7 which is much higher than that during treatment with Ac+NH3-PHSW at pH 7.3; it then gradually decreased, and became almost steady around pH 7.6. In all cases, pHj was basically steady about 5-10 min after the onset of treatment.

The effects of concentration in acetic acid and/or ammonia. We then studied the effects of concentration of ammonia and/or acetic acid on pHj change. As mentioned above, pHj was steady after 5-10 min of treatment; hence the mean pHj values of each sample at 10 min after the onset of treatment with Ac+NH3-PHSW are shown in Fig. 3. Their concentrations were 10, 20, 30 and 40 mM at three values of pH0, that is, 6.80, 7.30 and 7.80. At 20 and 30 mM, the values of pHj almost coincided with those of pH0. At 10 mM, the pH change was less than that at 20 or 30 mM and the standard deviation was larger; that is, pHj change varied in individual eggs. The concentration of 40 mM of acetic acid and ammonia was so high that eggs reacted abnormally.

In Fig. 4, the mean pHj values of each sample at 10 min after the onset of treatment with Ac-PHSW or with NH3-PHSW are shown. Ac-PHSW at the concentrations of 10, 20, and 40 mM of acetic acid at pH 6.8 and NH3-PHSW at the concentrations of 10, 20 and 40 mM of ammonia at pH 7.8 were applied. The pHj during treatment changed depending on the concentration of weak base or weak acid in PHSW. At 10 mM, the standard deviation was larger than that at the other concent-

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Fig. 2. Time course of pHj change in fertilized eggs of S. mirabilis. (a) Treatment with Ac+NH3-PHSW. Typical examples are shown in the cases of 20 mM Ac+NH3-PHSW at pH 6.80 (○), 7.30 (△) and 7.80 (□). (b) Treatment with Ac-PHSW or NH3-PHSW. Typical examples are shown in the cases of 20 mM Ac-PHSW at pH 6.8 (○) and 7.3 (▽) or 20 mM NH3-PHSW at pH 7.3 (△), and 7.8 (□).
Fig. 3. Relation between pHj and concentration of acetic acid and ammonia in Ac+NH₃-PHSW in the case of fertilized eggs of *H. pulcherrimus*. The pHj 10 min after the onset of treatment is plotted against acetic acid and ammonia concentration of Ac+NH₃-PHSW (pH 6.80 (○), 7.30 (△) and 7.80 (□)). Each point of data represents the mean of 5-16 measurements and error bars correspond to the standard deviation. The lower, middle, and upper broken lines represent the curves calculated from appendix (2) in three conditions of pH of PHSW (6.8, 7.3 and 7.8, respectively) assuming the cytoplasm has 60 mM phosphate for intrinsic pH buffer. The pHj value of 7.32 obtained in this study is used for pHj before treatment.

Fig. 4. Relation between pHj and concentration of acetic acid in Ac-pHSW or ammonia in NH₃-pHSW in fertilized eggs of *S. mirabilis*. The pHj 10 min after the onset of treatment is plotted against acetic acid concentration of Ac-pHSW at pH 6.8 (○) or ammonia concentration of NH₃-pHSW at pH 7.8 (□). Each point of data represents the mean of 3-12 measurements and error bars correspond to the standard deviation. The lower or upper broken line represents the curve calculated from appendix (2) as a function of the concentration of acetic acid or ammonia, assuming the cytoplasm has 60 mM phosphate for intrinsic pH buffer. The pHj value of 7.34 obtained in this study is used for pHj before treatment.

Table III. pHj in fertilized eggs before and after the treatment with various pHSW

<table>
<thead>
<tr>
<th>Species</th>
<th>pHSW</th>
<th>pHj</th>
<th>n</th>
<th>pHj before treatment</th>
<th>pHj after treatment (10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hemicentrotus</em></td>
<td>Ac+NH₃-pHSW</td>
<td>6.80</td>
<td>6</td>
<td>7.32±0.02</td>
<td>6.78±0.01</td>
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<tr>
<td><em>pulcherrimus</em></td>
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<td>11</td>
<td>7.42±0.10</td>
<td>7.28±0.05</td>
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<tr>
<td></td>
<td>7.80</td>
<td>9</td>
<td>7.45±0.07</td>
<td>7.75±0.04</td>
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<tr>
<td><em>Scaphechinus</em></td>
<td>Ac+NH₃-pHSW</td>
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<td>5</td>
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<td>6.73±0.02</td>
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<td><em>mirabilis</em></td>
<td>6.80</td>
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<td></td>
<td>7.30</td>
<td>16</td>
<td>7.37±0.09</td>
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<tr>
<td></td>
<td>7.85</td>
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<tr>
<td></td>
<td>6.62</td>
<td>3</td>
<td>7.29±0.04</td>
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<tr>
<td></td>
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<tr>
<td></td>
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<td>7.90</td>
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<td></td>
<td>8.00</td>
<td>3</td>
<td>7.42±0.08</td>
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<table>
<thead>
<tr>
<th>Species</th>
<th>pHSW</th>
<th>pHj</th>
<th>n</th>
<th>pHj before treatment</th>
<th>pHj after treatment (10 min)</th>
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<tr>
<td><em>Ac-pHSW</em></td>
<td>7.30</td>
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<td></td>
<td>7.70</td>
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<td>8.00</td>
<td>3</td>
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</table>

n is number of samples.
trations; that is, pH change varied in individual eggs.

The dependency of pH on pH of PHSW. Judging from the above results, a concentration of 20 mM was suitable for treatment. We subsequently examined the relationship between pH and pH of PHSW at 20 mM concentrations. In Table III, pH values 10 min after the onset of treatment are summarized when fertilized eggs of S. mirabilis and H. pulcherrimus were treated with 20 mM Ac+-NH$_3$-PHSW, Ac-PSW or NH$_3$-PHSW. As shown in Fig. 5, in which the mean pH values of S. mirabilis eggs treated with 20 mM Ac+-NH$_3$-PHSW are plotted, pH nearly coincides with each pH$_0$. Moreover, it could be regulated in the pH range, including pH 7.3 which is the original pH of the fertilized egg.

In Fig. 6, the mean pH values 10 min after the onset of treatment with Ac-PSW or NH$_3$-PHSW are plotted versus pH$_0$. At every pH$_0$, Ac-PSW decreased pH and NH$_3$-PHSW increased pH. In both Ac-PSW and NH$_3$-PHSW, pH did not coincide with pH$_0$, especially around pH 7.3.

pH change after returning to normal seawater from PHSW. In order to test whether pH change is caused by the ammonia and/or acetic acid, we also examined the pH change after returning to normal seawater at 20 min of each PHSW treatment. As shown in Fig. 7, by returning from Ac+-NH$_3$-PHSW at pH 6.80, pH recovered gradually to the original pH before treatment with PHSW. By returning to normal sea water from Ac+-NH$_3$-PHSW at pH 7.30 and 7.80, pH suddenly drop-

![Fig. 5. Relation between pH and pH$_0$ (pH of Ac+-NH$_3$-PHSW) in fertilized eggs of S. mirabilis. The pH 10 min after the onset of treatment with 20 mM Ac+-NH$_3$-PHSW is plotted against pH$_0$. Data correspond to those in Table III. The broken line represents the curve calculated from appendix (2) as a function of pH of Ac+-NH$_3$-PHSW assuming the cytoplasm has 60 mM phosphate for intrinsic pH buffer. The pH$_0$ value of 7.34 is used for pH$_0$ before treatment. The solid line represents that pH$_0$=pH$_0$.](image1.png)

![Fig. 6. Relation between pH and pH$_0$ (pH of Ac-PSW or NH$_3$-PHSW) in fertilized eggs of S. mirabilis. The pH 10 min after the onset of treatment with 20 mM Ac-PSW (○) or NH$_3$-PHSW (□) is plotted against pH$_0$. Data correspond to those in Table III. The lower or upper broken line represents the curve calculated from appendix (2) as a function of pH of Ac-PSW or NH$_3$-PHSW, respectively, assuming the cytoplasm has 60 mM phosphate for intrinsic pH buffer. The pH$_0$ value of 7.34 is used for pH$_0$ before treatment.](image2.png)

![Fig. 7. Time course of pH$_0$ change in fertilized eggs of S. mirabilis after returning to normal sea water from Ac+-NH$_3$-PHSW. Typical examples are shown in the cases of returning from 20 mM Ac+-NH$_3$-PHSW at pH 6.80 (○), 7.30 (△) and 7.80 (□).](image3.png)
ped to near pH 7.0 and then increased, recovering to the original pH. By returning from NH$_3$-pHSW, pH$_i$ suddenly dropped to pH 7.0-7.1 and recovered to the original pH$_i$, in a similar manner to the case of treatment with Ac$^+$/NH$_3$-pHSW at higher pH. By returning from Ac-pHSW, pH$_i$ recovered gradually to pH$_i$ before the treatment. In all cases of returning to normal sea water, pH$_i$ recovered to the original pH$_i$.

2-2. pH$_i$ response during treatment with pHSW in unfertilized eggs

In the case of unfertilized eggs, the results similar to those in fertilized eggs were obtained with regard to the time course of pH$_i$ change and the effect of concentrations on pH$_i$ (data not shown).

In Table IV, pH$_i$ after 10 min treatment with pHSW is summarized when unfertilized eggs of *S. mirabilis* and *H. pulcherrimus* were treated with 20 mM Ac$^+$/NH$_3$-pHSW, Ac-pHSW or NH$_3$-pHSW. The results in both species were similar. Similar to the case of fertilized eggs, the pH$_i$ of unfertilized eggs after 5-10 min of treatment with 20 mM Ac$^+$/NH$_3$-pHSW also coincided with pH$_i$ and pH$_i$ could be regulated in the pH range including pH 6.8 (Fig. 8).

Unfertilized eggs were treated with Ac-pHSW at three pH values of 6.30, 6.80 and 7.30 and with NH$_3$-pHSW at three pH values of 6.80, 7.30 and 7.80. At every pH$_o$, Ac-pHSW decreased pH$_i$ and NH$_3$-pHSW increased pH$_i$ (Fig. 9 and Table IV). In both treatments with Ac-pHSW and NH$_3$-pHSW, pH$_i$ did not coincide with pH$_o$, especially around pH 6.8, which was the original pH$_i$ in unfertilized eggs.

By returning to normal sea water from pHSW, pH$_i$ recovered to pH$_i$ before the treatment in a similar manner to the case of fertilized eggs. Briefly, by returning from NH$_3$-pHSW or Ac$^+$/NH$_3$-pHSW in pH range of 6.80 to 7.80, pH$_i$ suddenly dropped to around 6.6 and then recovered to pH$_i$ before the treatment. By returning from

![Fig. 8](image)

**Table IV.** pH$_i$ in unfertilized eggs before and after the treatment with various pHSW

<table>
<thead>
<tr>
<th>Species</th>
<th>pHSW</th>
<th>pH$_o$</th>
<th>n</th>
<th>pH$_i$ before treatment</th>
<th>pH$_i$ after treatment (10 min)</th>
</tr>
</thead>
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<tr>
<td><em>Hemicentrotus</em></td>
<td>Ac$^+$/NH$_3$-pHSW</td>
<td>6.30</td>
<td>6</td>
<td>6.78±0.02</td>
<td>6.30±0.01</td>
</tr>
<tr>
<td>pulcherrimus</td>
<td>6.80</td>
<td>3</td>
<td>6.84±0.06</td>
<td>6.81±0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.30</td>
<td>3</td>
<td>6.79±0.03</td>
<td>7.30±0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.80</td>
<td>6</td>
<td>6.94±0.05</td>
<td>7.75±0.05</td>
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</tr>
<tr>
<td><em>Scaphechinus</em></td>
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<td>1</td>
<td>6.74</td>
<td>6.20</td>
</tr>
<tr>
<td>mirabilis</td>
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<td>6.38</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>6.80</td>
<td>1</td>
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</tr>
<tr>
<td></td>
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<td>6</td>
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<td>3</td>
<td>6.79±0.04</td>
<td>7.78±0.05</td>
<td></td>
</tr>
<tr>
<td><em>Ac</em>-pHSW</td>
<td>6.30</td>
<td>3</td>
<td>6.74±0.05</td>
<td>6.26±0.02</td>
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<tr>
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<td>6.80</td>
<td>5</td>
<td>6.81±0.07</td>
<td>6.44±0.02</td>
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<td>5</td>
<td>6.80±0.04</td>
<td>6.64±0.03</td>
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<tr>
<td><em>NH$_3$</em>-pHSW</td>
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<td>5</td>
<td>6.78±0.08</td>
<td>7.14±0.05</td>
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<td>6.76±0.06</td>
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<td>7.80</td>
<td>4</td>
<td>6.76±0.04</td>
<td>7.67±0.04</td>
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</tr>
</tbody>
</table>

n is number of samples.
M.S. Hamaguchi et al.

Fig. 9. Relation between pHj and pH0 (pH of Ac-pHSW or NH3-pHSW) in unfertilized eggs of S. mirabilis. The pHj 10 min after the onset of treatment with 20 mM Ac-pHSW (•) or NH3-pHSW (■) is plotted against pH0. The data correspond to those in Table IV. The lower or upper broken line represents the curve calculated from appendix (2) as a function of pH of Ac-pHSW or NH3-pHSW, respectively, assuming the cytoplasm has 40 mM phosphate for intrinsic pH buffer. The pHj value of 6.80 is used for pHj before treatment.

Ac-pHSW or Ac+NH3-pHSW at pH 6.10 and 6.30, pHj recovered gradually to the pretreatment value.  

2-3. pHj response during treatment with normal pHSW

In order to examine whether pHj change is caused by other components of sea water than acetic acid/or ammonia, pHj was measured during treatment with normal-pHSW. Normal-pHSW at pH 6.80 without acetic acid or ammonia was applied to fertilized eggs. This pHSW did not affect pHj within 20 min of treatment. By treatment with normal-pHSW at 6.30 on unfertilized eggs, pHj was not affected in most eggs, though the pHj of some eggs decreased slightly (ca. 0.05 pH unit) after 20 min treatment. Before use, the unfertilized egg was stored in normal sea water whose pHj was at about 8.2. Hence, the pHj of the unfertilized egg did not change in normal sea water within the pH range of 6.3–8.2, although by the treatment with normal sea water whose pHj was 9.0 for 1.5–2.0 h, the pHj was reported to increase (2).

3. pH buffering power of unfertilized and fertilized eggs of sea urchins

From a previous study (8), it can be inferred that the fertilized eggs have a strong pH buffering power and that the value of pHj change after treatment by pHSW is dependent on the buffering power of the cell. This is because the buffering power supplies H+ or consumes H+ in the cell in order to minimize pHj change which would be caused by acetic acid or ammonia permeating the cell membrane. Here we determined the pH buffering power of the cell experimentally. In principle, a constant volume of pH buffer solution that does not permeate the cell membrane is microinjected into the egg cytoplasm. As a result, pHj changes to a definite value. The value of pHj after injection would depend on the intrinsic buffering power and some other factors (refer to appendix (1)).

In this experiment, the eggs of H. pulcherrimus were used. As shown in appendix (1), the pH buffering power (the amount of [H+] necessary for changing one pH unit) was obtained from the difference in pH between pHj before and after injection. The pH buffering power of the egg was calculated to be 23 mM in unfertilized eggs and 32 mM in fertilized eggs. As the difference between the values of unfertilized and fertilized eggs is statistically significant (t-test, probability < 0.5%), the buffering power is deemed to increase after fertilization. Assuming the intrinsic pH buffer was phosphate, the unfertilized and fertilized eggs had 43 mM and 58 mM

| Table V. pHj difference between before and after injection of HEPES solution and estimation of the intrinsic pH buffer in the egg cytoplasm. |
|---------------------------------|-----------------|-----------------|
|                                | unfertilized    | fertilized      |
| △pH                            | 0.50            | 0.28            |
| buffering powerb (mmol·pH−1·liter−1) | 23              | 32              |
| intrinsic pH bufferc mean (mM)  | 42.5            | 58.0            |
| S.D.                            | 6.3             | 5.6             |
| range (mM)                      | 32.4–56.4       | 46.5–67.9       |
| sample number                   | 12              | 8               |

a △pH is the difference between pHj before and after 3% injection of HEPES solution (1 M, pH 8.0).

b Buffering power is the mean of △[H+]/△pH. △[H+] is the amount of [H+] which associates to HEPES by pH change (refer to appendix (1)).

c The intrinsic power is the concentration in mM when it is supposed to be phosphate.
of phosphate, respectively. These results are summarized in Table V.

**DISCUSSION**

To establish a method of pH\textsubscript{i} regulation and to understand the pH regulation mechanism in the cell, we investigated pH\textsubscript{i} response in unfertilized eggs and fertilized ones when they were perfused with sea water of various pH that contained the weak acid, acetic acid, and/or the weak base, ammonia.

**Measurement of pH\textsubscript{i} by pyranine**

For the above purposes, the accurate measurement of pH\textsubscript{i} is indispensable. In this study, the fluorescent pH indicator, pyranine, was employed for measuring pH\textsubscript{i} by microinjecting it into eggs. Various methods have been reported for measuring pH\textsubscript{i} in the eggs of sea urchins; pH electrodes (20), NMR (27), DMO (12), and pH indicators (8, 17, 22). Each method has some advantages and limitations, for example with respect to time resolution, spatial resolution, sample volumes, and so on (for reviews, 16, 18). Among these methods, the pH indicator enables the rapid and simultaneous measurement of pH\textsubscript{i} during perfusion. In the previous study (8), using phenol red, which is one of the pH indicators, pH\textsubscript{i} was measured by comparison with the colors of the pH reference solutions by eye. In this study, the photoelectric method using the more sensitive fluorescent pH probe, pyranine, was employed for pH\textsubscript{i} measurement.

Pyranine is suitable for measurement of pH around the neutral range (pK\textsubscript{a}=7.22, Wolfbeis et al. 1983, pK\textsubscript{a}=7.00±0.07 at 200 mM in ionic strength, in this study). It has been demonstrated that the measurable pH range of pyranine coincides closely with the pH range in eggs of sea urchins as mentioned above. The usefulness of pyranine has also been reported in the cell of some other species (4, 5, 13, 24). For pH\textsubscript{i} measurement of sea urchin eggs, pyranine has been found to be a more suitable indicator than BCECF (2',7'-bis(carboxyethyl)-4 or 5-carboxyfluorescein, pK\textsubscript{a}=6.97) which has been used frequently in some other species. Because BCECF may be absorbed very quickly into the granules in the egg cytoplasm, pH\textsubscript{i} of sea urchin eggs cannot be measured by BCECF (Hamaguchi, unpublished data, 15, 22), although BCECF-dextran, a modified BCECF, is used for the measurement of cytoplasmic alkalization during fertilization of sea urchin eggs (17, 22).

Absolute calibration of pH\textsubscript{i} is generally difficult in pH measurement. In this study, we made the calibration curve using reference solutions, whose ionic strength was 200 mM which was similar to that of the egg cytoplasm, considering the fluorescence ratio of pyranine is dependent on ionic strength (28). As a result, we obtained constant and reproducible pH values, which are consistent with previous reports.

**pH buffering power in the egg cytoplasm**

If there were no buffering power in the cytoplasm, pH\textsubscript{i} would be equal to pK\textsubscript{a} of the weak acid or weak base in spite of the pH\textsubscript{o} of the applied PHSW. At pH\textsubscript{i} level of pK\textsubscript{a}, cells are barely alive. However, they do not die when they are treated with weak acid or base, because pH\textsubscript{i} would be stabilized to a certain value between pH\textsubscript{i} before treatment and pK\textsubscript{a}, by means of intrinsic buffering power. Therefore, it is important to consider the pH buffering power in the cytoplasm on the pH\textsubscript{i} regulation. It had been suggested that the egg of sea urchins has strong pH buffering power (8). In this study, more precise results have been obtained.

**Regulation of pH\textsubscript{i}**

In this study, it was revealed that PHSW containing both acetic acid and ammonia could regulate pH\textsubscript{i} in the egg cytoplasm over a wide pH range around 7.0. This means that pH\textsubscript{i} could be regulated by a single solution system. The pH\textsubscript{i} after the treatment is predictable from pH\textsubscript{0}; the pH\textsubscript{i} was nearly identical to pH\textsubscript{0} (pH of the PHSW) during 5-10 min application of Ac\textsuperscript{+}NH\textsubscript{3}\textsuperscript{-} pHSW in both unfertilized and fertilized eggs.

From the results of pH\textsubscript{i} response, we assumed a physicochemical model that the cell membrane could be permeated by molecules of both acetic acid and ammonia but not permeated by the other molecules and ions, and that the buffering power in the egg cytoplasm would be phosphate (appendix (2)). It is noted that the pH\textsubscript{i} after application is affected by various factors, such as pH\textsubscript{0}, the concentration of weak acid or base, pH\textsubscript{i} before application, and the intrinsic pH buffer of eggs. After application of PHSW, this intrinsic pH buffer competes with the applied buffer such as acetic acid or ammonia, which can permeate the cell membrane and this results in pH\textsubscript{i} change. In other words, pH\textsubscript{i} after PHSW application is supposed to be determined by the competitive reaction between dissociation of H ions from acetic molecules (or association to ammonium molecules) and association of H ions to (or dissociation from) the intrinsic pH buffer inside the cell membrane. On these assumptions, pH\textsubscript{i} values can be calculated theoretically under various conditions according to appendix (2). In Figs. 3–6, the calculated curves were plotted assuming the cytoplasm has 60 mM phosphate for intrinsic pH buffer in fertilized eggs. In Figs. 8–9, the calculated curves were plotted assuming the cytoplasm has 40 mM phosphate for intrinsic pH buffer in unfertilized eggs. It was found that the pH\textsubscript{i} response fairly matched this physicochemical model.

However, there is considerable difference between the measured pH\textsubscript{i} and the estimated pH\textsubscript{i} in the treatment with the media containing ammonia. This difference may not be explained by the intrinsic pH buffer (Fig. 10a). Rather, it may be explained by the possibility that the cell membrane could be permeated by ions to some extent during perfusion.
Fig. 10. Estimated curves of pHj in fertilized eggs as a function of the concentration of ammonia in NH3-PHSW. The pHj value of 7.34 is used for pHj before treatment. The experimental data are also plotted (■). (a) Three cases of the concentration of intrinsic pH buffer. This is assuming that the cytoplasm has 40 mM (---), 60 mM (-----) or 80 mM (----) phosphate for intrinsic pH buffer. (b) Three cases of permeability of ammonium ions. pHj of fertilized eggs was calculated as a function of the concentration of ammonia in NH3-PHSW when some ammonium ions could also permeate the cell membrane besides ammonium molecules. Three cases of ion permeability constant (0=impermeable (----), 0.1 (-----) or 0.4 (----)) are shown.

extent (Fig. 10b). This has already been pointed out about ammonia in neuron, axon and muscle cells (1, 23; for a review, 18); not only ammonium molecules also ammonium ions could permeate the cell membrane in the high pH range of Ac+NH3-PHSW or NH3-PHSW. During application of pHSW containing ammonia, pHj decreased gradually during the plateau phase (see Figs. 2a and 2b). This may be because ammonium ions would enter slowly into the egg and dissociate in the egg. Moreover, when eggs were returned to normal sea water from pHSW containing ammonia, pHj decreased suddenly below pHj before application (see Fig. 7). The explanation for this phenomenon may be that ammonium molecules leave the cell earlier and subsequently excess ammonium ions remaining in the cell dissociate into NH3 and H+ leading to a decrease in pHj. From these results, it is thought that ammonium ions can permeate to some extent the egg membrane of sea urchins. We next consider how the ability of ammonium ions to permeate the membrane affects pHj. Figure 10b shows the relation of the concentration of ammonia and pHj, assuming that the permeability constant of ammonium ions across the cell membrane is 0, 0.1 and 0.4. The experimental results coincide with the calculated data when the permeability constant of ammonium ion is assumed to be 0.4.

In the application of the mixture of weak acid and base, pHj changed linearly against pH0 (Fig. 5 and 8). This result is also expected from appendix (2). Therefore, the application of the mixture of acetic acid and ammonia is a simple method for quantitatively regulating the pHj of the sea urchin eggs. The pHj values after 5-10 min application almost equaled pH0; however, there was some discrepancy with values estimated from appendix (2). The reason is unknown but one possible explanation is as follows. From the time course of pH changes after the application of Ac+NH3-PHSW, Ac-PHSW, and NH3-PHSW, it is conceivable that there may be a difference between the rate at which ammonia permeates across the cell membrane and that at which acetic acid does (Fig. 2b). That is, ammonia could permeate faster than acetic acid in the high pH range; conversely, acetic acid could permeate faster than ammonia in the low pH range. The pHj may thus exceed the expected value in the high pH range, and it may become lower in the low pH range. As a result, the pHj may coincide with pH0 after 5-10 min application (Fig. 5 and 8).

APPENDICES

All the calculations in Appendices (1) and (2) were done by a program written in BASIC.

(1) Measuring pH buffering power in the egg cytoplasm

pH buffering power in the egg cytoplasm was estimated as shown below. A constant amount of HEPES solution, whose pH is higher than intracellular pH (1 M, pH 8.0 in this study), is injected (3% of the egg volume in this study) into the egg cytoplasm. HEPES ions associate with H ions, and pHj (pH before injection) elevates to pHi. The pH difference between pHj and that after injection (ΔpH) is described as equation (1).

$$\Delta pH = pH_i - pH_j$$ (refer to Table V) (1)

Amounts of H ions that associate with HEPES ions (Δ[H]) are described as below when pH changes from 8.0 to pHj after injection.
Regulation of Intracellular pH in Sea Urchin Eggs

\[
\Delta [H] = \text{[HEPES]}_t - \text{[HEPES]}_a
\]
\[
= \text{[HEPES]}_t \times \frac{[H]}{[H] + K_0} - 10^{-4.8} / (10^{-4.8} + K_0)
\]
where \([H]_0 = 10^{-8}\text{pH}\).

HEPES \rightarrow \text{HEPES}^- + H^+ : K_0 = 2.82 \times 10^{-8}

\[[\text{HEPES}]_t\] is the total concentration of HEPES and HEPES ion (30 mM in this study by 3% injection of the egg volume), \([\text{HEPES}]_a\) is HEPES concentration before injection, and \([\text{HEPES}]_t\) is HEPES concentration after injection. Buffering power (\(\beta\)) is calculated as follows.

\[
\beta = \Delta [H] / \Delta pH.
\]

In this study we assume that only phosphate works as the source for the buffering power (\(P_t\), although there may be some other sources such as carbonate, ammonia, carboxylate, amino acid, protein and so on. If necessary, the equations would have to be modified to consider them.

\[
\text{[HPO}_4^{2-}\] + [HPO}_4^{3-}\] + [PO}_4^{3-}\] + \[PO}_4^{5-}\] = \text{Pt}
\]

\[
\text{H}_2\text{PO}_4^- \rightarrow \text{H}_2\text{PO}_4^- + \text{H}^+ : K_1 = 7.11 \times 10^{-3}
\]

\[
\text{H}_3\text{PO}_4^- \rightarrow \text{PO}_4^{3-} + \text{H}^+ : K_2 = 4.80 \times 10^{-13}
\]

H ions dissociate from phosphate and then the pH changes. The amount of H ions (\(\Delta [H]\)) that dissociate from phosphate is described as equation (8).

\[
\Delta [H] = 3[H]\text{HPO}_4^- + 2[H]\text{H}_2\text{PO}_4^- + [H]\text{HPO}_4^2^- (8)
\]

Here \(\Delta [H]\text{HPO}_4^-, \Delta [H]\text{H}_2\text{PO}_4^-, \text{and} \Delta [H]\text{HPO}_4^2^-\) are the differences between the values of [H]PO_4^-, [H]H_2PO_4^-, and [H]HPO_4^2^- before injection and those after injection, respectively. Therefore, according to (4)-(7) \(\Delta H\) is described as follows.

\[
\Delta [H] = \text{Pr} \times \left( \frac{[H]}{[H] + K_0} + 2[H]_0 + 3[H]_0^2 / K_0 \right)
\]

\[
- \left( \frac{[H]_0 / K_0 + 2[H]_0 / K_0 + 3[H]_0^2 / K_0}{1 + [H]_0 / K_0} \right)
\]

when \([H]_0 = 10^{-8}\text{pH}\).

Pt can be calculated from (2)=(8) as shown in Table IV. Considering the fact that the egg volume increases after injection, the calculation are the same after all.

(2) Physicochemical model for the cell membrane, and pH change by the application of pHSW

\(pH\) after pHSW application is supposed to be determined by the competitive reaction between the dissociation of H ions from acetic molecules (or association to ammonia molecules) and the association of H ions to (or dissociation from) the intrinsic pH buffer inside the cell membrane.

On this supposition, the calculation of \(PH\) is as given below. H ions are dissociated from acetic acid in the case of Ac-pHSW (dissociation constant of acetic acid; \(K_a = 1.74 \times 10^{-5}\)). In the case of NH_4-pHSW, \(K_b = 5.62 \times 10^{-10}\) is used as the dissociation constant of NH_4^+. Inside the cell membrane after application of pHSW, hydrogen ions produced from or consumed by the intrinsic pH buffer in the cell would become equal to the acetic ions (CH_3COO^-) and/or ammonia ions (NH_4^+), which are produced in the cytoplasm from the CH_3COOH or NH_3 that has entered the cell. Phosphate that exists in abundance in the cytoplasm is substituted for intrinsic pH buffer and \(K_a, K_0, \text{and} K_b\) of phosphate (4)-(8) in appendix (1) are used. Therefore, after modification of the equation (8), the equilibrium inside the cell after application of pHSW is attained according to equation (9), because it is assumed that CH_3COOH or NH_3 freely enters the cell from sea water, the concentration of extracellular acetic acid being equal to that of intracellular acetic acid.

\[
\text{[CH}_3\text{COOH]}_t = \text{[CH}_3\text{COOH]}_a
\]

\[
\text{[CH}_3\text{COOH]}_t + \text{[CH}_3\text{COO}^-] = \text{Ac}
\]

\[
\text{[NH}_3\text{]}_t = \text{[NH}_3\text{]}_a + \text{[NH}_4\text{]}_a
\]

where Ac or N is the total concentration of acetic acid or ammonia in applied pHSW, respectively. In this case, these equations,

\[
\text{[CH}_3\text{COO}^-] = \text{Ac[H]} / [H], [\text{K_a][H]}_t
\]

\[
\text{[NH}_4\text{]}_t = (N / K_b) [H]_t
\]

are put into (9), where \([H]\_t\) or \([H]\_a\) is the intracellular concentration of H ions measured before or after the pHSW application, respectively. Therefore, using equation (9), \(pH\) after application can be calculated in any case of Ac-pHSW, NH_4-pHSW, and Ac+NH_4-pHSW.

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REFERENCES


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