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Toxicities of pH buffer solutions to *Chara* internodal cells

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Tris-HCl (pH 7.0), Tris-maleate (pH 7.0) and HEPES-KOH (pH 6.8–8.2), CHES (pH 8.6–10.0) and CAPS (pH 9.7–11.1) pH buffer solutions are widely used in physiological, biophysical and biochemical experiments. However, their effect on cells has not been thoroughly examined. The results of this study show that these pH buffer solutions can stop the protoplasmic streaming of *Chara* internodal cells and kill them within one to several days at 10 mol m^{-3}, probably by destruction of the membrane functions. However, the cells can be kept alive by the addition of 0.5 mol m^{-3} Ca^{2+}; if this is done, the velocity of the protoplasmic streaming remains normal for more than 10 days in 10 mol m^{-3} Tris pH buffer solutions. The same toxic phenomenon was observed in 10 mol m^{-3} HEPES pH buffer solutions, probably due to the liberation of calcium bound to the cell membrane by the K^+ added as KOH to adjust the pH value.

Key Index Words: calcium ions—Chara australis—*Charophyta—cytoplasmic streaming—Good pH buffer—HEPES—protoplasmic streaming—salt (electrolyte) tolerance—Tris-HCl—Tris-maleate—Tris pH buffer.*

The pH buffer solutions of potassium phosphate, Tris-HCl, Tris-maleate and HEPES-KOH are often used in studies in cell physiology and biophysics as well as in biochemistry. However, the toxicity of potassium phosphate pH buffer solution at pH 7.0 to the *Chara* internodal cells was pointed out by Kiyosawa and Adachi (1990). This toxic effect on cells, cell membranes and membrane fragments needs to be examined in detail.

Recently, Kiyosawa and Adachi (1990) found that *Chara* internodal cells were killed even when exposed to 10-50 mol m^{-3} KCl, 10.0 mol m^{-3} $MgCl_2$ or $Mg(NO_3)_2$, 1.0 mol m^{-3} $BaCl_2$ or $Ba(NO_3)_2$, as found with NaCl by Katsuhara and Tazawa (1986). However, these cells could survive in 80 mol m^{-3} $CaCl_2$, $Ca(NO_3)_2$, $SrCl_2$ or $Sr(NO_3)_2$ for more than ten days. Furthermore, addition of Ca^{2+} or Sr^{2+} to the KCl, $MgCl_2$ and $BaCl_2$ enabled the *Chara* internodal cells to survive for more than a week.

These studies using calcium buffer solutions showed that the minimum effective concentration of the Ca^{2+} in the surrounding solution of the *Chara* internodal cell was between pCa 5 (1.0×10^{-5} mol m^{-3}) and pCa 6 (1.0×10^{-6} mol m^{-3}). However, the calcium buffer solution needed a pH buffer to stabilize its pH during dissociation and/or binding of the calcium ions and protons from/to EGTA (Ogawa 1968). Such pH buffers as Tris and one of the Good buffers, HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), themselves may be the cause of death of *Chara* internodal cells, independently of the calcium concentration.

The Good buffers are said to be not very permeable to the cell membrane and considered to be suitable for use in biochemical, biophysical and cell physiological experiments. These buffers have one or two sulfonic groups or one or two carboxyl groups in their molecules. Usually KOH or NaOH is added when these buffers are to be used as a pH buffer solution. Although the buffers themselves should be harmless to cells, the K^+ (Kiyosawa and Adachi 1990) and Na^+ (Katsuhara and Tazawa 1986) externally added to the bathing solution of Characean

cells can kill the internodal cells. Therefore, Good pH buffer solutions containing K^+ or Na^+ of considerably high concentrations might be harmful to *Chara* internodal cells.

Tunnicliff and Smith (1981) have reported that HEPES competitively inhibits Na-independent binding or γ-aminobutyric acid (GABA) binding to its receptor, and Hanrahan and Tabcharani (1990) have shown that HEPES supplied internally blocks the anion channel of PANC-1 cells. Thus, the present study examined the toxicities of Tris pH buffers and some Good pH buffers, focusing on Tris and HEPES which are most widely used in biochemical, biophysical and cell physiological experiments. The present experiments were done to test whether or not an externally supplied Tris or Good pH buffer can kill *Chara* internodal cells as a result of biophysical and biochemical interactions with the cell membrane, as found in the case of KCl, $MgCl_2$ and $BaCl_2$ (Kiyosawa and Adachi 1990).

In this study, the toxicities of Tris and HEPES-KOH pH buffers were examined. *Chara* internodal cells in toxic electrolyte solutions, Tris and HEPES pH buffer solutions were found to show a gradual decrease in the velocity of their protoplasmic streaming with time, followed by its stopping. After this, the turgor pressure was lost, signifying plant cell death, at 1 or sometimes 2 days after the protoplasmic streaming had stopped. The velocity of the protoplasmic streaming of the *Chara* cells in Tris-HCl pH buffer solution was examined as a function of time and in relatioin to the loss of turgor pressure.

Also studied were the antagonistic effects of externally added Ca^{2+} and Sr^{2+} on the survival of *Chara* internodal cells in Tris pH buffer solutions and the antagonistic effects of externally supplied Ca^{2+} on the survival of *Chara* internodal cells in HEPES, one of the Good pH buffers.

These studies suggested that Tris and HEPES pH buffers, and other Good pH buffers, CHES (2-cyclohexylamino-ethanesulfonic acid) and CAPS (3-cyclohexylamino-1-propanesulfonic acid), disturb the normal membrane transport processes. This can be prevented by externally supplied Ca^{2+}. Therefore, the leakage of K^+, Ca^{2+} and Mg^{2+} from *Chara* internodal cells in 10 mol m^{-3} Tris-HCl (pH 6.9) and Tris-maleate (pH 7.1) solutions was also examined. The effects of CHES and CAPS on *Chara* internodal cells were studied to clarify the effect of HEPES on *Chara* internodal cells.

Materials and Methods

Uncalcified internodal cells of *Chara australis* were used. They were cultured in polyethylene buckets containing tap water and soil several centimeters thick at the bottom. Some of the buckets were exposed to the sun, and others were kept out of the sun with covers which permitted a little sunlight to pass through. Internodal cells were isolated from adjacent cells one or a few days before the experiments and incubated in artificial pond water (APW: 0.400 mol m^{-3} KCl, 0.100 mol m^{-3} NaCl, 0.300 mol m^{-3} $CaSO_4$ and 0.100 mol m^{-3} $MgSO_4$; pH ca. 5.3).

Each internodal cell (n=10) was put in a plastic vessel containing 30-40 cm^3 of the test Tris, HEPES, CHES or CAPS pH buffer solutions or deionized water of 17-18 MΩ cm^{-1} specific resistance. The cells were incubated in test solutions without agitation and observed every day for a week or 10 days. Cell death was judged from the loss of turgor pressure. This was done by slowly raising the *Chara* internodal cell with forceps from the test solution after confirming that the protoplasmic streaming had stopped. The changes in arrangement, shape and color of the chloroplasts were also observed with a microscope. If the *Chara* internodal cell bent easily on the forceps, the cell was regarded as being dead. The test solutions in plastic vessels were exchanged for newly prepared ones at 3-day intervals. The percentages of survival of the *Chara* internodal cells in Tris buffer solutions differed between those grown in the shade and in the sun, with those grown in the shade generally showing weaker tolerance to Tris buffer solutions. Thus, the per-

centages of survival of the *Chara* internodal cells for the respective test solutions were obtained from at least two experiments: one from *Chara* internodal cells (n=10) grown in the shade and the other from those (n=10) kept in the shade for one or a few weeks after having been exposed to the sun for more than several weeks or a month.

Since Tris buffers seemed to disturb the normal membrane transport processes, we examined whether or not leakage of K^+, Ca^{2+} and Mg^{2+} from the *Chara* internodal cell occurs in 10 mol m^{-3} Tris-HCl (pH 6.9) and Tris-maleate (pH 7.1) solutions. Apparent leakages of K^+, Ca^{2+} and Mg^{2+} from the *Chara* internodal cell to 10 cm^3 of 10 mol m^{-3} Tris-HCl, 10 mol m^{-3} Tris-maleate and deionized water of 10 cm^3 by 14 h after transfer of the internodal cell from APW to the respective solutions were measured by the atomic absorption method (Jarrell-Ash AA-845). The amounts of leakage of the respective ions were expressed in terms of averaged decrease in the ion concentrations of the internodal cell calculated from the volume of the internodal cell and the measured changes in the ion concentrations in the 10 cm^3 solutions. The volume of the internodal cell was calculated from the diameter measured with an optical microscope equipped with an eyepiece micrometer calibrated with an objective one, and the length was measured with a ruler. Ion leakage from *Chara* internodal cells in HEPES pH buffer solution was not measured because the HEPES pH buffer solution contained a large amount of K^+ which would have disturbed the determination of ion leakage from the *Chara* internodal cells by the atomic absorption method.

Experiments and incubation were conducted at 25±0.2°C and, unless otherwise stated, under a 12 h-12 h light-dark cycle. The light intensity was 3.4 W m^{-2}.

The velocity of the protoplasmic streaming, which is sensitive to the Ca^{2+} concentration in the cytoplasm of the *Chara* internodal cells (Williamson 1975, Tominaga and Tazawa 1981, Williamson and Ashley 1982, Tominaga *et al.* 1983), in Tris pH-buffer solutions was measured with an optical microscope equipped with an eyepiece micrometer calibrated with an objective one in continuous light because the velocity gradually decreased and attained almost equal values in a few days irrespective of differences in the types and concentrations of calcium salt solutions used, such as APW, 10 mol m^{-3} $CaSO_4$, 10 and 80 mol m^{-3} $CaCl_2$, or 10 and 80 mol m^{-3} $Ca(NO_3)_2$ solutions in the dark. However, the protoplasmic streaming did not stop in such concentrated calcium salt solutions in continuous light or in the dark (data not shown).

Results

Percentages of survival in Tris pH buffer solutions—All of the *Chara* internodal cells immersed in 5.0, 10.0 or 20.0 mol m^{-3} Tris-HCl pH buffer solution (pH 7.1) died within a few or several days (Fig. 1). The higher the concentration, the faster the drop to zero percent survival. The same results were obtained with Tris-maleate buffer solutions (pH 7.1; Fig. 2).

K^+, Mg^{2+} and Ba^{2+} tolerance of the *Chara* internodal cells increased with addition of Ca^{2+} or Sr^{2+} (Kiyosawa and Adachi 1990). Na^+ tolerance of *Nitellopsis* also increased on addition of Ca^{2+} (Katsuhara and Tazawa 1986). Figs. 3 and 4 show similar increased Tris tolerance of *Chara* internodal cells on addition of 0.5 mol m^{-3} Ca^{2+}. Addition of Sr^{2+} to Tris pH buffer solutions did not significantly increase the tolerance at a final concentration of 5.0 mol m^{-3} (Figs. 3 and 4).

The velocity of the protoplasmic streaming of the *Chara* internodal cells gradually decreased with time in 10.0 mol m^{-3} Tris-HCl buffer solution (pH 7.1), followed by the stopping of the protoplasmic streaming and death of the cell (Fig. 5). In the experiment of Fig. 5, all of the *Chara* internodal cells (n=5) in 10.0 mol m^{-3} Tris-HCl buffer solution (pH 7.1) died within a day. On the other hand, the velocity of the protoplasmic streaming of the *Chara* internodal cells in 10.0 mol m^{-3} Tris-HCl+0.5 mol m^{-3} $CaSO_4$

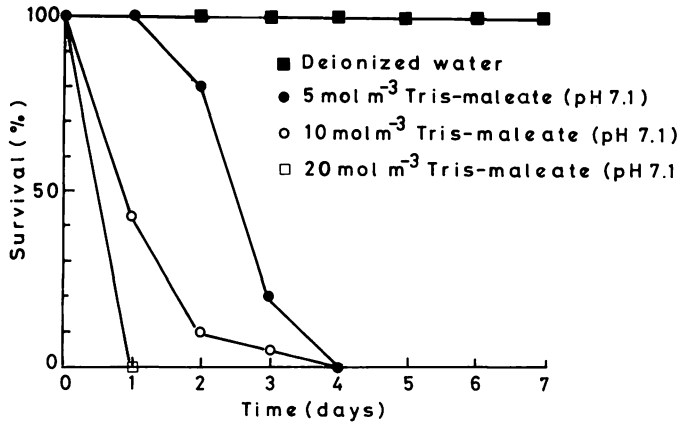

Fig. 1. Survival percentage of *Chara* internodal cells in 5.0 mol m^{-3}, 10.0 mol m^{-3} and 20.0 mol m^{-3} Tris-HCl as a function of time.

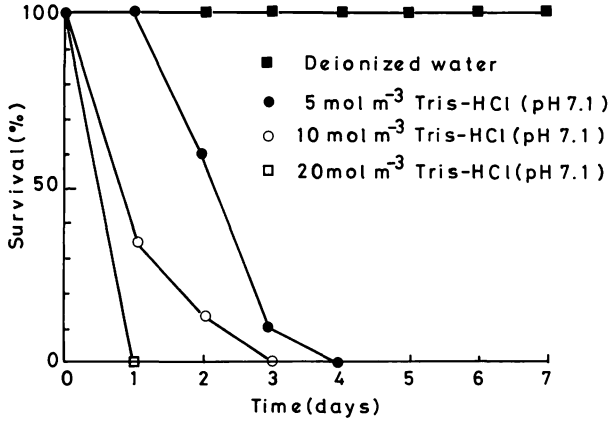

Fig. 2. Survival percentage of *Chara* internodal cells in 5.0 mol m^{-3}, 10.0 mol m^{-3} and 20.0 mol m^{-3} Tris-maleate as a function of time.

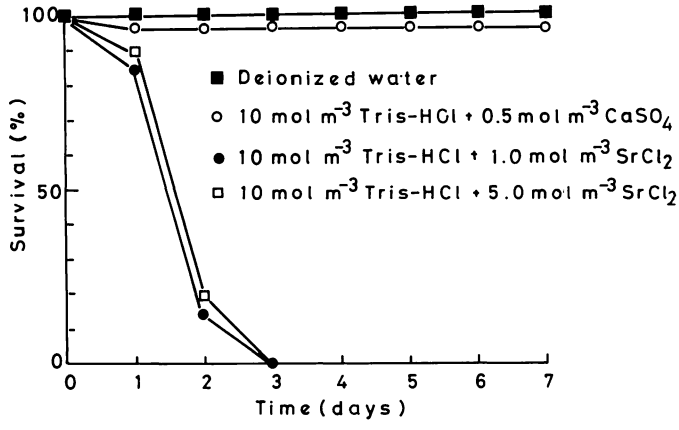

Fig. 3. Effects on survival percentage of *Chara* internodal cells of addition of 0.5 mol m^{-3} Ca^{2+} or 1.0 mol m^{-3} or 5.0 mol m^{-3} Sr^{2+} to 10.0 mol m^{-3} Tris-HCl.

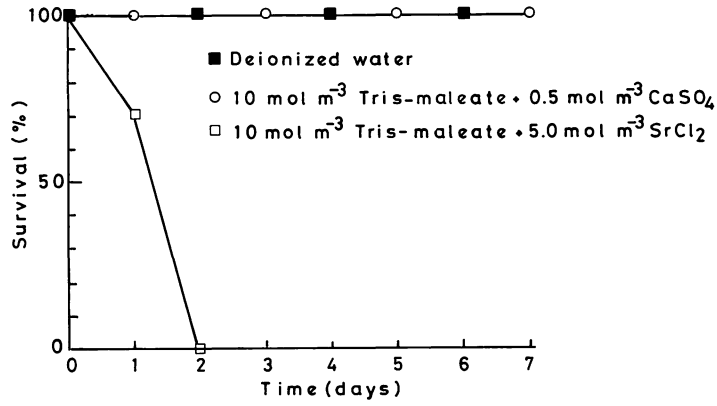

Fig. 4. Effects on survival percentage of *Chara* internodal cells of addition of 0.5 mol m^{-3} Ca^{2+} or 5.0 mol m^{-3} Sr^{-2} to 10.0 mol m^{-3} Tris-maleate.

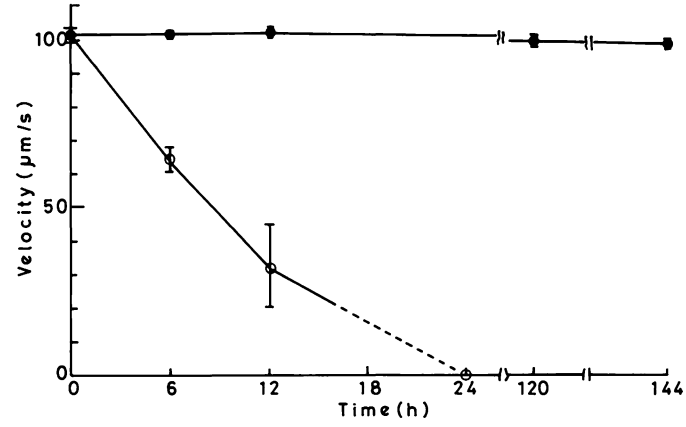

Fig. 5. Averaged values of velocity of the protoplasmic streaming of the *Chara* internodal cells in 10.0 mol m^{-3} Tris-HCl (pH 7.1) (○) and in 10.0 mol m^{-3} Tris-HCl + 0.5 mol m^{-3} $CaSO_4$ (●) as a function of time. The velocity of the protoplasmic streaming of the *Chara* internodal cells in 10.0 mol m^{-3} Tris-HCl (pH 7.1) decreased gradually with time, while that of the protoplasmic streaming of the *Chara* internodal cells in 10.0 mol m^{-3} Tris-HCl (pH 7.1) + 0.5 mol m^{-3} $CaSO_4$ remained constant for at least 144 h. All *Chara* internodal cells (n = 5) died by 24 h after immersion in 10.0 mol m^{-3} Tris-HCl, while all *Chara* internodal cells in 10.0 mol m^{-3} Tris-HCl + 0.5 mol m^{-3} $CaSO_4$ remained alive for at least 144 h. Velocities of the protoplasmic streaming are indicated with the mean ± standard error. Standard errors are shown with bars.

remained constant for at least 144 h (Fig. 5). The same results were observed with Tris-maleate and HEPES pH buffer solutions (data not shown).

The apparent leakages of K^+, Ca^{2+} and Mg^{2+} from the *Chara* internodal cell to 10.0 mol m^{-3} Tris-HCl (pH 7.1), 10.0 mol m^{-3} Tris-maleate (pH 7.1) solutions and deionized water are tabulated in Table 1. Apparently, K^+, Ca^{2+} and Mg^{2+} leaked from the *Chara* internodal cell in Tris pH buffer solutions, resulting in apparent decrease in their intracellular concentrations amounting to 6.5–8.8 mol m^{-3}, 3.9–4.6 mol m^{-3} and 0.71–0.64 mol m^{-3}, respectively.

Percentages of survival in simple HEPES solution—Until the 4th or 5th day after transfer from APW to the simple HEPES solutions of 10, 20 and 50 mol m^{-3} (pH 5.26), high percentages of survival were maintained irrespective of differences in HEPES concentrations, but were followed by a steep decrease in the

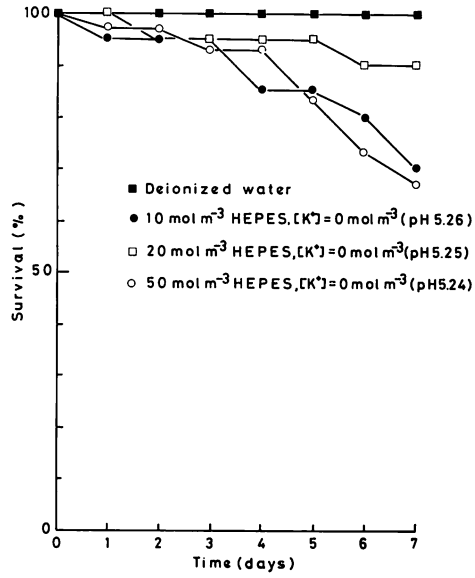

Fig. 6. Survival percentage of *Chara* internodal cells in simple 10, 20 and 50 mol m^{-3} HEPES solutions.

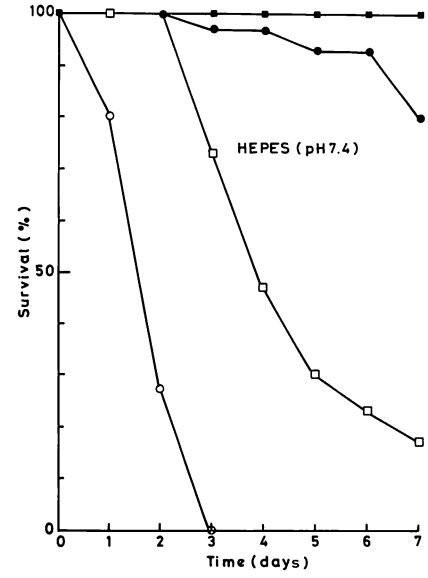

Fig. 7. Survival percentage of *Chara* internodal cells in 10 mol m^{-3} ($[K^+]$=5.0 mol m^{-3}; ●), 20 mol m^{-3} ($[K^+]$=10.0 mol m^{-3}; □) and 50 mol m^{-3} ($[K^+]$=25.0 mol m^{-3}; ○) HEPES pH buffer solutions at pH 7.4, and deionized water (■).

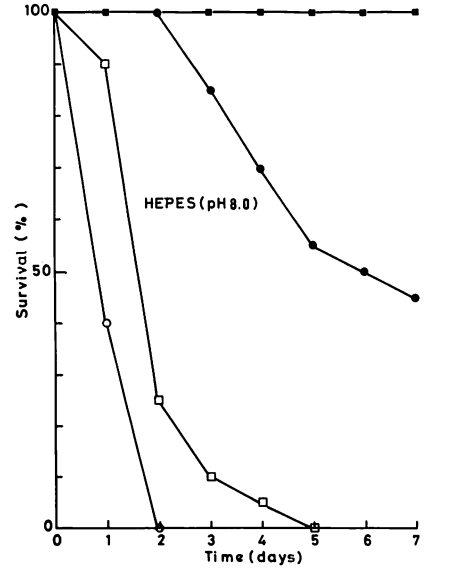

Fig. 8. Survival percentage of *Chara* internodal cells in 10 mol m^{-3} ($[K^+]$=8.9 mol m^{-3}; ●), 20 mol m^{-3} ($[K^+]$=17.8 mol m^{-3}; □) and 50 mol m^{-3} HEPES ($[K^+]$=44.5 mol m^{-3}; ○) pH buffer solutions at pH 8.0, and deionized water (■).

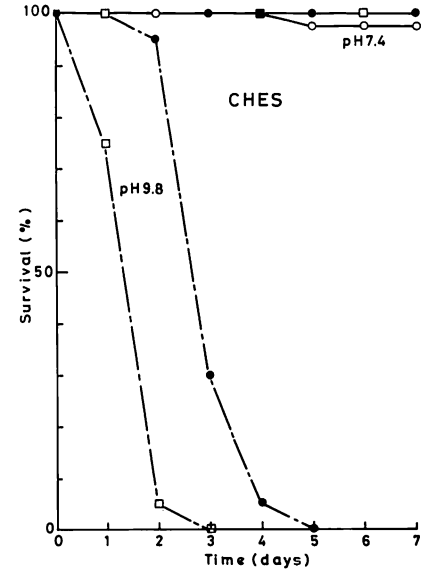

Fig. 9. Survival percentage of *Chara* internodal cells in 10 mol m^{-3} ($[K^+]$=0.1 mol m^{-3}; —●—), 20 mol m^{-3} ($[K^+]$=0.2 mol m^{-3}; —□—) and 50 mol m^{-3} ($[K^+]$=0.5 mol m^{-3}; ○) CHES pH buffer solutions at pH 7.4, and those in 10 mol m^{-3} ($[K^+]$=8.4 mol m^{-3}; ——●——) and 20 mol m^{-3} ($[K^+]$=16.8 mol m^{-3}; ——□——) CHES pH buffer solutions at pH 9.8, and deionized water (■).

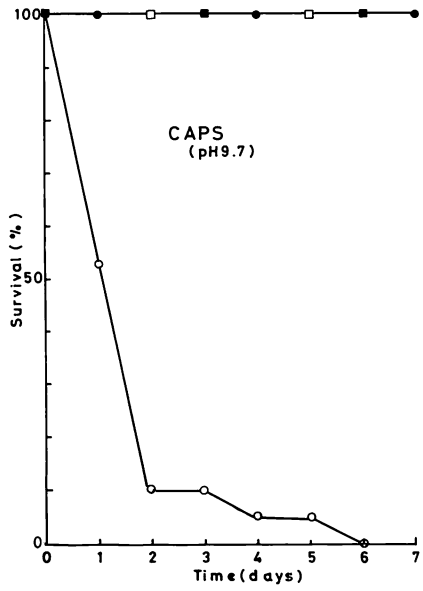

Fig. 10. Survival percentage of *Chara* internodal cells in 10 mol m^{-3} ($[K^+]=1.8$ mol m^{-3}; ●), 20 mol m^{-3} ($[K^+]=3.6$ mol m^{-3}; □) and 50 mol m^{-3} ($[K^+]=9.0$ mol m^{-3}; ○) CAPS pH buffer solutions at pH 9.7, and deionized water (■).

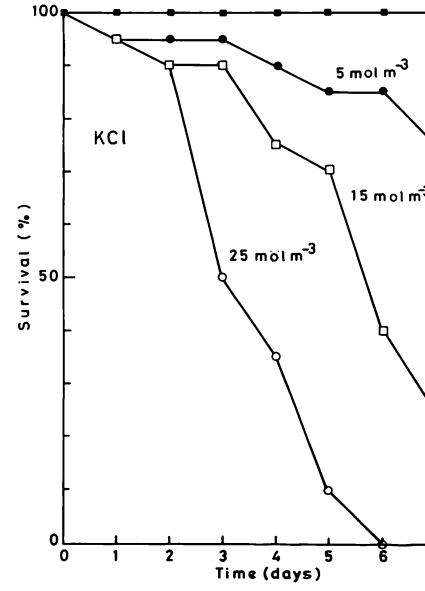

Fig. 11. Survival percentage of *Chara* internodal cells in 5.0 mol m^{-3} (●), 15.0 mol m^{-3} (□) and 25.0 mol m^{-3} (○) KCl solutions (pH ca. 5.3), and deionized water (■).

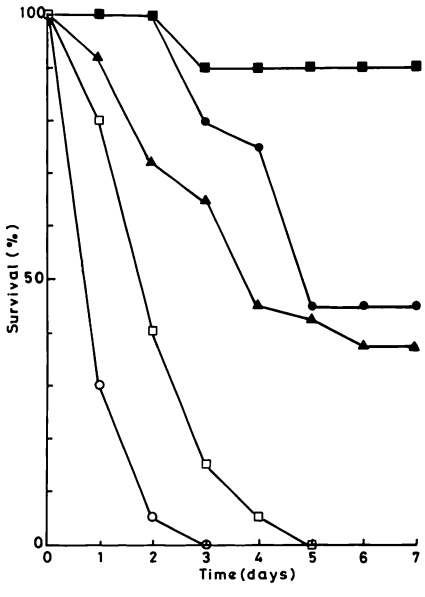

Fig. 12. Effects on survival percentage of *Chara* internodal cells in 20 mol m^{-3} HEPES (pH 8.0, □) and 20 mol m^{-3} CHES (pH 9.8, ○) pH buffer solutions on addition of 0.5 or 1.0 mol m^{-3} Ca^{2+} to their solutions; ■: 20 mol m^{-3} HEPES+0.5 mol m^{-3} $CaSO_4$; ●: 20 mol m^{-3} CHES+0.5 mol m^{-3} $CaSO_4$ and ▲: 20 mol m^{-3} CHES+1.0 mol m^{-3} $CaSO_4$.

percentage of survival after 4 days (Fig. 6).

Percentage of survival in HEPES pH buffer solution (pH 7.4)—The decrease in the percentage of survival of the *Chara* internodal cells in 10, 20 and 50 mol m^{-3} HEPES pH buffer solutions at pH 7.4 became steeper as a function of time with increase in the concentration of the HEPES pH buffer solution (Fig. 7). The K^+ concentrations from the KOH used to adjust the pH of the respective HEPES pH buffer solutions were 5.0, 10.0 and 25.0 mol m^{-3}.

Percentage of survival in HEPES pH buffer solution (pH 8.0)—The percentage of survival of the *Chara* internodal cells in 10, 20 and 50 mol m^{-3} HEPES pH buffer solutions at pH 8.0 decreased more steeply with time than those in the HEPES pH buffer solutions at pH 7.4 of the corresponding concentrations (Fig. 8; cf. Fig. 7). The K^+ concentrations from the KOH used to adjust the pH of the respective HEPES pH buffer solutions at pH 8.0 were 8.9, 17.8 and 44.5 mol m^{-3}. The higher the pH and the K^+ concentration, the steeper was the decrease in the percentage of survival as a function of time.

Table 1. Average leakages of K^+, Ca^{2+} and Mg^{2+} from *Chara* internodal cells (ΔC in mol m^{-3}) 14 h after transfer of the internodal cells from artificial pond water (pH ca. 5.3) to 10 mol m^{-3} Tris-HCl pH buffer solution (pH 7.1), 10 mol m^{-3} Tris-maleate pH buffer solution (pH 7.1) or deionized water.

Solution	ΔC^*		
	K^+	Ca^{2+}	Mg^{2+}
Tris-HCl	6.5±3.0	3.9±0.2	0.71±0.06
Tris-maleate	8.8±2.7	4.6±0.2	0.64±0.00
Deionized water	0.0±0.0	0.0±0.0	0.00±0.00

ΔC^*: Averaged decrease in the concentration of respective ion in *Chara* internodal cells (n=6) due to leakage in terms of mol m^{-3}.

Percentages of survival in CHES and CAPS—Fig. 9 shows the percentage of survival of the *Chara* internodal cells in 10, 20 and 50 mol m^{-3} CHES pH buffer solutions at pH 7.4, and those in 10 and 20 mol m^{-3} CHES pH buffer solutions at pH 9.8. The K^+ concentrations of 10, 20 and 50 mol m^{-3} CHES pH buffer solutions at pH 7.4 were only 0.1, 0.2 and 0.5 mol m^{-3}, respectively, and the CHES pH buffer solutions of such concentrations were almost nontoxic to the *Chara* internodal cells. CHES pH buffer solutions of 10 and 20 mol m^{-3} at pH 9.8 contained 8.4 and 16.8 mol m^{-3} K^+, respectively. At pH 9.8, the survival percentage of the *Chara* internodal cells decreased more steeply with time with an increase in the concentration of the CHES pH buffer solution. Also, the survival percentage of the cells at pH 9.8 decreased much more steeply as a function of time than those in CHES pH buffer solutions at pH 7.4 of the same concentrations.

In CAPS pH buffer solutions of 10 and 20 mol m^{-3}, all of the *Chara* internodal cells survived at pH 9.7 for more than a week (Fig. 10). This indicates that CAPS itself is not strongly toxic and a high pH of 9.7 is not one of the main causes of *Chara* internodal cell death. However, 50 mol m^{-3} CAPS containing 9.0 mol m^{-3} K^+ was very toxic.

Percentage of survival in KCl solutions—KCl solutions of 5.0, 15.0 and 25.0 mol m^{-3} (pH ca. 5.3) could kill the *Chara* internodal cells (Fig. 11). The higher the concentration of KCl, the more steeply the percentage of survival decreased with time.

Effects of Ca^{2+} on the percentages of survival in HEPES (pH 8.0) and CHES (pH 9.8) pH buffer solutions—All of the *Chara* internodal cells immersed in 20 mol m^{-3} HEPES pH buffer solution (pH 8.0), which was much more toxic than 10 mol m^{-3} HEPES pH buffer solution (pH 8.0), died within several days. However, addition of 0.5 mol m^{-3} Ca^{2+} to 20 mol m^{-3} HEPES pH buffer solution (pH 8.0) increased the percentage of survival of the *Chara* internodal cells to 90% (Fig. 12). Addition of 0.5 or 1.0 mol m^{-3} Ca^{2+} to CHES pH buffer solution (pH 9.8) also delayed the decrease in the survival percentage of the *Chara* internodal cells as a function of time. However, the effect of Ca^{2+} on the suvival percentage of the *Chara* internodal cells in CHES pH buffer solution was weaker than that on the survival percentage in 20 mol m^{-3} HEPES pH buffer solution (Fig. 12).

Discussion

Tris and Good pH buffers have been widely used in physiological, biophysical and biochemical studies. However, this has been done without checking their direct effects on the biochemical molecules in question or their toxic effects on the cells used. The present experiments showed that Tris and one of the Good pH buffers, HEPES, are toxic to the *Chara* internodal cells even at 10 mol m^{-3} when the cells are externally exposed to them. This indicates that Tris and HEPES pH buffers can affect the cell membrane.

The protoplasmic streaming of *Chara* internodal cells did not stop in calcium salt solutions of high concentrations such as 10 mol m^{-3} $CaSO_4$, 10 and 80 mol m^{-3} $CaCl_2$, and 10 and 80 mol m^{-3} $Ca(NO_3)_2$, and even in long-term plasmolysed *Chara Braunii* cells in $Ca(NO_3)_2$ or $CaCl_2$ solution of high concentrations (Hayashi and Kamitsubo 1959) kept under continuous light as well as in the dark. The concentration of the Ca^{2+} in the cytoplasm of *Chara* internodal cells is thought

to be as low as below 10^{-6} mol m^{-3}, but as high as ca. 10 mol m^{-3} when measured by a direct chemical method using the atomic absorption method (Okihara and Kiyosawa 1988). The electrochemical potential difference across the *Chara* cell membrane for Ca^{2+} calculated from the measured Ca^{2+} concentrations inside and outside the *Chara* internodal cell, and the electrical membrane potential difference indicates that the Ca^{2+} in APW should be forced to enter the internodal cell and stop the protoplasmic streaming of the cell. The fact that protoplasmic streaming of intact *Chara* cells in APW and calcium salt solutions of various types at high concentrations continues for a long time indicates that the normal function of the *Chara* cell membrane is to prevent a large amount of Ca^{2+} from entering the cell, which would stop the protoplasmic streaming, but to allow enough Ca^{2+} to enter to instantaneously stop the protoplasmic streaming on excitation by some stimulus, such as an electrical current (Barry 1968, Hayama *et al.* 1979, Kikuyama and Tazawa 1983, Lunevsky *et al.* 1983). The gradual decrease in the velocity of protoplasmic streaming of *Chara* internodal cells, followed by its stopping, in Tris buffer solutions or HEPES pH buffer solutions without any special stimulus indicates that the normal functions of the *Chara* cell membrane are disturbed by Tris and HEPES pH buffers.

Recently, Katsuhara and Tazawa (1987) showed that internal ATP at 1 mol m^{-3} was necessary for the cell membrane of tonoplast-free *Nitellopsis* cells to maintain salt (NaCl) tolerance in 100 mol m^{-3} NaCl in the presence of external 10 mol m^{-3} Ca^{2+}. The velocity of the protoplasmic streaming of *Chara* internodal cells immersed in a Tris pH buffer solution (pH 7.1) gradually decreased with time, followed by cell death (Fig. 5). These phenomena were observed in the HEPES solutions as well. Externally added 0.5 mol m^{-3} $CaSO_4$ could keep the survival percentage of the internodal cells at 100% and maintain the normal velocity of the protoplasmic streaming (Fig. 5). This fact suggests that Tris pH buffers cause disturbance of the normal membrane transport processes, and induce leakage of ions and some biochemical components including ATP (Williamson 1975, Shimmen 1978) responsible for maintaining the normal protoplasmic streaming and keeping the *Chara* cell alive, and that externally supplied Ca^{2+} and intracellular ATP prevent Tris pH buffers from inducing the leakages of ions and some biochemical components including ATP (cf. Katsuhara and Tazawa 1987).

From the viewpoint stated above, we examined whether or not the leakage of K^+, Ca^{2+} and Mg^{2+} from the *Chara* internodal cell occurs in 10.0 mol m^{-3} Tris-HCl (pH 7.1) and Tris-maleate (pH 7.1) solutions. Our findings (Table 1) together with those of previous work (Kiyosawa and Adachi 1990) show that the leakage of K^+ is from the cytoplasm (Katsuhara and Tazawa 1986) which contains much K^+ (MacRobbie 1962, Spanswick and Williamson 1964, Kishimoto and Tazawa 1965, Tazawa *et al.* 1974, Okihara and Kiyosawa 1988). However, most of the calcium ions liberated will be from the cell wall, to which a considerably large amount of calcium is bound (Kiyosawa and Adachi 1990, Reid and Smith 1992), and/or the cytoplasm. The liberated magnesium ions are thought to come from the cell wall and/or the cytoplasm. Leakage of the intracellular ATP has not been measured yet.

When *Nitellopsis* cells are transferred from artificial pond water (APW′: 0.1 mol m^{-3} KCl, 0.1 mol m^{-3} NaCl, 0.1 mol m^{-3} $CaCl_2$; pH ca. 5.3) to 100 mol m^{-3} NaCl+APW′, the concentration of K^+ in the cytoplasm decreases while that of Na^+ increases immediately after the transfer. This effect of the external NaCl of 100 mol m^{-3} on the cytoplasmic K^+ and Na^+ concentrations can be nullified by addition of 10 mol m^{-3} $CaCl_2$ to the external 100 mol m^{-3} NaCl (Katsuhara and Tazawa 1986). The same effects of the external 70 mol m^{-3} NaCl on the survival of *Chara corallina* internodal cells have been reported together with a decrease in the K^+ concentration and an increase in the Na^+ concentration in the vacuole occurring a few days or several

days after transfer from artificial pond water (APW″: 1.0 mol m^{-3} NaCl, 0.05 mol m^{-3} K_2SO_4, 0.1 mol m^{-3} $CaSO_4$, 5.0 mol m^{-3} HEPES titrated to pH 7.0 with NaOH) to 70 mol m^{-3} NaCl+APW″ (Tufariello *et al.* 1988). In this case, the coexistence of 7.1 mol m^{-3} Ca^{2+} is enough to prevent an increase in the vacuolar Na^+ concentration and a simultaneous decrease in the vacuolar K^+ concentration.

Cramer *et al.* (1985) and Lynch *et al.* (1987), measuring the fluorescence of Ca^{2+}-chlorotetracycline from intact cotton root hairs and protoplast suspension of corn roots, reported that externally supplied Na^+ reduced the amount of calcium binding to the plasmalemma of cotton root cells and of corn root protoplasts, respectively.

Although we have no direct and clear evidence as to whether the externally supplied alkali metal and alkali earth metal ions affect the calcium bound only to the outer surface of the cell membrane, or even the calcium inside the cell membrane, the available experimental results (cf. Katsuhara and Tazawa 1986, Tufariello *et al.* 1988, Kiyosawa and Adachi 1990) including the present ones indicate that externally supplied Ca^{2+} can prevent the disturbance of membrane integrity by externally supplied alkali metal ions, some of the alkali earth metal ions and Tris ion. These observations suggest that the externally supplied Ca^{2+} affects the cell membrane itself and can help maintain normal membrane functions by suppressing calcium liberation from the cell membrane in electrolyte solutions of alkali metal ions, some alkali earth metal ions (cf. Kiyosawa and Adachi 1990) and Tris ion.

Kiyosawa and Adachi (1990) have shown that KCl, $MgCl_2$ and $BaCl_2$, which killed the *Chara* internodal cells even at considerably low concentrations, caused liberation of almost all of the calcium bound to the *Chara* cell wall within an hour (cf. also Reid and Smith 1992). This also occurred with $SrCl_2$ added at 80 mol m^{-3}, which could keep the *Chara* internodal cells alive for more than two weeks, but did not occur with externally supplied Ca^{2+} at 80 mol m^{-3}. Also, externally supplied Ca^{2+} of 0.5-1.0 mol m^{-3} more or less inhibited liberation of the bound calcium in KCl, $MgCl_2$ and $BaCl_2$ solutions. The findings suggested that Sr^{2+} could maintain membrane integrity in a manner different from that of Ca^{2+} (Kiyosawa and Adachi 1990) or that Sr^{2+} could suppress the calcium release from the cell membrane by K^+, Mg^{2+} or Ba^{2+} but its action differed from that of externally supplied Ca^{2+}.

Externally supplied Sr^{2+}, which can maintain *Chara* cell membrane integrity in KCl, NaCl, $MgCl_2$ and $BaCl_2$, is not effective at 5 mol m^{-3} in Tris pH-buffer solutions (Fig. 3). Thus, if the viewpoint is taken that externally supplied Sr^{2+} can suppress the calcium release from the cell membrane caused by K^+, Mg^{2+} or Ba^{2+}, this observation can be simply and reasonably explained. Tris pH buffers liberate the calcium bound to the *Chara* cell membrane so effectively that it cannot be suppressed by externally supplied 5 mol m^{-3} Sr^{2+}, but can be by externally supplied 0.5 mol m^{-3} Ca^{2+}, as shown in Fig. 3. Further studies using *Chara* cell membrane or cell membrane fragments of other plants are needed to verify this.

On interpreting the effects of Good pH buffer solutions on *Chara* internodal cells obtained in the present study, together with those of previous work, the following can be considered to be important: (1) pH value, (2) K^+ concentration, and (3) the concentration of the Good buffer itself.

Chara internodal cells can survive in an acidic APW at pH 4.72 (Kiyosawa 1990). Thus, the pH of 5.24 of a simple HEPES buffer solution should not be low enough to kill *Chara* internodal cells (Fig. 6). It must be the action of the HEPES itself that kills that *Chara* internodal cells (cf. Fig. 6). As shown in Fig. 6, an increase in the number of dead *Chara* internodal cells in simple HEPES solutions after the 5th day suggests that HEPES interacts directly with the cell membrane components or channels, not mainly via lowering of the pH of the bathing solution, as reported for the isolated semicircular canal of the frog (Norris and Guth 1985), *Helix* neurons (Witte *et al.*

1985), and cultured *Drosophila* neurons (Yamamoto and Suzuki 1987).

Fig. 10 shows that the *Chara* internodal cells can survive in 10 mol m^{-3} and 20 mol m^{-3} CAPS pH buffer solutions at pH 9.7. These findings together with an earlier one (Kiyosawa 1990) indicate that *Chara* internodal cells can survive in solutions where the pH is as low as 4.72 to as high as 9.7. Therefore, in the present experiments, the pH of the test solutions probably was not the main determinant of the percentage of survival of the *Chara* internodal cells, although it may have been a contributing factor. Compared with the results of Fig. 11, the fact that the percentage of the survival of the *Chara* internodal cells decreased at a more rapid rate with time in the Good pH buffer solutions of higher concentrations seems to be explainable mainly in terms of the higher K^+ concentration in the Good pH buffer solutions. One of the main actions of the K^+ in HEPES and CHES seems to be liberation of the calcium bound to the *Chara* cell wall (cf. Kiyosawa and Adachi 1990, Reid and Smith 1992) and the cell membrane. From this point of view, the survival percentage of the *Chara* internodal cells was examined in 20 mol m^{-3} HEPES (pH 8.0)+0.5 mol m^{-3} $CaSO_4$, 20 mol m^{-3} CHES (pH 9.8)+0.5 mol m^{-3} $CaSO_4$ and 20 mol m^{-3} CHES (pH 9.8)+1.0 mol m^{-3} $CaSO_4$ in comparison with those in 20 mol m^{-3} HEPES alone (pH 8.0) and 20 mol m^{-3} CHES alone (pH 9.8) (Fig. 12).

The results of Fig. 12 clearly show that the toxicity of 20 mol m^{-3} HEPES (pH 8.0) pH buffer alone, which was more toxic than 10 mol m^{-3} HEPES pH buffer solution alone, was almost nullified when 0.5 mol m^{-3} Ca^+ was present for more than 7 days, as shown with Tris pH buffers (Fig. 3). These observations further suggest that the blocking effects of HEPES on the ion channels themselves (cf. Witte *et al.* 1985, Yamamoto and Suzuki 1987, Hanrahan and Tabcharani 1990) may have been reduced by addition of Ca^{2+} to the solution as well. These observations lead to another important conclusion that all of the pH buffers which contain K^+ (KOH), Na^+ (NaOH) or ions capable of liberating the calcium binding to the cell membrane may be toxic to cells and modify the membrane fragments.

However, the toxicity of a simple 20 mol m^{-3} CHES (pH 9.8) pH buffer, which was very toxic to *Chara* internodal cells, could not be nullified, although it was slightly reduced by addition of 0.5 or 1.0 mol m^{-3} Ca^{2+}. In the case of 20 mol m^{-3} CHES pH buffer solution to which 0.5 mol m^{-3} or 1.0 mol m^{-3} $CaSO_4$ was added, many crystals of rectangular prisms, flower-like hexagons, dumb-bells, and ellipsoid and indefinite forms, were observed at the bottom of the vessel, on the internodal cell, and on the surface of the solution. These crystals were also observed in solutions of 20 mol m^{-3} CHES (adjusted at pH 9.8 by KOH or NAOH)+1.0 mol m^{-3} $CaCl_2$ or $Ca(NO_3)_2$. However, no crystals were observed in 20 mol m^{-3} CHES (to which no KOH or NaOH was added)+5.0 mol m^{-3} $CaSO_4$ solution. In other words, even when 0.5 mol m^{-3} or 1.0 mol m^{-3} Ca^{2+} was added to 20 mol m^{-3} CHES pH buffer solution, the concentration of the Ca^{2+} in the CHES solution was lower than calculated, due to the formation of CHES-Ca crystals.

A previous paper (Kiyosawa and Adachi 1990) reported that *Chara* internodal cells kept in 10 mol m^{-3} HEPES pH buffer solution remained alive for more than 10 days. This may have resulted from the use of *Chara* internodal cells grown in the sun which had calcium-rich cell walls and also because the internodal cells had been immersed in the same HEPES solution for a week. These findings and considerations led to the conclusion that Tris ions and HEPES-KOH interact with the *Chara* cell membrane and the cell wall to induce leakage of K^+, and probably Ca^{2+} and Mg^{2+}, from inside the cell and also liberation of Ca^{2+} and Mg^{2+} bound to the cell wall and probably to the cell membrane. This finally leads to stopping of the protoplasmic streaming and death of the *Chara* internodal cell. Leakages of such biochemical components as ATP-Mg, some inorganic and organic ions

responsible for keeping the cytoplasm and the cell membrane normal and maintaining the normal protoplasmic streaming may also occur from inside the cell through the cell membrane in HEPES as well as Tris pH buffer solutions. Thus, when Tris or HEPES pH buffer solution is used with or without other electrolyte(s), a moderate amount of Ca^{2+} should be added to the solution to prevent modification of the biomembranes in such solutions.

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清沢桂太郎：車軸藻節間細胞に対する pH 緩衝液の毒性

これまで膜生理学，生物物理学及び，生化学でかなり頻繁に用いられてきた Tris-HCl (pH 7.0), Tris-maleate (pH 7.0), 及び，Good の pH 緩衝液である HEPES (pH 6.8–8.2) の車軸藻節間細胞に対する毒性を調べた。各 pH 緩衝液は，10–20 mol m^{-3} の濃度で，1 日から 4 ～ 5 日の間に車軸藻節間細胞の原形質流動を徐々に減速させ，停止させた後，同節間細胞を死に至らしめた。しかし，0.5–1.0 mol m^{-3} の Ca^{2+} を加えると，上記 pH 緩衝液の車軸藻節間細胞に対する毒性をなくした。Good 緩衝液の毒性は，同緩衝液の pH を合わせるために加える KOH の K^+ が，Tris 緩衝液の場合は Tris イオンが，細胞膜に結合している Ca^{2+} を遊離させて細胞膜の機能を損なうためと推定された。(560 豊中市待兼山町1–1　大阪大学基礎工学部生物工学科)