The strategic use of Good buffers to measure proton gradients around growing pollen tubes

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ABSTRACT

The use of non-invasive vibrating probes for studying proton and bicarbonate flux into and out of cells depends on a knowledge and measurement of local pH. Without buffer the pH surrounding a living cellular system is constantly changing based on the active secretion of protons as well as bicarbonate. Stability of the experimental arena is most easily accomplished if there is a functional buffer in the medium. However, the existence of a buffer will destroy protons and sharpen proton gradients, in some cases to extinction. We have studied the operation of the non-invasive proton probe in the presence of several Good buffers as well as in unbuffered conditions to establish the ability of the probe to detect proton fluxes from artificial and natural sources. We are able to recommend a Good buffer system for pH ranges and proton fluxes which maximize the ability to detect and extrapolate the measured flux back to its origin in order to estimate the transmembrane flux of protons. We have tested the effectiveness of our approach by measuring the proton flux across the growing pollen tube.

1. Introduction

The intention of this essay is to familiarize the ion probe user with the complications, benefits and drawbacks of using pH buffers while trying to estimate the direction and strength of proton flux vectors in the medium outside cells or tissues. The particular use of the Good buffers [1, 2, 3] in this essay reflects their common use in biology based on their relatively inert behavior with regard to affecting cell functions.

While this advantage of the Good buffers in biology is well known, the advantages do need to be reiterated periodically in order to reevaluate the benefits in a changing technological background. This is particularly important in our dynamic measurement case, when one wishes to accurately inventory flux components which will be used to calculate ionic balance. Facilitated diffusion is a phenomenon in which a proportion of the proton flux is absorbed by a buffer and hidden from traditional measurement with pH electrodes. Buffers effectively change the apparent rate at which protons are able to disperse from a source. The buffer absorbs local protons, allowing them to escape the source more rapidly. However, the protons are slowed in their physical exit of the locale of the source since they are tied up in the more slowly diffusing buffer molecule. When allowed to diffuse associated with the unique buffer, water, protons exhibit an anomolously high flux rate dependent on the hydrogen bonding properties of water (Fig. 1A). This rapid dispersal of protons is lost for that proportion of protons associated with other buffers.

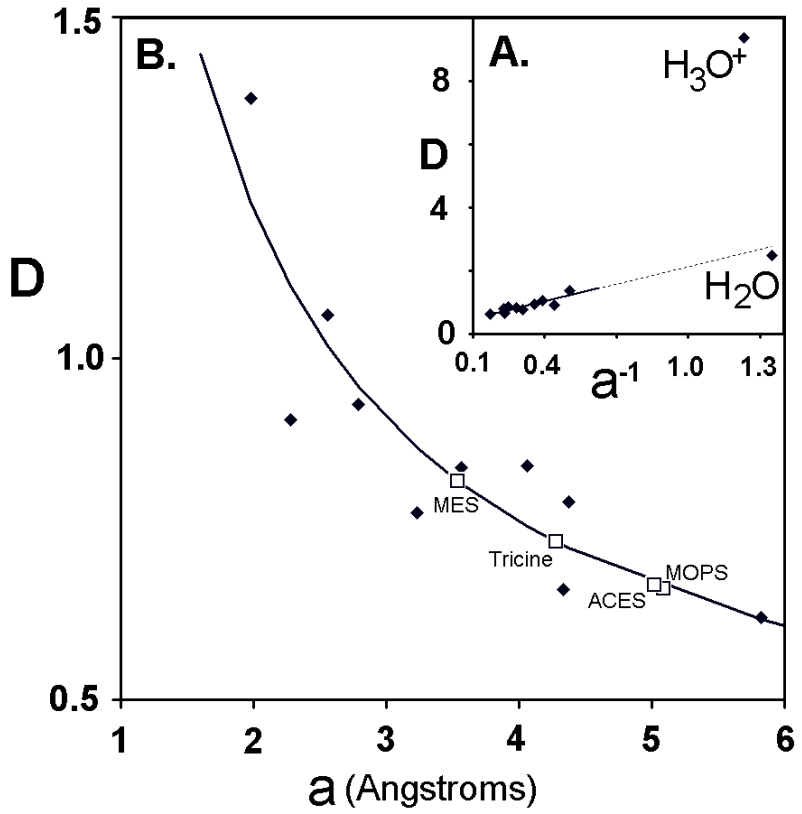

Fig. 1. Diffusion of weak electrolytes of known diffusion coeficient, **D** (cm^2/sec), and Stokes radius, **a** (Angstroms). **A.** Confirmation of water (**H_2O**) behaving as a weak electrolyte and the hydronium ion (**H_3O^+**) behaving anomalously with a high apparent **D** The regression of electrolytes with known **D** and **a** (filled diamonds) includes alpha-alanine, beta-alanine, citric acid, glycine, glycyl-glicine, HEPES, meta-, ortho-, and para- amino-benzoic acids, and urea. **B.** Estimation of **D** (open squares) for several Good buffers based on their weak electrolyte properties and an estimate of their Stokes radius from molecular models. The linear regression of weak electrolyte **D** vs **1/a** derived from panel **A** is plotted and used to estimate the expected **D** for the buffers MESS, Tricine, ACES and MOPS.

The intrinsic inertness of most Good buffers is in contrast to naturally occurring buffers, such as phosphate, carbonate and citrate. The later may participate in the cellular metabolism and may be actively transported into or out of cells, such that their concentration may change at the cell surface and that change may have unexpected local physiological consequences aside from the focus of the experiment. As an example, phosphate is historically a buffer frequently used to achieve pH stability in biochemical reactions but an unnaturally high titer of inorganic phosphate can have dramatic effects on living cell physiology. Other naturally occurring buffers such as citrate will chelate ions that may be important to cell function and thus should be avoided or closely regulated when these ions are needed or can not be avoided. In addition, some synthetic buffers, Tris in particular, are known to have inhibitory effects on particular enzyme reactions that can distort results [1]. The Good buffers have by now a history of use and known properties in many experimental systems. The large size and formal charges on the Good buffers usually preclude their entry into cells unless injected. Caution is of course advised whenever applying a buffer to a new situation. We will proceed cognizant of these warnings and benefits as if they were given and accepted assumptions when using the Good buffers.

However, the general strategy of buffer usage in the field of ion probe technology and ion gradient measurement, in particular, is in need of more thoughtful explanation in order that their appropriate use gains more widespread appreciation. All

the effects that we present and demonstrate here are certainly logical and could be predicted from first principles that include the buffer concentrations, dissociation constants and diffusion coefficients. However, if we avoid going through the rationale by which these buffers affect ion probe measurements around artificial sources, we are less likely to use the buffers effectively in our experiments with cells. Many of the principles demonstrated can also be applied to ions other than protons, which may operate in buffered states and are in need of careful measurement as well. In particular the phenomenon of facilitated diffusion is of general interest to the movement of any ligand that may be bound by another molecule. In generalized facilitated diffusion, a localized buffer or chelator removes a ligand molecule from activity in solution locally. As a result, the local concentration or activity of the free ligand is reduced creating a concentration gradient, encouraging diffusion of free ligand from the surrounding environment or local source. In this way a buffer may increase the rate at which a ligand leaves a cell even though a buffered amount of ligand remains physically close to the cell chelated by buffer molecules. The apparent concentration and local activity of the ligand depends on its ability to react in its bound state and on the concentration and diffusion coefficient of the buffer. We will test the rules of thumb that have been suggested for use of buffers, particularly as they apply to the measurement of external proton gradients associated with growing pollen tubes [4].

2. The Mathematics of Buffer and Proton Flux Measurement

The total flux of protons (${}^{T}\mathbf{J}_{H}$) in the presence of buffers is calculated as the sum of a diffusional component (${}^{D}\mathbf{J}_{H}$) and a facilitated component (${}^{F}\mathbf{J}_{H}$) [5]. The diffusional component is calculated the traditional way using the flux equation (1),

$$ {}^{D}\mathbf{J}_{H} = \mathbf{D}_{H} \bullet (d[H^{+}]/dx) \qquad (1) $$

where $\mathbf{D}_{H}$ is the diffusion coefficient of the proton and $(d[H^{+}]/dx)$ represents the proton concentration gradient over the distance dx.

The facilitated component is carried by the facilitating buffer(s) and its calculation (2) is dominated by the diffusion coefficient and concentration of each buffer, (B_1, B_2, ..., B_n).

$$ {}^{F}\mathbf{J}_{H} = \mathbf{D}_{B1} \bullet (d[B_1]/dx) + \mathbf{D}_{B2} \bullet (d[B_2]/dx) + \ldots + \mathbf{D}_{Bn} \bullet (d[B_n]/dx) \qquad (2) $$

After algebraic simplification and some substitution [5] we are able to calculate (3) buffering capacities, **ß**, of each buffer,

$$ \mathbf{ß}_{Bi} = ([B_i]/[H^{+}]) \bullet (f/(1+f)^{2}) \qquad (3) $$

where $f = K_d / [H^{+}]$ and K_d is the dissociation constant or log pK_a of buffer Bi. These coefficients, $\mathbf{ß}_{Bi}$, vary as indicated with buffer concentration and pH, and thus they must be calculated point by point where they are applied, but they will allow us to calculate the total flux (4) more directly,

$$ {}^{T}\mathbf{J}_{H} = (\mathbf{D}_{H} + \mathbf{D}_{B1} \bullet \mathbf{ß}_{B1} + \mathbf{D}_{B2} \bullet \mathbf{ß}_{B2} + \ldots + \mathbf{D}_{Bn} \bullet \mathbf{ß}_{Bn}) \bullet (d[H^{+}]/dx) \qquad (4) $$

using only the measurement of local pH, the pH differential and the known concentration of each buffer. The only missing information in this calculation is the

diffusion coefficient for the buffers. Such diffusion coefficients are not always available in the literature. However, they can be estimated by calculation from the measured Stokes radius of the buffer molecules and a calibration equation of known diffusion coefficients of several weak electrolytes (Fig. 1A) whose diffusion coefficients were obtained the hard way by our physical chemist forebears. This same figure demonstrates the anomalously high diffusion coefficient for protons, which assume the form of hydronium ions in aqueous solution. The higher than expected diffusion coefficient for protons is however not shared with water, as measured by the dispersion of water's oxygen, which behaves like a typical weak electrolyte. This enigma is solved by noting that protons are part of the reverberating hydrogen bonding structure of the hydronium ion, which encourages the phenomenon of tunneling similar to electron tunneling in a conductor wire.

Table 1. Physical properties of the Good [1, 2, 3] buffers. The maximum distance between atomic centers of each buffer was measured from a 3-D model and used as an estimate of its molecular Stokes radius (a). The same was done for weak electrolytes with known diffusion coeficients (D). Linear regression of D vs 1/a for the weak electrolytes was used to predict the expected value of the Ds (E[D]) of the Good buffers. Atomic distances were measured using the Chem-3D plugin from CambridgeSoft Corporation (www.camsoft.com) in combination with 3-D data models of the compounds available from the online ChemFinder Database.

Buffer	**pKa**	**Atomic dist**	**2a [Å]**	**E [D]**
MES	6.15	O_7-H_{20}	7.08	0.820
ACES	6.90	H_{12}-H_{13}	10.03	0.668
MOPS	7.15	H_{14}-H_{21}	10.15	0.664
HEPES	7.55	O_{14}-H_{16}	11.41	0.618
TRICINE	8.15	H_{13}-H_{16}	8.56	0.731
TRIS	8.30	H_{11}-H_{15}	6.28	0.886

Using the linear regression of D vs 1/a from Fig. 1A, we can estimate the unknown diffusion coefficients for the Good buffers we worked with in this study: ACES, Tricine, MES and MOPS (Fig. 1B, Table 1). By examining the equations for flux it is clear that the diffusional flux, calculated by considering only the pH gradient and proton diffusion, can under-report the total flux by a large amount, particularly when substantial buffer concentrations are used and one is operating in the operational range of the buffers. The under-reporting needs to be augmented by the diffusion of buffer components available to combine with protons, which are sharpening the local gradients and carrying a latent flux of protons along with the buffer molecule. Thankfully, the calculation of total proton flux given (4) is a function (5) of known and measureable values,

$$^{T}\mathbf{J}_{H} = \text{fn}(\text{pH } d[H] \mid dr\ [B_i]\ pKa_{Bi}\ D_{Bi}) \qquad (5),$$

known constants for the current experiment dr and $[B_i]$, physical constants pKa and D_{Bi} of each buffer, and the two time-dependent variables, pH and d[H], which are measured by the ion probe at each queried point. The time dependency of this result is a major complication that should be taken seriously when investigating living systems. One needs to realize that the buffering capacity of the medium has adjustable limits and an investigator needs to plan whether the buffer will be functioning in its useful buffer

range near the cell surface and if not, how far away from the cell one must get until a buffered condition will exist. The following exercises in measuring artificial proton sources will help understand how to achieve these objectives. Ultimately we will see how this approach applies to a growing pollen tube.

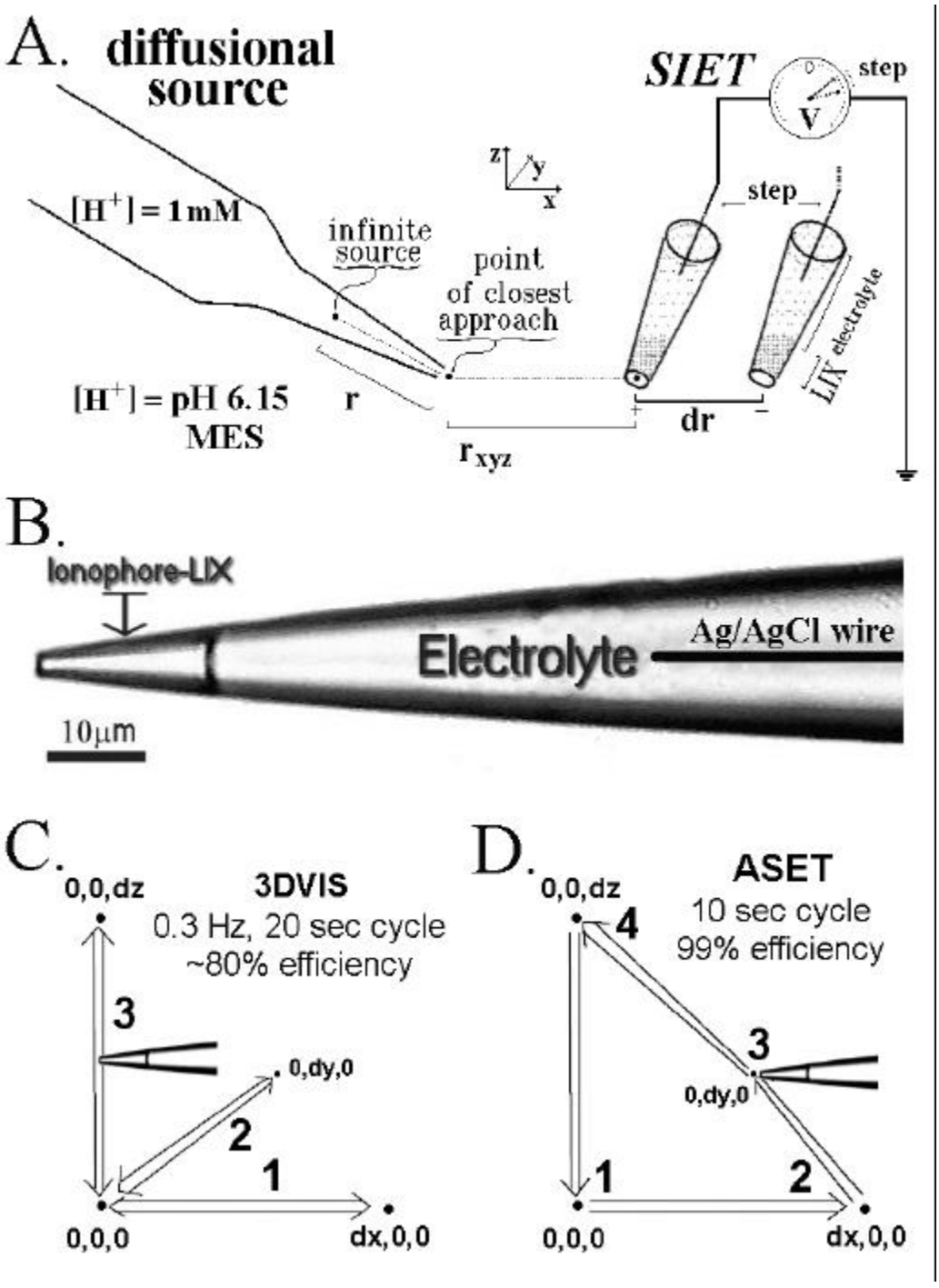

Fig. 2. Ion selective microelectrode. **A.** Artificial diffusional source arena for calibrating a scanning ion-selective electrode (SIET). **B.** Probe tip with ion-selective liquid ion exchanger (LIX), electrolyte and Ag/AgCl wire in place. **C.** Old 3DVIS 3-D sampling protocol using oscillating motion. **D.** New ASET 3-D sampling rule approach.

3. Dynamic measurement of proton flux using non-invasive ion probes

The use of liquid ion exchangers (LIX) in glass microelectrodes to measure the concentrations of specific ions in solution is a well-established methodology [6].

Traditionally this is done with an electrode that is stationary and can reach its appropriate Nernstian voltage in response to a concentration of the target ion in a reasonable amount of time; 10's of seconds would be acceptable for most uses. However, when using these microelectrodes in dynamic approaches (Fig. 2) to measure the flux of an ion in solution adjacent to a cell, time is often of the essence and a second or fraction of a second is the preferred time scale for measurement. The most rapidly responding LIXs are used for dynamic self-referencing techniques [7, 8]. Modern self-referencing ion selective scanning electrodes move programmatically from one position to another a short distance away (5-10 µm) under the control of a computer driven motion control system, stop at each point, wait briefly for gradients to reestablish themselves and then measure the ion concentration at that point. One is able to calculate the expected flux of the ion between the two points visited based on the concentrations measured by the probe and the laws of physics. Improvements in hardware and software have made the efficiency of measuring proton flux close to 100% (Fig. 2C, D). While this approach is basically simple, complications arise when the ion measured is the proton. The proton probe technology was initially applied in an idealized setting in which there were few complications, i.e., no buffers. Any compound, or structure which binds the ion of interest is a complication. A buffer will bind the ion reversibly, taking a fraction of the ions out of free solution and changing the apparent concentration of the ion of interest and, by removal from free solution, change its apparent reactive and apparent physical properties. Protons associating with water have an apparent diffusion coefficient of $9.4\ 10^{-5}\ cm^2/sec$, while when associated with the Good buffer MES they diffuse 12-fold slower, $0.8\ 10^{-5}\ cm^2/sec$ (Fig 1). The net results of the buffering are complicated and take considering the concentration, the activity and the buffered reserve of the ligand into account. When possible, to minimize the number of variable compounds that need to be followed, no buffer, or a low concentration of a Good buffer which is not itself changed in concentration during the experiment should be used. Clearly, simple unbuffered or low buffered conditions can not be maintained in all experimental arenas. Bicarbonate and phosphate are components of many established Ringer solutions and culture media, which were designed to maintain the pH at a prescribed level. Bicarbonate is also secreted in substantial amounts by highly metabolic cells and phosphate may be transported into the cell, depleting its concentration immediately adjacent to the cell. How does one deal with such situations using ion probe technology? Does it matter?

4. Effect of Buffer Strength and pH on Proton Flux

One might ask, why use a buffer at all if it presents unfriendly complications? First, buffers produce a stable environment that one can work in for a sufficient amount of time such that the experiment can be reasonably replicated under similar conditions. Without a buffer the proton gradients around a cell or tissue may change continuously and be ill-defined. This may not be a problem from the point of view that one is seeking to discover how the cell is modifying its environment. However, it can be technically difficult establishing if the probe is working properly when there is no place to go locally with the probe where a stable background pH is measurable. Confidence in the functioning of the electrode is extremely important in the use of these non-invasive electrodes over extended periods of time. Also, buffer strength is critical for being able to localize a proton source or sink associated with a region of a cell or a particular cell within a tissue. This can be demonstrated by examining an artificial source of the ion with predictable geometry in an arena configured as shown in Figure 2.

We demonstrate how buffers sharpen gradients allowing sources and sinks to be localized more easily (Fig. 3). Researchers have used various concentrations of buffers for culturing or examining their cells (Table 2) and each of these situations needs to be considered as far as its effects on proton flux. A look at the facilitation factor in this table shows that while using the same concentration of HEPES, the adjustment of the pH by 1 unit results in a greater than 5 fold difference in the facilitation of proton flux by this buffer. This is in addition to the change in proton diffusional flux due to the higher (or lower) gradient imposed by the 7.2 to 8.2 pH shift.

Table 2. The buffer molarities used by investigators in non-invasive ion probe studies of biological systems with a computed facilitation factor ($^{D}F/^{T}F$) of increased proton flux due to that level of buffer at the prevailing or potential pHs.

Buffer pK	molarities, pH	$^{D}F/^{T}F$	references
MES	15 mM, pH 5.5	63	pollen tube growth
6.15	1 mM, pH 6.0	22	pollen tube growth and Ca flux [9]
	50 µM, pH 6.15	3	pollen tube growth and H flux [4]
HEPES	0.001, pH 8.2	1495	gastric oxyntic cells [5]
7.55	0.001, pH 7.2	230	*Periplaneta* oocytes [10]
Bicarbonate	0.005, pH 8.2	840	gastric oxyntic cells [5]
6.13	0.0024, pH 7.8	395	*Xenopus* oocytes [10]
	0.0048, pH 7.8	789	
	0.0024, pH 6.13	104	
Boric acid	0.0016, pH 6.0	1	pollen tube growth Ca flux [9] and H flux [4]
9.14	0.0016, pH 7.0	16	
	0.0016, pH 8.0	1281	
	0.0016, pH 8.2	2976	

In general, the facilitated diffusion factor is additive and does not become significant until the product of buffer capacity and buffer diffusion coefficient, $\mathbf{D}_B \cdot \mathbf{ß}_B$, approaches or exceeds the proton diffusion coefficient. This will only happen when the local pH is within the buffer's functional buffering range and buffer concentration is high enough to achieve significant buffer capacity to accept or donate protons. Of note here is the potential participation of the essential plant growth ingredient, boric acid, in the facilitated diffusion process. Boric acid, like many weak acids, can behave as a buffer for the range pH 8-10.8, however it has potential buffering effects down as low as pH 5.5 if the amount of boric acid is high enough. A 100 mM solution of boric acid is intrinsically pH 5.2. Table 2 shows that there is no significant facilitated component of proton flux carried by boric acid for pH 6 with the 1.6 mM boric acid included in the culture media used by several investigators. But, if the pH is raised 1 or 2 units, boric acid becomes a significant carrier of proton flux, rivaling that of similarly sized bicarbonate. Such pHs may very well be achieved in small extracellular regions adjacent to metabolically active cell surfaces. Figure 3 also demonstrates the importance of adjusting the buffer strength to the source strength; in this case the 60 µM buffer is too weak and the 7.5 mM buffer is too strong to delineate this diffusional point source. If the probe were not brought within about 10 µm of the source in 7.5 mM buffer, the source might be totally missed despite the rather strong nature of this particular source. Searching for proton pumping cells in an epithelium or patches on a cell surface should not be done with such highly buffered media. At the other extreme, in low (60 µM) buffer, details of a complex pattern might easily be obscured due to the long distance effects of prominent large features. Of some interest is the fact that at this source strength, time scale, pH and buffer strength there is very little apparent facilitated

diffusion component to the flux of protons (Fig. 3) in the examined region of the strong artificial source.

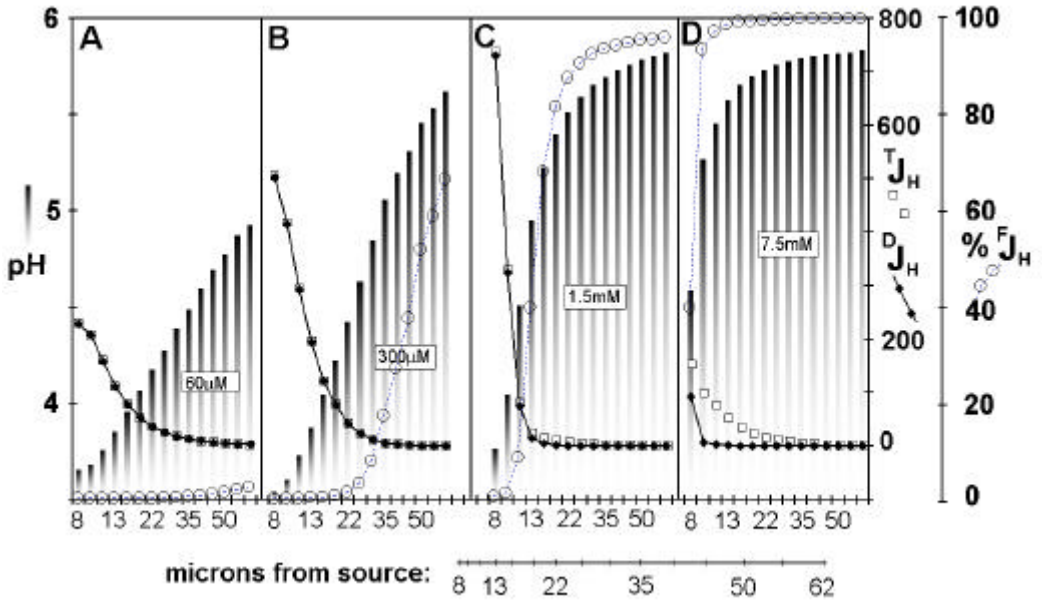

Fig. 3. Diffusional, $^{D}J_{H}$, and facilitated, $^{F}J_{H}$, components of total flux, $^{T}J_{H}$, are affected differently by increasing concentrations of the Good buffer MES. An artificial source of protons is scanned with the same probe in different bath solution buffer concentrations. Note the increased steepness of the diffusional flux curves as the buffer strength is increased from 60 µM to 1.5 mM MES (inset labels). The only significant facilitated diffusion is detected at the high buffer concentration and at this concentration the diffusional flux is all but extinguished. Flux is given in picomoles per cm^2 per sec. The bath solutions used included: x µM MES, 100 µM KCl, 200 mM Sucrose. The artificial source consisted of a capillary drawn to a 2 µm opening, filled with bath solution which lacked buffer, was adjusted to 0.01 N HCl and gelled with 0.08% agarose.

However, using an artificial proton source that is closer to physiological (Fig. 4), the strength of the buffer becomes more important. In the lower buffer strengths (60 and 300 µM), the region close to the source is still effectively unbuffered and the protons are diffusing essentially as hydronium ions without any facilitation by the buffer. Substantial facilitation (close to 100%) is occurring at 1.5 mM buffer and at that buffering level the source could easily be missed if one did not make the effort to find it and compute the facilitated component. At the intermediate (300µM) buffer strength, the flux vectors are stronger close to the source and reasonably measurable out to 15 µm from the source; this is the desirable sharpening effect that buffers afford. Beyond that the measurable flux declines toward zero and the fraction of the proton flux being carried by the buffer increases rapidly toward 100%. The facilitated proton flux is calculated from the measurable proton differences over short distances divided by the predictable fraction which is measurable. Therefore it follows that when the buffer strength extinguishes any departures of the pH from that set at the outset, as is seen at 7.5 mM buffer (Fig. 4), any calculated total flux will have a strong noise component since it is obtained by dividing an instrument noise level at close to zero flux by a small and decreasing fraction of one as one recedes from the source.

These diffusing proton fronts depicted in figures 3 and 4 are snapshots taken at about 15 minutes after the diffusion process was initiated. The process in these inert artificial circumstances is dynamic and one can envision these fronts of pH proceeding to the right as time passes. It is clear that a similar time dependent process is happening in experiments with live material with each patch of cell membrane acting as a point source with its own strength and kinetics. This delineates the problem of measuring proton gradients around cells and why attention to buffer strength is important if the pattern and balance sheet of ionic flux is to be established in particular cases.

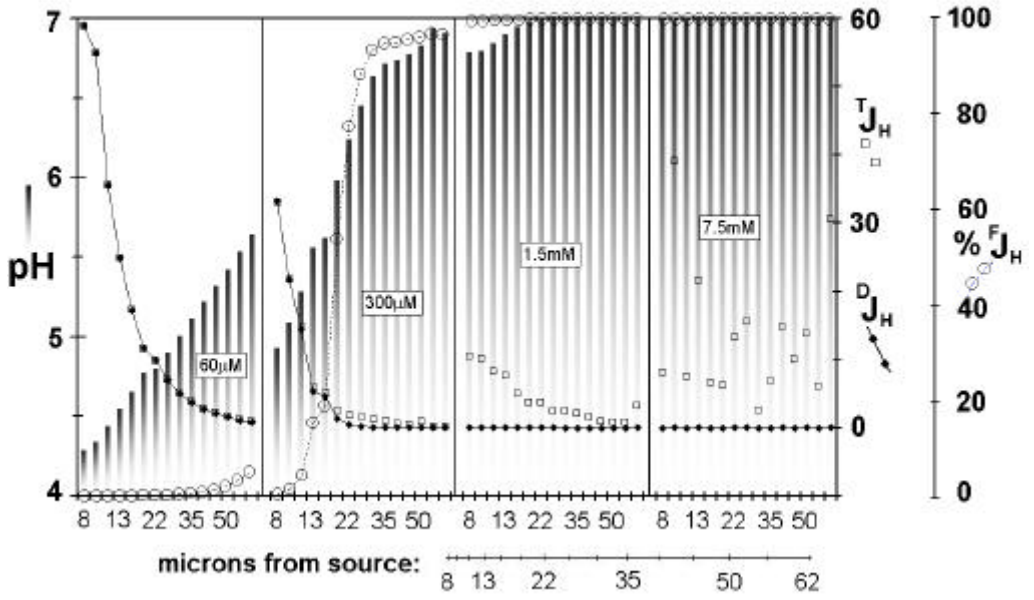

Fig. 4. Diffusional, $^{D}J_H$, and facilitated components, $^{F}J_H$, of total flux, $^{T}J_H$, are affected differently by increasing concentrations of the Good buffer MOPS. An artificial source of protons approximately 1/10 the strength of the source in Fig. 3 is scanned with the same probe with different bathing buffer concentrations. The percent of the proton current that is facilitated (% ^{F}J) is plotted to demonstrate the increased proportion of facilitated diffusion within the range of the buffer and the increased error in estimating the total flux associated with high buffer concentrations. Flux is given in picomoles per cm^2 per sec. The buffers used included: x µM MOPS, 100 µM KCl, 200 mM Sucrose. The artificial source consisted of a capillary drawn to a 2 µm opening, filled with bath solution which lacked buffer, was adjusted to 0.001 N HCl and gelled with 0.08% agarose.

5. Effect of other Buffer components on Point Sources of Protons

Although many components of culture media, like boric acid, are not normally considered buffers, many are weak electrolytes, which have a buffering capacity, although the pK may be far from the media's pH. Amino acids are weak electrolytes and often make up substantial proportions of culture media. Two examples of culture media that have been used to measure proton fluxes using ion probe technology are listed in Table 3 along with the modified medium that was devised for focusing on proton flux. These media are used for historical illustration only and are not recommended as final answers to any future study. Many other examples exist in the literature and we will provide the tools for making comparisons of similar nature with any such choice. The unmodified media in both cases was developed for long term growth which require large amounts of buffer as well as growth supporting substances. The strong buffer and growth supporting substances could potentially be eliminated in designing a physiological saline that will support function for the shorter periods of time needed to complete an experiment on ionic flux. Table 3 briefly compares the critical components of the two media and their improved versions with respect to their role in conducting protons in solution.

The two media differ most dramatically in the reduction of the number of amino acids that they contain and in their concentrations. The first example, Landureau's medium, is known for its use in insect cell culture including neuronal growth. Its components include concentrations of amino acids and TCA cycle intermediates which when examined and compared in table form to modern media, have not changed significantly since their original discovery as essential for culturing by Silver Wyatt in 1960. Grace's medium was developed from the original Wyatt's medium with identical

molarities of many components and is the universally referenced historical medium for insect cell culture. I used Landureau's medium modified for cockroach cell culture for many years in examining protein endocytosis by isolated oocytes. This medium allowed maintenance of close to normal growth of the oocytes in organ culture for up to 24 hours. Being warned of possible complicating factors when using ion probes with complex media, I eliminated the amino acid and TCA intermediates and reduced the buffer, phosphorous acid (H_2PO_3), by a factor of 10. Interestingly, phosphorous acid is listed by Good and Izawa [1] as an underutilized and unappreciated (potentially inert) buffer.

Table 3. Media weak-electrolyte-component molarities and computed percentages of proton flux ($J_{\%}$) by media component. Comparison of original media with media modified for examination of proton flux measurement.

Media, pH components	**[B] mM**	**J%**	**references**
Landureau's, pH 6.8			
H^+	-	0.06	
glu	10	1.2	
gly	10	0.5	
his	1.3	5.3	
lys	1.4	0.3	insect tissue culture medium [11]
H_2PO_3	11	92.1	
Landureau's(-aa), pH 6.8	-	0.7	modified for proton flux measurements [10]
H^+	1	99.3	
H_2PO_3			
DMA, 6.5			
H^+	-	0.52	
glu	10	6.55	
gly	10	1.09	
his	1.2	33.28	
lys	1.4	0.67	
met	1.7	0.43	
PIPES	1	24.16	*Achlya* growth medium [12]
H_3PO_4	1	32.21	
DMA (deficient), 6.5			
H^+	-	0.90	
glu	1	0.86	
met	1	0.43	
PIPES	1	41.5	*Achlya* medium for proton flux study [12]
H_3PO_4	1	55.4	

As Table 3 shows, the reduction in the buffer produces a medium in which proton flux measured as diffusing as protons is amplified 11 fold. The amino acids in

the original medium do not contribute substantially to proton flux at the nominal pH of the medium but complications could arise if the local pH were modified by action of the tissues. In a similar example from Table 3, a modified medium used by Darryl Kropf [12] for examining tip growth, protons and amino acid symport in the water mold, *Achlya*, shows dramatic changes in the mode by which protons move in the media. It should be noted that the investigators verified that tip growth was unchanged by the modifications to the medium. In the original medium, DMA, the amino acid histidine carries 33% of the proton current diffusing in the media. In the modified medium, 97% of the proton flux is carried by two components, PIPES and phosphate. There was no reduction in the buffer concentrations and thus the measurable flux of protons using the ion probe remains low (0.9%). One suggestion might be to test whether the phosphate level could be lowered without adversely affecting growth. Reduction of phosphate to 100 µM reduces the percent of protons carried by phosphate from 55% to 11%. Reduction to 50 µM reduces its percent participation to 6% while increasing the measurable flux with an ion probe by 2 fold over the modified medium. Reduction of PIPES would similarly enhance the measurable flux and might provide adequate buffering at a substantially reduced molarity for the required time of the experiment.

6. Application of Good buffers to Measuring Natural Sources of Protons

As an example of applying the buffer approach to a natural source we have taken data from the lily pollen tube. This one example of using two buffer strengths on measuring 3-D vectors emanating from a growing pollen tube is meant to illustrate the calculation of diffusional flux and the total flux of protons measured in a transect away from the base of the growing pollen tube, Fig. 5A. In the transect away from the pollen grain under the lower buffer concentration regime, 60 µM MES, the percentage of the proton flux carried by facilitated diffusion varies from 22 to 40 percent, Fig. 5B. At the higher buffer concentration the pH is regulated better all the way into the pollen grain surface and the proportion of proton flux that is carried by the buffer remains between 84 and 90 percent. This lower buffer concentration is not absolutely overwhelmed close to the pollen grain; the pH is still inside the recommended zone about the pK of MES. The pH measurements approach 5.5 close to the pollen grain and pH 5.8 at the furthest distance measured in a transect away. Further out from the pollen grain the pH eventually reaches the pH 6.15 to which the buffer had been adjusted. If one was mainly interested in proton secretion, one might adjust the pH of the MES to a higher value than the pK, which would provide a greater proton buffering capacity from the source side. Using the weaker buffer the fraction of the flux which is facilitated and needs to be estimated is small enough that the flux vectors measured are reasonably close to the total flux, particularly close to the pollen tube surface (Fig. 5C). The stronger buffer strength, 300 µM MES, controls the pH more rigorously close in to the pollen grain, pH 5.96 is the lowest pH measured by our probe close to the pollen grain, while further away the pH approaches the maximum attainable background, pH 6.15. The pH closer to the cell is likely to be substantially higher as the cell continuously acidifies its environment. One must imagine the pHs that are approached in the confined space in which the pollen tube naturally grows in order to appreciate the potential role of pH gradients in pollen tube development. A disadvantage of the higher buffer strength is that the only directly measurable quantity is the pH difference, which results in an estimate of diffusional flux. The total flux is computed as a multiple of the measured diffusional flux.

At high buffer concentrations the pH gradient is small and the measurable flux approaches the noise level not far distant from the pollen grain, Fig. 5C. The computed total flux thus includes a larger fraction of noise, multiplied by an increasing factor as the buffer concentration increases. This is similar to the artificial source example (Fig. 4.) in which the total flux illustrated for 7.5 mM MOPS has a large component of noise. Similarly the 300 µM MES buffer results illustrated in Fig. 5C show how, at 200 to 300 µm from the pollen grain, the small measured vectors of diffusional proton flux can be magnified by the facilitation factor of the media to make the vectors seem significant. Without the 3-D spatial context of the surrounding vectors one might jump to wrong

conclusions about the significance of these measurements.

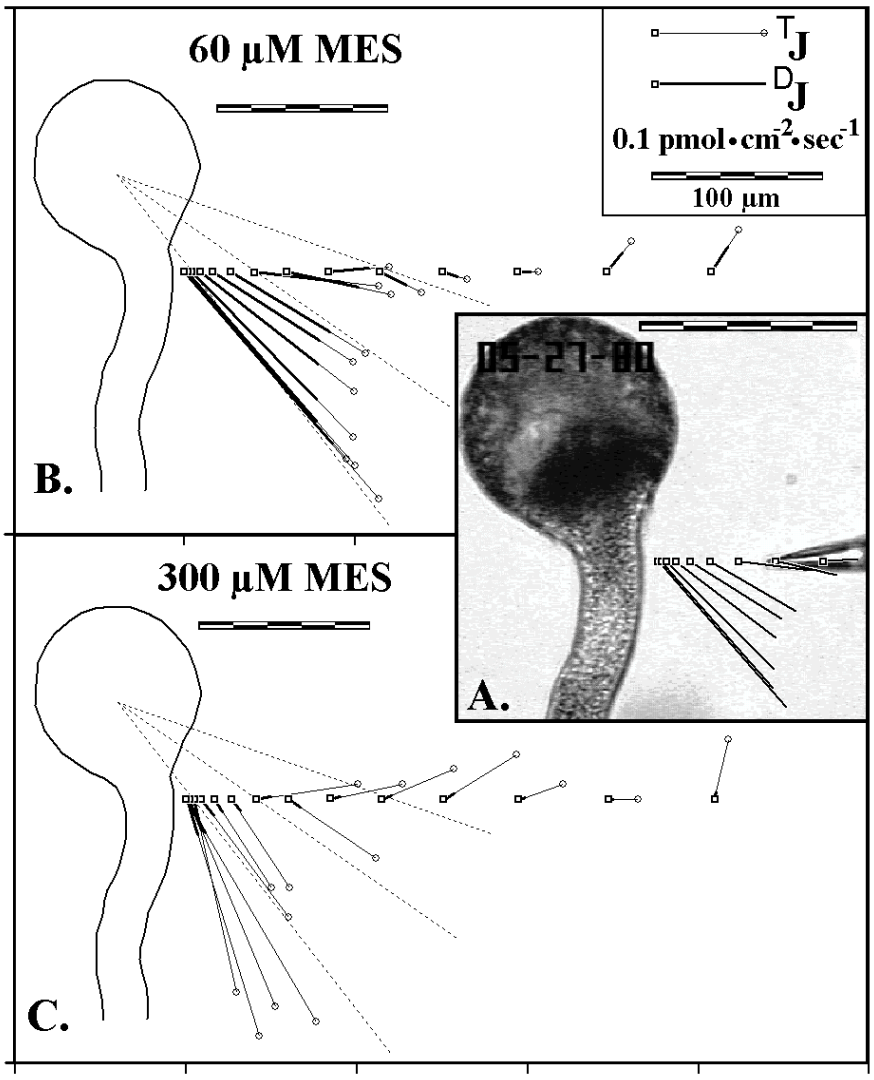

Fig. 5. Lily pollen proton flux measured in two concentrations of MES, pH 6.15. **A.** Raw µV differences measured at positions marked by squares. **B.** Computed diffusional flux and facilitated flux in 60 µM MES. **C.** Computed diffusional flux and facilitated flux in 300 µM MES. The probe is sensative to more global proton flux in the lower concentration MES. Noise becomes a larger contributor to total flux in the higher concentration MES because the measureable information (µV difference over 10 µm excursion) becomes a smaller proportion of total proton flux. The media used to examine the pollen tube growth contained 1.0 mM KCl, 0.05 mM $CaCl_2$, 1.6 mM H_3BO_3, x µM MES and 5% sucrose [4].

7. Discussion

The major recommendations which derive from this exercise on learning to measure proton flux in the face of buffering potential are: (1) keep the culture media as simple as possible; (2) reduce the buffer components to as low titer as possible; and (3) measure the 3-dimensional components of flux in order to be assured of the rational consistency of flux vector strengths and directions relative to a source or sink. Keeping the media simple may involve removing components that are not needed for short-term culture. Kropf and coworkers [12] noted that their deficient-DMA media produced equivalent tip growth of *Achlya* while perhaps undergoing less branching. Nonetheless, the deficient-DMA buffer provided sufficient components to study the phenomenon at hand which was proton enhancement of amino acid transport that accompanies tip

growth. The effort to simplify media will pay off when one needs to start modeling the different diffusing components. Although the equations that were presented above are simple to apply for idealized situations such as the diffusion of protons from a point source, a realistic model of a pollen tube with multiple sources and sinks of protons is still a challenge. If one wishes to include a temporal progression of proton flux starting with a change of new media, it remains a daunting task to compute the patterned progression, even when the number of buffer components carrying the protons is small. By paying attention to the buffer capacity and how fast it is overwhelmed, one can also get an idea of the molar work that is being done in the media. Part of our future calculations may include using the "buffered memory" of protons produced by the growing tube. Currently we throw this information away as the background upon which we must measure an immediate flux; but the background may be able to be used to calculate the latent recent history of the local region.

The tools for calculating diffusion coefficients of weak electrolytes based on their molecular dimensions are available over the internet [13]. It is recommended that anyone working with estimating proton flux into or out of cells should examine the potential facilitated flux component in the media or saline that are planned for use.

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