Feature essay 3.1 - Using thought experiments to strengthen critical thinking at universities

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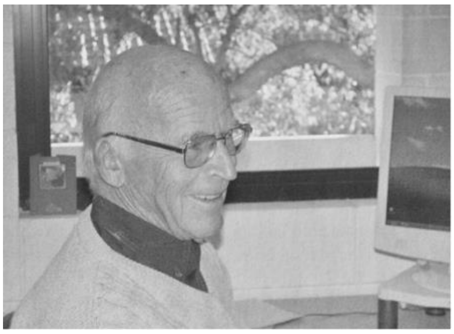
An approach to strengthening critical thinking in university students and young researchers by using 'thought experiments'. It includes an example of a 'thought experiment' using principles of membrane transport of ions (see Section 3.6) and pH regulation in plant cells as the background material necessary to 'solve' the experiment.



Contents

'Fundamentally, university teaching is there for people to take ownership of their own learning' (Frank Carrigan, Macquarie Law School, Sydney).

This paper is based on my experience running University undergraduate courses in environmental plant biology at 3rd - 4th year level. A similar approach might well be possible in the last two years of secondary school and as coursework modules throughout postgraduate research degrees. While this is written as a reflection on teaching in my own field of research, this approach has not been intrinsically geared to plant biology; it can just as well be applied to any area of experimental research. Indeed, mathematics and theoretical physics rely overwhelmingly on thought experiments. This approach builds upon the innate curiosity of many students and the assumption that if provided with sufficient knowledge, they will become innovators. Critical thinking and innovation are listed as key criteria for decisions on acceptance or rejection in a comprehensive survey of requirements for scientific publication (Setter et al. 2020).

My specific purpose was to foster the intellectual independence that comes with putting the principles of the 'scientific method' into practice. The overarching principle is the ability to think critically. The development of critical thinking needs a theme as a framework for discussions. I chose aspects of environmental plant biology, my area of expertise; another lecturer might focus, for example, on organic chemistry. Not surprisingly, I personally think that environmental plant biology provides themes particularly suited for this mode of University education.

Most students need substantial preparation in the subject matter chosen for the course before practicing their critical thinking skills in applying the scientific method. This preparation can begin to foster a student's independence by encouraging questions and answers in tutorials, so allowing the student to take 'ownership of their own learning'. Once students had sufficiently mastered the background to the topic, the key component of my courses consisted of students formulating and solving thought experiments to test hypotheses set by the academic and by the students themselves.

My courses focussed on mechanisms of acclimation and adaptation of plants to adverse environments, such as high salinity and waterlogging. These events will be major determinants of food security as saline soils and floods have ever greater impact on crops. By the same token, a better understanding of the scientific method should substantially benefit our world, which now is so dependent on the technologies arising from scientific inquiry. Tangible examples include a deeper critical evaluation of the arguments around immunisation or climate change, and the most efficient ways to mitigate their severity and ameliorate their consequences. So, thought experiments build valuable intellectual skills as well as confronting real problems for humanity.

The themes of my courses, regulation of solute fluxes and cytoplasmic pH in plants, are key components of acclimation and adaptation to high salinity and flooding. Sufficient background material on these themes is provided in this paper to illustrate the principles of a thought experiment. This material would be suitable as a base for academics and students to formulate many further thought experiments.

1. Development of study program and its rationale

The objective was to strengthen the students' skills in critical reasoning. In this study program, these skills were fostered by students conducting and discussing thought experiments to solve real problems.

What's the difference between this and a normal question-and-answer tutorial? A normal tutorial discussion tests whether students have understood ideas as conveyed by textbook and/or an academic 'expert'. Thought experiments on the other hand are fundamentally distinct from this approach; they entertain multiple solutions to test each hypothesis and therefore require the iteration of ideas between students and their tutors. That is, they encourage intellectual engagement by stimulating curiosity and initiative. Thought experiments are the difference between simply understanding current theories and developing the skills and confidence to use this knowledge to make one's own contributions.

1.1 Design of the study program

The phases of the study program are set out in Table 1. The course began with an introductory lecture to outline the approach, providing the groundwork to motivate students to take '*ownership of their own learning*' (Carrigan, 2018). The remainder of the course was based on my prepared text, which included both references to key papers and quiz questions. The text was supplemented by tutorials that focused on questions from students prompted by the text and its related material. These together provided the background and 'lead-in' for the thought experiment to follow. The questions from students could be either handed in ahead of the tutorial or raised during the tutorial. The final step was the formulation by the tutor and students of the thought experiment to be tackled individually by the students over several days. Optionally, students could hand in their solutions to the thought experiment or could raise them during a tutorial-seminar set for this purpose.

Table 1. Design of courses implemented by Hank Greenway to 'empower' students to 'take ownership of their own learning' and practise the scientific method. [1]Students were encouraged to submit questions and comments on a particular theme in writing 24 h before a tutorial. These questions from students guided tutors on the themes to tackle and how best to maximise student participation in tutorials. [2]Results of the survey ranged above 8 in a scale out of 10. While participants tended to respond that no improvements were needed, tutors had several suggestions that improved the study guide and discussion within the tutorials.

Phase	Purpose	Description
1	Understanding of subject, e.g. solute transport	Study guide (text), including suggested reading, contains quiz questions and answers to enhance student understanding.
2	Enhancing understanding of subject	Tutorials on questions and comments handed in by students[1] prompted by the study guide and associated literature.
3	Critical thinking phase	Poses thought experiments which participants tackle individually, preferably with their solution submitted before the tutorial / seminar to follow.
4	Practising the scientific method	Tutorials / seminars to debate submitted solutions and new solutions that might arise during discussion, leading to formulation of new hypotheses.
5	Adding an experimental component	To complement the course, students might reach the stage of testing their own hypotheses in a laboratory (see section 3 of this paper).
6	Evaluation and assessment of the students by staff	Students were evaluated by their contributions to solving of thought experiments in written form and during discussion at tutorials.
7	Evaluation of the course by students	Anonymous assessment of the study course was obtained by asking students to rate the course from 1 to 10 and to suggest improvements[2].

Description of a thought experiment

A thought experiment is the practice of 'thinking through' the consequences of a hypothesis (https://en.wikipedia.org/wiki/Thought_experiment). In this paper, the focus is on testable hypotheses. Section 2.4 gives an example based on a publication by Ullrich and Novacky (1990) concerning ion movements associated with pH regulation.

A thought experiment can take the following form:
(i) A plausible hypothesis (this might be found in the literature or generated by students during tutorials);
(ii) The 'expected' result might sometimes be observed, but sometimes the opposite might occur, so creating an enigma;
(iii) A new hypothesis is developed bringing together concepts to explain the discrepancy in results;
(iv) An experiment is proposed to test the new hypothesis and resolve the enigma (the solution).

In practising with these thought experiments the students are encouraged to find their own solutions. *After the students feel they have found the solution they are encouraged to formulate a new hypothesis.* It is very important to emphasize that in contrast to the more usual 'question-and-answer' that test knowledge, students should not aim at a quick answer and then check the solution, or whether they are right: that approach will not develop their critical, innovative thinking. Rather, participants should try hard to find their own solutions, put the problem aside for some days, do some more reading and ponder about the enigma. Then, they often will get the broad outline of the solution in a flash. For example, when they are swimming, walking or having a drink. This insight may show the hypothesis being considered is not sufficient, or on the other hand, that it is worth testing in the laboratory. Only when the participants feel they have a rational solution can they write a statement which can be handed out and discussed during a tutorial / seminar.

There are many general papers on the development of critical thinking and its use in education (http://www.criticalthinking.org/) including a seminal paper on cognitive learning and the 'hierarchy of thinking levels' by Bloom (1956). Here we have described a plant sciences course focused on developing critical thinking in 3rd to 4th year university students, which is distinguished by its inclusion of thought experiments created by the academic, but also by the students after they have understood the subject and can evaluate the data. At this level, practice of a thought experiment relied heavily on the initiative of students, although more structured guidance might be required for students in the final years of secondary school. Crucially, thought experiments were discussed by the participants during tutorials / seminars, so encouraging them not only to engage in higher level critical thinking but to have the confidence to put forward and debate their reasoning. In my experience posing of questions to be solved by thought experiments has been the key to achieving the independence of the students, with the measure of independence being the expression of innovative ideas, some of which have led to published experimentation (Colmer et al. 1998).

1.2 Reasons why the study program was developed

Carrigan's statement quoted above that '*University teaching is there for people to take ownership of their own learning*' seems so obvious. Yet, it took me years at The University of Western Australia to develop a design that did motivate the students to become independent and skilled in debating their views. To amplify the importance of this change in teaching approach, I give a brief description of the various stages of my University teaching career.

When I started as an Associate Professor in 1968, at the School of Agriculture of The University of Western Australia, my courses were centred on lectures. I used this classical teaching approach even though I had early on read the sage advice of an experienced academic: '*Before giving a lecture ask yourself if a lecture is really the best way to stimulate the interests of the students*'. Fortunately, with time, I became disillusioned with the approach based on lectures. This in particular when I marked examination papers: even the brightest students showed little evidence they really understood the principles taught during the year. The turning point came when one of my students chose a biochemistry course about which he was very enthusiastic. So, I went to the biochemistry lecturer for advice (Dr Patty Weaver, UWA). She also had found her courses based mainly on lectures did not work, so she had developed a mode based on study guides and tutorials. Her explanation is worth recounting in some detail: marking examination papers she found only few principles had been grasped. Then she looked at the exam results of a course of one of her colleagues, who was renowned as an orator. Surprisingly, she found the same dismal results, so she concluded that when even her 'orator' colleague failed, lectures would remain ineffective for her and she developed a new type of course. These were based on texts written by herself with references to relevant sections from textbooks and scientific journals. It should be emphasized that tutorials were an integral part of these courses.

I realise the emphasis on the tutorial in teaching is not ‘rocket science’. For a long time, tutorials have been used, among others, by the University of Oxford and the University of Cambridge as a core teaching method (http://www.ox.ac.uk/admissions/undergraduate/, https://www.undergraduate.study.cam.ac.uk/courses/). These tutorials are based around ‘conversations’ between a small number of students and their tutor, a specialist in the topic. The University of Cambridge lists this system of ‘personal tuition’ as one of its greatest strengths (https://www.undergraduate.study.cam.ac.uk/courses/how-will-i-be-taught). Closer to home, the benefits of encouraging student ownership of their learning experience have been recognised by The University of Western Australia through its support of a ‘flipped’ classroom model. This model encourages on-line content delivery, with face-to-face in-class time used to engage students in collaborative, complex problem-solving activities (http://www.worldclasseducation.uwa.edu.au/eeu/flipped-classroom).

It is timely to comment here on the use of the internet in teaching. Of course, with the ability to use the internet, the question of whether a classical lecture course is still warranted has become more acute. In my view, so-called self-pacing courses based on scripts on the internet without suitable backup by tutorials are not much better than lectures, and possibly inferior.

Assessment by participants

About 150 students participated in the course I describe here over a 4-year period at UWA, while around 50 participated in workshops in Southeast Asia. Years later at reunions, students continued to rate the courses as a substantial success, with several graduates commenting to me: ‘*in your courses you always strived to make us think for ourselves*’. I used the same approach during workshops lasting 2 - 3 weeks among others in Southeast Asia. In Thailand, the background of the participants ranged from agricultural college to PhD graduates. The response was most encouraging; students from across these different backgrounds came up with innovative solutions. A visiting English academic commented: ‘*We often say it is difficult to get Thai students to talk and express their own views. However, your courses show that need not to be so at all*.’

The outcome of this type of approach was remarkable. Many students designed astute and sometimes original thought experiments, and tutorials were lively and stimulating, as described in the words of one colleague, Tim Colmer, who experienced this approach as an undergraduate and used it successfully in workshops in Southeast Asia.

Testimonial by Tim Colmer, University of Western Australia

I have been extremely fortunate to have experienced Hank Greenway's teaching style, both as an undergraduate student at the University of Western Australia (third year unit on Environmental Plant Physiology, 1988), and then as a young academic as part of the team assembled by Hank to deliver Training Workshops on Experimental Techniques in Plant Physiology in Thailand (1996) and Vietnam (1997). As undergraduate students, we soon realised that we were experiencing what at that time was an 'unusual approach' - there were only a couple of lectures (to help orientate us), readings from a well-prepared booklet and of a published review, and two tutorials per week. We soon become active learners, bring our questions to the tutorials at which Hank and/or team members from his laboratory would enthusiastically engage us in wide-ranging discussions and encourage us to find answers from our own reading of other literature and increasingly from thought experiments. A highlight was that mid-way through the semester we formed small groups and each group *conducted an experiment - designing an experiment to test our own hypothesis - learning about the scientific method and other valuable skills such as team work, critical thinking, data presentation, and the written and oral communication of our results (as well as plant stress physiology). This was an experience that several other graduates still talk about when we occasionally meet and reminisce, as well as being an excellent learning experience and preparation for our final year's honours research projects.*

As a young academic, I enjoyed being part of Hank's team for an Australian Crawford Fund Training Workshop in Thailand. I observed the careful planning; the selection of the pre-reading papers, the workshop booklet, a few key lectures, tutorial discussion topics, and hands-on experiments. A great strength of the approach was to cater for the interests of the participants, within the overall workshop theme of Plant flooding stress. Groups conducted biochemical experiments, studied nutrient uptake, evaluated responses to ethylene, or quantified aerenchyma and root radial oxygen loss. Participants gained theoretical knowledge, applied the scientific method and gained skills in some laboratory techniques. Although the time was short, the experiments produced valuable data sets, and even resulted in a joint publication by one of the groups (Colmer et al. 1998).

The success of the workshop in Thailand resulted in the Vietnamese participant suggesting a similar workshop held in Vietnam. Hank enthusiastically applied for funding again from the Crawford Fund and he organised the Workshop. Unfortunately, a health issue meant that Hank was not able to participate. Tim Setter and I lead the workshop following the 'Greenway Recipe', and once again achieved in-depth discussions, participant-centred learning with various hypotheses and thought experiments discussed, and some experiments conducted by small groups. I have recently been fortunate to meet again each of the main in-country hosts of these two workshops; we reminisced fondly what enjoyable learning experiences we had together and how these had influenced our subsequent careers.

Finances

One obvious practical problem can be the scarcity of funding for tutors required when the number of participants is higher than say 12 - 14 (the optimal numbers for a tutorial), and indeed, the design of university education described here was in my experience often quickly dismissed with: '*there are no funds for tutors*'. I was able to overcome this financial hurdle by involving PhD students. During the period in the 1980s when I developed this approach, the PhD students were Brian Atwell, Ed Barrett-Lennard, Miles Dracup and Tim Setter. These students received no stipend for their contribution. Feedback indicated that they felt they had benefitted from the stimulation endowed in guiding the less advanced students, together with reinforcement of their own understanding of physiological principles and their exposure to methodologies to encourage critical thinking in others. More recently, all these contributors have commented that giving these tutorials has benefitted their later careers.

There must be other ways to overcome this financial problem and academics surely should be innovative enough to find solutions, particularly in this new age where *in vivo* lectures can be readily replaced by on-line presentations (lectures and appropriate texts). As one example, the online textbook 'Plants in Action' (http://plantsinaction.science.uq.edu.au) is a free resource available globally, which can be used as suggested reading in the early phases of courses such as the one outlined here.

2. An example of a thought experiment

This section provides an example of a thought experiment. First, however, some essential background is given on solute transport and factors determining pH in plant cells to allow those who are less familiar with these themes to appreciate this exercise. Independently, students in the biological sciences are likely to find these sections particularly relevant and useful. In my experience, the background is also excellent for biological science teachers who wish to develop their own thought experiments, while the example will illustrate to those from the more fundamental sciences why this approach is rigorous. Even so, it is important to note that these sections are not a comprehensive review. Instead, they give some interesting facets of solute transport and pH regulation that can be used to solve the thought experiment and might be helpful in formulating other thought experiments.

Why choose solute transport and pH regulation? My reasons for choosing these themes were partly because they are particularly suited to foster innovative and independent thought, and partly because I am myself fascinated by them. Another reason is that many principles of solute transport and pH regulation are well defined but understanding how they interact in plants under stress can be fraught. Furthermore, the theme of solute transport and pH regulation is a critical component of the physiology of animal as well as plant cells. As an example, during a personal experience I found criteria that were used to judge recovery of patients in intensive care are remarkably similar to those used by many plant scientists for health of plant cells, e.g. pH, energy-dependent transport and K^+/Na^+ regulation.

Solute transport is a key process in nearly all biological cells, and pH regulation to a large extent depends on solute fluxes. Further, pH regulation in plant cells is central to metabolism, with nearly all metabolic processes in one way or another regulated by pH. Because they are key processes, some readers are likely to be familiar with the topic and might wish to skip sections 2.1, 2.2 and 2.3 in which we describe the basic background necessary to participate in the thought experiments. For those not so familiar with ion relations in biological cells I hope they find the topic as fascinating as I did when I came across it during my career in environmental plant biology.

I will now focus on material essential to understanding the thought experiment. This will include some characteristics of the plant cell, and of solute transport across a membrane, including transport along and against a free energy gradient, membrane proton pumps, co-transport systems and very briefly, K^+ channels. I will then focus on factors that determine pH in biological cells before turning to a detailed description of a thought experiment.

2.1 Some characteristics of the plant cell

The plant cell is a small volume separated from its environment by a lipid bilayer embedded with proteins, forming a semi-permeable membrane called the plasma membrane. A specific feature of plant cells is that only meristematic cells are similar to animal cells, being mainly cytoplasm. All more mature plant cells are highly vacuolated, i.e. a cytoplasmic shell surrounds a large central vacuole, that contains mainly electrolytes, but can also contain organic acids and sugars. These ‘compartments’, the cytoplasm and vacuole, are separated from each other by a semi-permeable membrane called the tonoplast. At steady-state, the environment and cytoplasm and vacuole are in dynamic equilibrium, that is, in flux equilibrium (Dainty, 1962). The cytoplasm in most plant cells has a very high surface area to volume ratio, which helps to explain the very rapid effect that solute transport across its bounding membranes has on concentrations within the cytoplasm.

Plant tissues contain a large number of solutes including inorganic electrolytes, such as K^+ and Cl^-, and organic metabolites, such as sugars, amino acids and organic acids, some of which carry a charge at physiological pH. The fluid in the cytoplasm of a cell can differ greatly in composition from that in the vacuole, with the pH of the cytoplasm usually ranging between 7.2 - 7.5, while the vacuole is in most cases between 4.5 - 5.5. In most plant cells under optimum conditions, K^+ is a major cation, with the concentration of K^+ in the cytoplasm typically around 80 mM, while the concentration in the vacuole is more variable depending on the concentration of K^+ in the environment (Leigh, 2001). The balancing anions vary, consisting of Cl^-, NO_3^- and, if their sum in equivalents is lower than K^+, dissociated anions of weak organic acids make up the deficit. An imbalance between inorganic cations and anions can be met by changes in organic acid metabolism, with organic acids synthesised to provide balancing anions when increases in concentration of inorganic cations exceeds that of inorganic anions, and *vice versa*, with organic acids catabolised when increases in inorganic anions exceed cations. For example, organic acids increased substantially in excised roots of 6-day old barley plants upon the addition of K_2SO_4 to the bathing solution containing 0.2 mM $CaSO_4$ (Hiatt 1967). Uptake of cations from this treatment solution would be much larger than of anions, leading to synthesis of organic anions by the root cells. Conversely, organic acids decreased in excised roots when $CaCl_2$ was added to the bathing solution (Hiatt 1967). Here, cation uptake from solution would be less than anion uptake leading to catabolism of organic anions. Table 2 shows that the changes in cations and anions in excised roots were of the same order of magnitude. In this example, the solutions in which plants were raised and the treatments applied contained $CaSO_4$, so providing the calcium ions needed to protect membrane integrity of the plant tissue.

Table 2. Changes in concentration of K^+, Cl^- and organic acids (μmol g^{-1} fresh weight) over 6 h in excised roots of 6 day-old barley plants raised in 0.2 mM $CaSO_4$.

Treatment	K^+	Cl^-	Organic acids
KCl	+39	+36	+1
K_2SO_4	+22	negligible	+18
$CaCl_2$	negligible	+11	–10

How do these changes in organic acid metabolism impact on pH? According to Stewart (1983), the H^+ concentration is not determined directly by such proton-producing or consuming processes but rather by the strong ion difference (SID) between cations and anions to which the dissociated anions of organic acids contribute (see section 2.3). As such, any changes in pH during organic acid metabolism will reflect the changes in SID. For further insights into pH regulation see section 2.3 of this paper, and Smith and Raven (1979).

Unless stated otherwise, our focus is on the cytoplasm of the plant cell and the transport of solutes across the plasma membrane that separates the cytoplasm from the environment surrounding the cell. Comparable transport mechanisms occur at the tonoplast.

2.2 Solute transport across a membrane

The fluxes of solutes across a membrane can be diffusive, that is along a gradient of free energy, or energy-dependent, that is occurring against a free energy gradient (Nobel, 1974).

Before discussing solute fluxes across a membrane, there are two key concepts that need to be understood with regard to fluxes of electrolytes. First, movement of electrolytes will alter and at the same time determine the electrical potential across the membrane. At a steady membrane potential, the flux of positive and negative electrolytes across the membrane is equal. Second, in all solutions bounded by a cell membrane, the number of negative charges is balanced by the number of positive charges (see Plants in Action Chapter 3.6.3, http://plantsinaction.science.uq.edu.au/content/363-ions-charge-and-membrane-voltages). The membrane potential reflects a tiny imbalance in the concentration of cations and anions on either side of the membrane, the membrane potential difference arising from the unequal transport of charge by diverse ions through the membrane (Dainty 1962). In a healthy plant cell, the normal resting potential across the plasma membrane is around –115 mV. The capacitance (C) of biological membranes is very small, at around 1 mFarad cm^{-2}, and so the charge (q) imbalance at the membrane need only have changed by 1 pmol of univalent ion per cm^2 of membrane to see a 100 mV change in membrane potential (V), where $V = q/C$. That is the equivalent of 12 nmol L^{-1} of univalent ion averaged over a cell volume of about 650 pL (see Chapter 3.6.3). Another way of looking at it, given the cytoplasm is only 10% of the cell volume and assuming a cytoplasmic concentration of ~80 mM, the concentration of a univalent ion in the cytoplasm only needs to change by about 1.5 millionth, a tiny amount, to change membrane potential by 100 mV. The very small membrane capacitance and the tiny change in charge imbalance at the membrane that is associated with a change in membrane potential helps to explain the rapid response of membrane potential to perturbation. Interestingly, a similarly high sensitivity applies to the magnitude of the differences between cations and anion concentrations to enact changes of pH in the physiological range.

(a) Transport along a free energy gradient

Consider first the energy-independent transport of a neutral solute along its concentration gradient. For a neutral solute, this can be expressed as:

$$da/(dx.R_1)$$

where da/dx is the chemical free energy gradient arising from the difference in solute activity, da, over distance, dx, and R_1 is the resistance of the membrane to flow of the solute concerned. In many cases the solutes have close to full activity and therefore the activity coefficient is 1. Then the activity gradient, da, becomes the concentration gradient, dC, of the solute concerned. As expressed in Fick's law, where D is the diffusion co-efficient, and, C is the solute concentration:

$$\text{net flux} = D\,(dC/dx) \qquad (1)$$

Electrolyte flux has a similar form but with the free energy gradient including not only a concentration component but also an electrical component, expressed as:

$$E_m/R_2,$$

where E_m is the membrane potential and R_2 is the resistance to flow through the membrane for that particular electrolyte. That is, the total potential for net movement of an electrolyte arises from the sum of its chemical potential gradient and its electrical potential gradient; an electrolyte moves under the influence of its electrochemical potential gradient. For both neutral solutes and electrolytes, passive flow is nearly entirely through channel proteins (Dainty, 1962; Plants in Action Chapter 3.6.3). The big advantage of such a system *versus* flow through the lipid part of the membranes is that the proteins confer selectivity, and resistance to transport through channels can be closely regulated.

Assuming a solute activity coefficient of close to 1, the chemical free energy gradient arises from the concentration gradient, dC, of the solute concerned. The electro- part of the gradient is related to the electric field, i.e. the gradient in voltage. If the cell is negative relative to the medium, cations will accumulate in the cell and anions will be excluded unless there is energy-dependent transport e.g. pumping Cl^- against the electrical gradient (see section 2.2 (b)). The voltage gradient can also drive influx or efflux of ions through ion channels as a passive process, but the voltage gradient itself is developed by an energy-dependent process.

A useful expression here is the Nernst potential, also called the equilibrium potential. If for a particular electrolyte there is no electrochemical potential gradient across the membrane, then there is no driving force on that electrolyte, and it is in passive equilibrium across the membrane. Although passive, this is a dynamic equilibrium in that the electrolyte will be moving across the membrane, but the influx and efflux will be equal. The Nernst equation describes the potential difference (mV) across a membrane that develops at the concentration gradient at which the electrolyte is in passive equilibrium, that is when influx equals efflux.

$$E_m = (RT/zF) \ln (C_o/C_i) \qquad (2)$$

where E = membrane potential (mV), R = gas constant (8.314 J.K^{-1}.mol^{-1}), T = temperature (K), z = valency, F = Faraday constant (96500 J.V^{-1}.mol^{-1}), C_o and C_i = outside and inside concentration (mol.L^{-1}) respectively. Solving for constants and substituting $\log_{10}$ for the natural log (ln), this can be rewritten as:

$$E_m = (58.2/z) \log_{10} (C_o/C_i) \qquad (3)$$

where $2.303RT/F$ at 20°C = 58.2 mV

This form of the Nernst equation illustrates that at an equilibrium membrane potential of –58 mV, the concentration of a monovalent, positively charged electrolyte will be 10-fold higher inside the cell than outside. So, at an E_m of –115 mV, typical of the plasma membrane of a plant cell, an external concentration of 1 mM KCl will be in flux equilibrium with a cytoplasmic concentration of K^+ of 100 mM at 20^{o}C. That is, the internal concentration of an electrolyte can be much greater (or smaller) than the concentration outside the cell even though the electrolyte is in passive equilibrium across the membrane. A higher internal concentration of an electrolyte is not evidence of energy-dependent transport of that electrolyte into the cell (Dainty, 1962; Chapter 3.6.3). In this example, the equilibrium concentration of Cl^- in the cytoplasm would be 0.01 mM (100-fold lower than the external solution).

The Nernst potential gives a useful criterion for determining whether energy-dependent transport is occurring. Taking K^+, if the measured resting membrane potential is less negative than the equilibrium potential (Nernst potential) calculated from the observed K^+ concentration gradient across the membrane there would be an efflux of K^+, so any K^+ uptake must require energy (Dainty, 1962). As an example, in barley roots the *measured* potential was –103 mV compared with the *calculated* equilibrium potential for K^+ of –125 mV (Walker et al. 1995), so these authors concluded to maintain flux equilibrium of K^+ at –103 mV, there must have been energy-dependent influx of K^+.

(b) Transport against a free energy gradient

Ions can be transported against an electrochemical potential gradient and neutral solutes against their chemical gradients, provided there is a source of energy.

As an example, we consider the very clear case of low Cl^- concentrations outside a cell. In this case, cells as a rule accumulate Cl^- against an electrochemical gradient since cells are usually negatively charged relative to their environment. So, with no energy-dependent transport, internal Cl^- concentration would be exceedingly low according to the Nernst equation. In fact, at modest external Cl^- (say 0.5 - 5.0 mM) the internal concentration usually becomes much higher than the external concentration.

Returning to K^+, whether the influx is passive or requires energy depends not only on the free energy gradient arising from the difference in K^+ concentration across the membrane but also on the prevailing membrane potential. If the measured membrane potential is less negative than the equilibrium potential predicted by the Nernst equation from the known internal and external K^+ concentrations, energy-dependent K^+ influx is indicated (Dainty, 1962; Walker et al. 1995).

(c) Solute/H^+ co-transport

In plants, energy-dependent transport is often by solute/H^+ co-transport. In this type of transport system, there are primary and secondary transporters. A primary transporter, taking for example the plasma membrane H^+- ATPase, uses energy to pump H^+ from the cytoplasm to the environment, creating a voltage (*electro*) and pH (*chemical*) gradient. The H^+ can flow back along its *electrochemical* gradient e.g. from environment to cytoplasm, and the energy released during the influx of H^+ can be used in secondary solute/H^+ co-transport to transport solutes against their free energy gradient (Figure 1). Since this type of transport occurs so frequently it is worth drawing a useful analogy. We can say the inward H^+ flow along its electrochemical gradient is *downhill* and the solute co-transport is *uphill*. Uphill transport can also be referred to as 'pumping'. Examples are Cl^-/H^+ transporters (H^+ influx - Cl^- influx) and K^+/H^+ transporters (H^+ influx - K^+ influx) called symports (Figure 1; Ullrich and Novacky, 1990), and Na^+/H^+ transporters (H^+ influx - Na^+ efflux) called antiports (Atwell et al. 2017). The simplest example is for a neutral solute, such as a sugar. A sugar/H^+ symport (H^+ influx - sugar influx) can drive sugar uptake against its concentration gradient, that is, where the ratio of the concentration of sugar in the cytoplasm / sugar in medium is greater than 1.0 such as occurs when sugars are 'loaded' into the phloem.

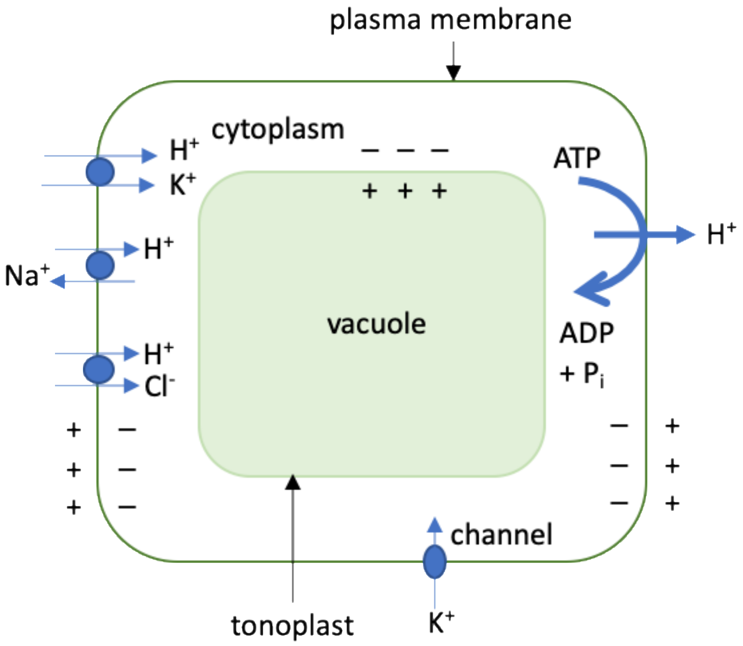


Figure 1. Solute / H^+ cotransport at the plasma membrane. The plasma membrane H^+ -ATPase pumps H^+ against an electrochemical gradient out of the cell. H^+ enters the cell along its electrochemical gradient, providing energy for co-transport. For example, Cl^-/H^+ symport, where Cl^- is taken up by the cell against its electrochemical gradient; Na^+/H^+ antiport, where Na^+ is pumped out of the cell against its electrochemical gradient. K^+ can be co-transported via a K^+/H^+ symport (high affinity); uptake can also be substantial through K^+ channels (low affinity). The membrane potential across the tonoplast is about 30 mV more positive than that across the plasma membrane (Chapter 3.6.3) because of transport processes across this membrane that are not discussed here.

There is one other membrane proton pump in plant cells, the H^+ -PP_iase. The energy for the pumping is in this case provided by the high energy compound PP_i rather than ATP. In this regard, the ATP and PP_i can be considered as two different currencies (Atwell et al. 2017).

(d) Fluxes of different ions

Ions like K^+ and Cl^- are known to be in dynamic equilibrium with their environment. K^+ fluxes in particular have a critical role in modifying membrane potentials and presumably pH over very short time periods (Dainty 1962; Chapter 3.6.3 for membrane potentials). Evidence for this includes the short half-times of exchange of the cytoplasm for K^+ in barley, being 16 - 25 min for the high-affinity transport system (low external K^+ concentrations, 0.1 mM) and only 8 - 9 min for the low-affinity transport system (high external K^+ concentrations, > 1.5 mM) (Szczerba et al. 2006). The high-affinity transport system occurs via a K^+/H^+ symport with the inward flux of K^+ against an electrochemical gradient, while the low-affinity transport system, which dominates at a higher external K^+ concentration, is via the activity of K^+ channels with flow occurring along the electrochemical gradient. Exchange rates of a third phase, presumably the vacuole, were much slower (Szczerba et al. 2006).

Membranes have different permeabilities for different ions because of the transport proteins that are expressed in different cell types. Fluxes of K^+ across membranes tend to be very high, as are Na^+ fluxes in several halophytes, with much lower fluxes for Ca^{2+} and Mg^{2+}. For anions, Cl^- and NO_3^- fluxes are much higher than SO_4^{-2} (Cram 1973). K^+ is usually the most permeable ion, with a rate of flux across the membrane typically 10 - 100 fold faster than Cl^-.

The dominance of K^+ and to a lesser extent of Na^+ and Cl^- in ion fluxes across biological membranes can be seen in the Goldman equation which predicts the equilibrium membrane potential E_m taking into account all the ions that permeate the membrane. This equation is a function of the permeabilities of all diffusible ions and the concentrations of each of these ions on either side of the membrane. For a detailed description of the Goldman equation see Chapter 3.6.3. Only the permeabilities of K^+, Na^+ and Cl^- tend to be included in the prediction of the equilibrium potential by the Goldman equation (Dainty 1962), which was developed for situations where it was known that the permeabilities of SO_4^{-2} and Ca^{2+} were very small relative to K^+, Na^+ and Cl^-. The same has since been assumed for other situations.

As K^+ fluxes are usually much larger than Cl^- fluxes, longer term K^+ uptake is ‘held back’ by the rate of uptake of the balancing ion, Cl^-. However, if there is no readily absorbable anion in the external solution (e.g. when K^+ is balanced by SO_4^{2-} or a colloid or non-penetrating organic acid), K^+ uptake can still take place by producing organic acids in the cell, which in their dissociated form function as counter ions of K^+, providing H^+ is pumped out. In general, where cation and anion uptake differ substantially, the biological pH stat comes into play, with either syntheses or catabolism of organic anions (see section 2.1 and 2.3).

2.3 pH in biological cells

Understanding factors determining pH is always useful in view of the widespread importance of pH, for example a basic knowledge of pH is required to understand why, at increased CO_2 in the atmosphere, waters of the seas and oceans will acidify.

We chose the regulation of cytoplasmic pH for the present course since changes in pH of biological cells are suited for several ‘thought experiments’.

(a) Factors determining pH

Stewart (1983) has stated that pH, an expression of the concentration of protons in solution, is a dependent variable. He then argued that the pH of solutions is determined by:

(1) The strong ion difference (SID) between cations and anions, excluding H^+ or OH^-. Here, we refer to cations as $*C^+ = (C^+ - H^+)$ and to anions as $*A^- = (A^- - OH^-)$;
(2) The content of weak acids and bases; these will influence pH since they dissociate, yielding anions or cations respectively;
(3) The partial pressure of CO_2, which will determine the amount of H_2CO_3 in aqueous solution. This is just a special case of a weak acid, but is particularly relevant to ecology, photosynthesis and ocean acidification.

That the concentrations of protons and hydroxyl ions in solution are dependent, not independent variables, gives a counter-intuitive dictum. That is, the H^+ concentration of a solution is not determined by proton-producing or importing processes directly but via changes in the difference between $^*C^+ = (C^+ - H^+)$ and $^*A^- = (A^- - OH^-)$, with the pH increasing when $^*C^+ - {}^*A^-$ increases (i.e. $^*C^+$ increases relative to $^*A^-$) and decreasing when $^*C^+ - {}^*A^-$ decreases (i.e. $^*A^-$ increases relative to $^*C^+$).

pH is a dependent variable because of the very low dissociation constant of water. In pure water at pH 7, the hydrogen ion concentration is 10^{-7} M = 0.0001 mM. Therefore, according to Stewart (1983), very slight changes in the balance between cations and anions are responsible for changes in pH we see in the physiological range, with H^+ concentration fluctuating, depending on the imbalance between cations and anions. Consequently, the same applies to changes in membrane potential, where an increase in membrane potential of 100 mV, i.e. from –200 mV to –100 mV, would require an increase in $^*C^+ = (C^+ - H^+)$ relative to $^*A^- = (A^- - OH^-)$ of between 10^{-5} to 10^{-6} mM. For that to be achieved by H^+ concentration alone, the pH of the cytoplasm would have to decrease to ~ 5, a level of acidity too low to allow metabolic reactions to occur.

(b) Example of a biochemical pH stat in anoxic rice coleoptiles

In cells growing under optimal conditions, longer term effects of treatments on pH are usually complicated by the many-fold reactions that occur. Fewer complications can be expected for anoxic rice coleoptiles since, while still healthy, as judged by rapid regrowth after return to aeration, their metabolism is very slow (Greenway et al. 2012). At external pH 6.5, coleoptiles elongated and took up K^+ slowly, matched by equivalent amounts of malate synthesis. When these cells were put under an additional acid load by transfer to pH 3.5, malate and succinate decreased over 18 h by 0.3 and 0.1 µmol g^{-1} fresh weight $hour^{-1}$ respectively to just below 50% of their previous levels, with levels of both declining more slowly thereafter to near exhaustion. These losses were matched by leakage of K^+, initially in excess of 0.5 µmol g^{-1} fresh weight $hour^{-1}$ in the period immediately following transfer to pH 3.5, with K^+ leakage dropping to 0.5 µmol g^{-1} fresh weight $hour^{-1}$ in the period 10 - 18 h after transfer (Greenway et al. 2012). In interpretation, at low external pH, H^+ leaked into the cell and was incorporated into the organic anions, which were catabolised to ethanol and CO_2 and lost to the medium, while the balancing cation, in this case K^+, leaked to the medium as the organic anions declined (Greenway et al. 2012). The long term data confirmed organic acids played a major role in the biochemical pH stat (see section 2.1).

To sum up, biological cells need a very close balance of cations and anions. We have seen that a substantial electrical potential gradient across the plasma membrane results from a very slight imbalance between the concentrations of cations and anions. Likewise, a change in difference between strong cations and strong anions in a solution need only be minute to generate substantial changes of pH within the physiological range. Otherwise the balance would have to be provided by H^+ or OH^- and pHs would develop that can’t be tolerated by metabolic reactions.

2.4 A thought experiment

Experiments by Ullrich and Novacky (1990) are used here to illustrate a successful thought experiment, which in this particular case was actually tested in the laboratory. With knowledge gained from reading through sections 2.1, 2.2 and 2.3, readers may wish to try their hand at this thought experiment, after reading the information in the next two paragraphs.

Ullrich and Novacky's experiments concerned a key biological issue, namely, in what way and by what mechanism does the ubiquitous proton-pumping at the plasma membrane affect pH_{cyt}. As background to this question, the research literature describes the effects on pH_{cyt} of fusicoccin, a stimulator of the plasma membrane H^+-ATPase that uses energy to transport protons out of the cell. Some experiments supported the plausible hypothesis that increased proton-pumping would, by removing H^+ from the cytoplasm, increase cytoplasmic pH_{cyt}. However other investigators reported an 'unexpected' result; rather than increasing pH_{cyt}, stimulation of proton-pumping decreased pH_{cyt}.

Ullrich and Novacky then used their knowledge of solute transport and pH regulation to hypothesise that the changes in pH_{cyt} were related to changes in the strong ion difference (SID; section 2.3 (a)) and that stimulation of H^+ export from a cell stimulated a solute/H^+ co-transport system, i.e. there were two processes happening in sequence over short periods (likely to be in seconds). So, they devised a cunning thought experiment that extended a methodology used by Hiatt (1967) and involved placing plant roots in a basal solution of $CaSO_4$ then applying two treatments plus and minus fusicoccin. At the start of the experiment, the first treatment received K_2SO_4 resulting in a bathing solution from which the cation (K^+) was taken up by the tissue faster than the anion (SO_4^{2-}), while the second treatment received $CaCl_2$, from which the anion (Cl^-) was absorbed faster than the cation (Ca^{2+}). They predicted that pH_{cyt} would increase when cation uptake exceeded anion uptake and, *vice versa*, pH_{cyt} would decrease when anion uptake exceeded cation uptake. This is a testable hypothesis and typically, would end the thought experiment.

However, in fact, Ullrich and Novacky were fortunate in having the equipment and skill to test their hypothesis, the outcomes of which supported their predictions (Table 3).

Table 3. Changes in pH_{cyt}[1] resulting from supply of K^+ or Cl^- plus or minus fusicoccin[2] (FC) in root hairs of *Limnobium stoloniferum*[3]. The observations were taken ~ 10 min after starting the treatments (based on Ullrich and Novacky, 1990).[1] pH_{cyt} is measured with a pH microelectrode; [2] fusicoccin is the chemical that stimulates the H^+ excreting proton pump; [3] the plant material was chosen as very suitable for measurement of pH_{cyt} using microelectrodes, but otherwise is not of relevance to the experiment.

Solution	Treatment	Change in pH_{cyt}
1 mM K_2SO_4	-FC	+0.30
"	+FC	+0.43
1 mM $CaCl_2$	-FC	–0.25
"	+FC	–0.32

(a) Resolving the thought experiment

The thought experiment is key to our goal of stimulating and fostering independence in students, so here the Ullrich and Novacky narrative is reiterated with a different emphasis to show how the 'unexpected' result was resolved.

The original hypothesis, that H^+ export from the cell reduces internal H^+ concentration, i.e. increases pH_{cyt}, cannot explain the result observed with $CaCl_2$ where pH_{cyt} decreases. So, we need to develop a new hypothesis that accommodates both the results observed with K_2SO_4 and with $CaCl_2$, as follows:

(1) In $CaCl_2$, extra proton pumping stimulated by fusicoccin in turn stimulates influx by the H^+/Cl^- co-transporter;
(2) Little Ca^{2+} is taken up;

(3) So the SID changes, with an increase in $*A^-$ relative to $*C^+$. This means that the concentration of the dependent variable (H^+) increases, and pH_{cyt} decreases even though proton extrusion was increased by the fusicoccin.

Thus, we can formulate a new hypothesis. That H^+ export from the cell leads to changes in the difference between cations and anions with *consequent* changes in proton concentration.

This hypothesis also accommodates the K_2SO_4 result. K^+ is much more readily taken up than SO_4^{2-}, and so the SID changes with an increase in $*C^+$ relative to $*A^-$, which means that the H^+ concentration will decrease, so in this case the pH_{cyt} increases.

The students can then be asked to formulate a reasonable hypothesis as a further thought experiment. One suggestion might be the transport of a neutral solute when one would predict there would be no change in pH_{cyt}. To check this hypothesis, apply a non-metabolised sugar in the form of 3-0-methyl glucose and there should be no effect on SID, that is, on pH. Note that glucose itself would add a new complexity, because as a metabolite it may stimulate uptake metabolism and hence uptake of a cation or anion.

Once the concept of thought experiments is adopted, most science disciplines abound with examples. Two such, using themes very different from those used here, can be found in Dixon and Grace (1984) on the impact of wind speed on evaporation from a leaf, and at a whole plant level, in Turner et al. (2020) on suckering and plant size in plantains.

3. An experimental component

Short-term experiments as part of a study program have already been described briefly in Tim Colmer's testimonial (section 1.2).

A crucial requirement for any such experiments associated with a study program of this sort is that they are short term and require simple equipment. In the experiment at the Prachinburi (Thailand) workshop, a cylindrical O_2 electrode together with 'cunning' manipulation of environmental conditions led to a key finding that early in flooding a barrier to radial O_2 movement formed in the roots, preventing loss of O_2 derived from the shoots to the anaerobic environment around the roots (Colmer et al. 1998). At UWA we used simple tools to achieve rewarding results. Students took part in the planning but were given choices from a list of useful plant material by the tutors depending on suitability and available equipment. For example, meaningful experiments can be done with a ruler to measure elongation of coleoptiles (i.e. the first shoot organ of germinating cereals). Atwell et al. (1982) based nearly an entire research publication on elongation of rice coleoptiles in various oxygen regimes to show differential sensitivity to oxygen deficits during early growth phases. Similarly, elongation of roots can be used as a simple but meaningful criterion, allowing emphasis on designing and testing critical experiments. Rice coleoptiles are particularly useful, since if required the seedlings can be grown in stagnant solutions in the dark, i.e. under very simple conditions. Another possible object of study is duckweed (*Lemna*), which can be grown in stagnant solution on a windowsill. A sub-species *Lemna gibba*, is described in Wikipedia (https://en.wikipedia.org/wiki/Lemna_gibba) as merely having a (green) thallus and a single root. Simply counting the number of thalli (fronds) formed per day would be an easy way to test hypotheses. Similarly for whole plants, leaf numbers vs leaf area can be used to provide a good guide to the contrasting effects of limiting factors on development and growth.

Another suitable experimental set up was used in practicals by the late Professor Michael Pitman (pers. comm. Steve Tyerman). A root of a K^+-deficient barley plant was immersed in a dilute solution of $CaSO_4$, along with an immersed pH electrode. This system allows measurement of net proton changes in solution with different treatments e.g. K_2SO_4 was added and the changes of K^+ and H^+ in solution measured. Students who have participated in the course described here should have little trouble suggesting possible outcomes and further experiments and, if time permitted, in carrying out several intriguing experiments using simple set ups.

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Hank dedicates this work 'to Trish who for years tolerated my preoccupation with teaching and research'.

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