Problems in Frequency Sweep Measurements of Stress Relaxation in Cell Walls of Living, Turgid Plant Tissues

This is an online addendum to **Mechanical properties of plant cell walls probed by relaxation spectra** by SL Hansen, PM Ray, AO Karlsson, B Jørgensen, B Borkhardt, BL Petersen and P Ulvskov. It provides an in depth discussion of Table 1 in the main paper and analyses a paper by Alverez and Canet (1998) in this context.

We consider here phenomena that can cause turgor pressure (*P*) in potato tissue disc cells to change under the conditions of frequency sweep rheological measurements. This includes both processes that could influence relaxation spectra directly, by being induced by the oscillating strain that the rheometer imposes to make modulus measurements, and also processes that could alter cell *P* over the course of the ca. 20 min measurement period. These latter, because the tissue's elastic rigidity varies with *P,* would affect the *G*' and *G*" values that the rheometer reports for any given oscillation frequency. This appears to be principally responsible for the observed large variations in these values, which correlate with variations in the compressive "normal force" (NF) that is imposed on the tissue specimen by the rheometer's pressure plates that clamp and hold it for oscillatory straining (Fig. 1). These processes, and the conclusions that we here deduce about them, are summarized in the published paper (section "Problems in stress relaxation measurements").

Issues and problems similar to those discussed here will affect rheometer measurements made on other kinds of tissues, but the details that need consideration will differ depending on the form of the tissue fragment used, its anatomical structure, and the type of rheometer and tissue clamping method. These details must be analyzed on a case-by-case basis; the present analysis for potato tissue discs and the type of rheometer we used will provide hopefully a guide to what needs to be considered for other tissues and instruments.

A. **Processes that could directly contribute to relaxation spectra.**

1. "***In vivo* stress relaxation**" (Cosgrove 1985, 1987). In the absence of an absorbable water supply (as with the tissue discs in our rheometer), the walls of turgid cells that are capable of growth can be expected to undergo a stress relaxation that is related to the irreversible extension that they undergo when the cells can take up water. Such a relaxation could also be provoked by the artificial strain that the rheometer imposes. This relaxation would be additive to that from reversible, viscoelastic relaxation processes in the walls and could in principle yield its own relaxation-spectral peak whose position, on the τ scale, depends upon the cells' normal growth rate and their walls' elasticity.

Isolated discs of potato tuber tissue in water grow only slowly (~4% volume increase in 48 h) if incubated without auxin (Hackett & Thimann 1952). By the principles of *in vivo* stress relaxation (Cosgrove 1985), this growth rate corresponds to a relaxation time of about 6 x 10^5 s, far outside the range of our relaxation spectra. However, Hackett & Thimann's potato discs were pretreated in water for 24 h before growth measurements, to take them beyond the "initial phase" post cutting (Brauner *et al*., 1940), a phase that could involve more substantial irreversible cell enlargement. We therefore checked gravimetrically the short-term growth behavior of potato discs immediately after cutting (as were employed for our rheometer measurements). Weighed discs similar to those used in the rheometer were incubated in water or in 0.1 or 0.2M sucrose, followed, after either 20 or 120 min by 0.28 M sucrose which was osmotically strong enough to just restore their initial *P* and thus reverse the elastic expansion that they undergo in water. Re-weighing showed that they enlarged irreversibly at a rate of about 3% h^{-1} in water and about 1.5% h^{-1} in the 0.1M medium (as recorded at both 20 and 120 min), and hardly at all in 0.2M. This corresponds to a relaxation time of about 1.6 x 10^4 s. This lies well outside the reliable range of our spectra and thus apparently could not be responsible for either of its principal peaks.

From a shear stress creep test performed on live potato tissue, Alvarez & Canet (1998) reported a substantial "Newtonian", *i.e*. simple viscous, strain component in the material. This is contrary to the cell growth principles referenced above: Newtonian flow occurs under any stress except zero, whereas irreversible extension of cell walls has invariably been found to occur only above a definite yield stress (Cosgrove 1987, 1993). If cells in an intact potato had a significant capacity for irreversible extension, during post-harvest storage in the absence of a water supply their walls would eventually relax down to their yield stress.

As explained in Appendix I, for multiple reasons Alvarez & Canet's (1998) data do not, in our opinion, support the interpretation of a Newtonian creep in their potato material.

2. **Simple osmotic relaxation**. Intuition might suggest that the cells' *P* would increase as a result of the strain imposed by the rheometer. This would bring *P* above the value that exists at the osmotic equilibrium that prevails, in the absence of mechanical

perturbation, between the cell contents and the cell wall. An osmotically driven relaxation of *P* toward its equilibrium value would follow, and since the elastic modulus of a plant tissue varies with *P* (Falk *et al*. 1958; Niklas 1988; Davies *et al*. 1998), the rheometer would register this as a mechanical relaxation. Its dynamics should be the same as those of the relaxation after a step change in *P* imposed in the pressure-probe technique (Hüsken *et al*. 1978, Zimmerman & Hüsken 1979). For potato cells a half-time of 5 s has been reported (Michael *et al*. 1997, Table IVB); this corresponds to a relaxation time (τ) of about 7 s. This falls right at the low point between the principal peaks in our relaxation spectrum (Fig. 3), so apparently could not be responsible for either of them.

Moreover, simple osmotic relaxation actually appears not to affect the present dynamic measurements at all, because the oscillating strain that the rheometer imposes is a shear strain, which does not change a material's volume or, consequently, the cells' *P*. However, it could be a problem for the method by which potato discs are clamped between our rheometer's plates for measurements, to be discussed below, for which some basic considerations regarding strain-induced *P* changes in cells need to be given.

The *P* in a pressurized, liquid-filled chamber changes if an externally imposed strain tends to change the chamber's volume, because the contained liquid volume cannot change (at least immediately) so its *P* must change just enough to prevent the change in chamber volume (ΔV) that would otherwise occur (Hepworth & Bruce 2000). This latter ΔV will tend to occur under an imposed uniaxial strain if the Poisson's ratio of the chamber's wall material (the ratio of its transverse contraction [or expansion] to lengthwise extension [or compression] under a uniaxial tensile [or compressive] stress) is less than 0.5 (as is true of most materials, including cell walls). An imposed compressive stress then tends to decrease the chamber's volume, resulting in an increase in *P*, whereas an imposed tensile stress tends to increase the chamber's volume, resulting in a decrease in *P* if the chamber's contents are already under *P*, as are a plant cell's contents.

We verified, using a model spherical cell (a water-filled, pressurized, spherical rubber balloon coupled to a simple hydraulic manometer), that the *P* within it increased when it was subject to uniaxial compression and decreased under uniaxial extension, but did not change at all when the model was deformed by a shear. This is reasonable since this type of strain, as noted above, does not cause a change in volume.

B. **Changes in *P* when tissue is clamped between rheometer plates**

To hold a tissue disc between the rheometer's plates firmly enough that shear strain could be imposed on it without its surface slipping between the plates, these have to apply a compressive force to the specimen (the normal force or NF). This compression must increase the cells' *P* above its pre-existing, equilibrium level, so as explained above, an osmotic relaxation must then occur, involving efflux of water through each cell's plasma membrane into its cell wall, until the cell contents and walls regain water-potential equilibrium. This relaxation would show itself as a relaxation of the NF between the tissue disc and the pressure plate surfaces. Since, as noted above, the elastic moduli of a plant tissue depend strongly on *P*, relaxation of *P* would decrease the measured *G* values, which could cause them to correlate with NF, as in our published paper's Fig. 1.

1. **Simple osmotic relaxation.** The NF of about 100 g that develops initially (during automated advance of the movable rheometer plate in the first ca. 15 s of our 60 s pre-measurement "equilibration" period) corresponds to a stress of about 5.7 kPa on the disc as a whole. In the abovementioned cell model experiments mentioned above, at small strains an externally imposed compressive stress increased *P* by about 1/3 of the imposed stress' value (imposed force divided by the entire model's cross-sectional area). For potato cells the increase would probably be greater, because cell wall material has a smaller Poisson's ratio than rubber, but in the disc tissue as a whole (not its surface cells, which will be considered shortly) the increase could not exceed the over-all imposed stress, because the rheometer plates lack any mechanical advantage comparable to that of a narrow piston in a hydraulic system.

By the classical osmotic method we determined the average *P* of potato cells to be about 0.38 MPa, whilst Michael *et al*. (1997) reported a *P* of 0.48 MPa from *P*-probe measurements on potato cells. Thus the initial NF could cause at most a 1-2% increase in the average cell's *P*. Since potato tissue's elastic modulus changes by about 3 times a change in its *P* (Nilsson *et al*. 1958), the initially imposed NF could have caused at most about a 5% increase in its *G* values. But the actual variation in *G* values that correlates with NF greatly exceeded 5%, so simple osmotic *P* relaxation resulting from compression of the tissue by the NF cannot account for the observed variation.

Furthermore, as noted above, this type of P relaxation should have a τ of about 7 s, and thus should actually have gone virtually to completion within the 60 s equilibration period that preceded our first rheometer measurement with each disc. So even had the compressive increase in P been larger than just estimated, its relaxation should not have influenced the measurements. In agreement with this, Alvarez & Canet (1998, Fig. 6a) reported that compression of turgid potato tissue caused a (probably osmotic) stress relaxation with a half-time of about 4.2 s.

Contrary to expectation for simple osmotic relaxation of P, a rapid relaxation of NF often continued beyond the 60 s equilibration period for up to 100-200 s more. This "tail" was followed by a much slower relaxation, thereafter, throughout the ca. 20 min duration of the tests on any one disc (Fig. 2).

2. **Ordinary viscoelastic relaxation.** The early, rapid NF relaxation must be due at least partly to whatever process is responsible for the longer-τ peak at about 100 s in our relaxation spectra (Fig. 3). However, because this relaxation's magnitude could be at most only a fraction of the stress imposed on the cells by the NF, and this stress was, as noted above, small compared to the cells' P, this relaxation could not cause the large effect on P that is needed to explain the large variations in G' and G'' with NF.

3. **Cell compression and collapse by pressure plate knurls.** The surfaces of our rheometer's pressure plates, between which a tissue disc is held during measurements, are knurled to counteract a tendency of a tested specimen to slip relative to the plates. The knurls, or outwardly projecting pyramidal bumps, are disposed in an orthogonal array of rows 0.5 mm apart in both directions. They are about 0.15 mm high, above the bottoms of the grooves between the rows, into which grooves the sides of the knurls slope linearly. Since the average diameter of potato cells is about 130 μm (Michael *et al*. 1997, Davies *et al*. 1998), at a target plate separation of 2.8 mm between the tips of the knurls (see Methods) they should penetrate into each side of a 3 mm thick tissue disc by almost one cell diameter, severely compressing or collapsing the impacted cells. Rows of depressions visible on both surfaces of the discs after a set of measurements was completed, showed that cell compression or collapse did occur. Besides the cells that are directly impacted by the tips of the knurls, adjacent cells out to a distance of about 0.13 mm from the tips must become strongly compressed. From the numbers just given one

can estimate that about 25% of the surface cells on either side of a potato disc must become compressed by 50% of their initial volume or more. Compression would also strongly affect at least the second layer of cells beneath the directly impacted ones, since potato tissue contains a negligible fraction of intercellular air channels that could absorb the ongoing compression.

The initial compression of these cells must generate a large stress on them, and a large increase in their *P*, probably exceeding their own initial *P*. This would probably not rupture their cell walls, since the walls can support at least twice the cells' normal (within the tuber) *P* when potato tissue is placed in, and osmotically equilibrated with, water. The severely compressed cells must instead rapidly lose, osmotically, most of their water and shrink greatly, probably over a period of a few hundred s at most (Alvarez & Canet [1998, Fig. 6a] found a half-time of about 4 s for stress relaxation in strongly compressed potato tissue). This collapse is probably largely responsible for the initial, rapid phase of NF relaxation noted earlier. Water made available to other cells, in their common cell wall space, by collapse of the directly impacted ones, would be expected to raise *P* in parts of tissue remote from the knurl impact areas, since their initial *P* of about 0.4 MPa lies well below that required for osmotic equilibrium with water (about 0.7 MPa, the cells' average solute osmotic potential). This rise would increase the tissue's rigidity and contribute to the unusually high initial values of *G* and of NF (far above the set target value) obtained in the first measurement that was made, in a number of the measurement runs.

4. **Solute leakage** from cells compressed by pressure plate knurls. Water loss from cells impacted by the knurls must raise these cells' internal solute concentration far above normal. This will very likely lead to substantial solute leakage through their plasma membranes into the cell wall space, both as a result of membrane damage that they probably will experience during their sudden compression, as well as simply high-concentration-driven efflux. As these solutes diffuse into nearby cells' wall space, lowering its water potential, this will force these cells to lose water and reduce their *P*. This effect would gradually extend, by solute diffusion, throughout the entire disc after cell collapse is completed. The *P* of potato tissue strips that had a 1 x 1 mm cross section, equilibrated osmotically with changes in external mannitol concentration with a

half-time of about 3 min (Virgin 1955). For our potato discs this half-time should be more than doubled, because our tissue was 3 times as thick as Virgin's, and solute would have diffused into our discs' interior from just 2 sides rather than from their strips' 4 sides; cell solutes' weighted mean diffusion coefficient may well also be smaller than mannitol's. The resulting decline in *P* would thus be expected to continue throughout the 20 min measurement period. It would contribute to the gradual decline in NF and *G* values that occurred during most of the 20 min measurement period, after the rapid initial NF relaxation "tail".

A rough estimate of the probable magnitude of this effect is as follows. From their diameters and approximately isodiametric shape one can estimate that the cells that must be severely compressed on both sides of a disc by the pressure plate knurls constitute at least 2% of the disc's volume. If most of the solutes in these cells were to enter, and diffuse throughout, a free space amounting to perhaps 5% of the remaining tissue volume, the osmolarity in that space would become about 40% of the initial osmolarity of the potato cells, prior to the leakage of water that would occur into it from the un-collapsed cells throughout the rest of the disc when subjected to so high an external osmolarity. This leakage should cause the *P* in cells throughout the rest of the disc to fall greatly. In the actual situation the directly collapsed cells might not leak all their solutes, but indirectly compressed or distorted cells beneath and to either side of them would probably leak some. It seems clear that a substantial decrease in *P* and thus in measured *G* values should occur, since at their normal *P* about half of potato cells' rigidity has been estimated to be due to their *P* (Davies *et al*. 1998).

In addition, it seemed possible that solute leakage into the free space from cells throughout the disc, provoked by the compressive stress that it is under, or by the (very small) shear strains to which it is subjected during the measurements, might occur and cause *P* to fall. However, tests we made of discs placed under even a 100 g compressive load, applied through a flat surface, indicated that their *P* did not fall any faster than that of unloaded discs, and indeed no faster than could be explained by evaporative water loss from their edges. Therefore, this possibility seems contra-indicated.

In about a quarter of our rheometer runs, the NF that was recorded at the end of the first *G'* and *G"* measurement, at less than 100 s after the equilibration period, was less

than 30 g, and only the second, slow phase of NF relaxation was seen over the length of the run, so the initial, rapid NF relaxation phase was evidently missing. These may be cases in which the disc thickness (see item C, below) was sufficiently less than 3.0 mm that, in advancing toward the target gap of 2.8 mm, the plate serrations' ridges did not penetrate into the disc far enough to cause substantial collapse of its surface cells. If so, the following two mechanisms may have been responsible for the modest NF relaxation that was observed in these cases.

5. ***In vivo* stress relaxation**. The *P* in the surface cells impacted by the pressure plate ridges very likely exceeded, initially, the potato cells' yield threshold, allowing some *in vivo* stress relaxation to contribute to the rapid NF decline early in a run. Since, according to the preceding analysis, the general *P* in the tissue disc would have fallen below its initial (pre-clamping) value, and hence below the yield stress, by the time that the more gradual, post-100 s decline in NF was occurring, *in vivo* stress relaxation should not have contributed to the latter decline. Not knowing how far the initial (post clamping) *P* rose above the yield threshold or how long it remained there, it is not possible to estimate how much of an effect this process could have had on the system.

6. **Evaporative water loss.** As clamped in the rheometer, both faces of a potato disc were protected, by the rheometer plates, from evaporation, but its edges were directly exposed to the air. From measurements that we made of evaporation from the edges of potato discs 3 mm thick into still air we calculate that a disc would have lost about 2% in volume from its edges during the measurement period, corresponding to a decrease in *P* of about 0.1 MPa. This is only a rough expectation, because atmospheric conditions and temperature, which affect evaporation rate, would not have been exactly the same in our evaporation tests as within the rheometer. But evaporative decrease in *P* would evidently have contributed to the correlation of *G* values with NF that we observed.

C. **Variations in initially imposed NF**. After a tissue disc was inserted into the rheometer, its movable pressure plate advanced toward the target NF and plate separation settings over about 10 s in an intermittent, "searching" fashion. The first NF values reported in different measurement runs varied considerably (Supplementary Fig. 1),

indicating that the compromise between these targets that was reached, at the time plate advance stopped, was not consistently repeatable.

Besides intrinsic, stochastic variations in the instrument's operation, a biological factor that would very likely contribute to initial differences in NF among discs is variation in initial disc thickness (some such variation was known to occur). This variation would obviously affect the NF value that would be reached if pressure plate advance were to stop at exactly the set target separation.

Initial variations in NF persisted during the subsequent decline in NF that occurred through each run (Fig. 2). Thus, although changes in NF in the course of this decline probably correlate well with changes in P, an absolute relation between NF and *P* values in different runs cannot be expected. This is because the relation between the two depends on the uncontrolled actual area of contact, and extent of penetration into the tissue, of the pressure plates' knurls (*cf*. Wei *et al*. 2001). Therefore variation, between runs, in the general level of NF values arising from initial differences in NF cannot be expected to correlate well with *P* or with reported *G* values. It would instead cause a scatter or imperfection in the correlation of *G* values with NF (Fig. 1) that would be additive to any variation in mechanical properties between different discs, plus any imprecision in the rheometer's mesurement of *G* values, in contributing to the "noise" that would remain in the data after they have been normalized to a standard NF value.

Appendix: Alvarez & Canet's (1998) report of a Newtonian compliance in potato tissue.

The viscosity that Alvarez & Canet (1998) reported for a Newtonian element in potato tissue, and the total elastic compliance that they gave for the tissue, would, if such a component is actually present, give a relaxation time of about 100 s in a stress relaxation test, *i.e*., closely similar to the longer-τ peak in our relaxation spectra. However, the creep time course that they published (Alvarez & Canet 1998; Alvarez *et al*. 1998) contains no linear portion which, according to their description of their method of analysis (Alvarez *et al*. 1998, p. 359 and Fig. 1), would be utilized by their computer program to find and subtract a Newtonian component from the creep curve. Thus it is difficult to see, following the description of their analytical method, how it could have

validly detected a Newtonian component. Furthermore, the approximate slope of the terminal portion (just before 2 min) of this concave-down creep curve is about one tenth of what would be predicted from the Newtonian viscosity that the authors reported. This is so even though at 2 min a retarded elastic component with a compliance ("J_1") of more than half the tissue's total elastic compliance would, according to their analysis, have been only about half way through its time course and would thus have been responsible for most if not all of the creep's slope at that time. Therefore, the creep time course does not support the occurrence of a Newtonian component. Since the post-unloading (*i.e.*, post 2 min) recovery curve (upper panel in Alvarez & Canet 1998, Fig. 7 and in Alvarez *et al*. 1998, Fig. 6) does indicate that some irreversible strain occurred during the 2 min creep period, the foregoing points suggest that this may have been a "permanent set" that occurred either upon loading or in less than 2 min. The repetitive stress relaxation curves that they also presented (Alvarez & Canet 1998, Fig. 6a) tend to support this, because these suggest that some kind of irreversible relaxation occurred, and was virtually completed, within 1 min. after first imposing a compressive strain on the tissue.

The upper panel in Alvarez & Canet's (1998) Fig. 7 (and in Alvarez *et al*. 1998, Fig. 6) implies that the instantaneous elastic recovery of strain upon unloading (at 2 min) substantially exceeded the instantaneous strain upon loading. If so, since it is very unlikely that a substantial change in instantaneous elastic modulus of the cell walls could have occurred in but 2 min, this would seem to indicate that the tissue's *P* fell substantially during this period, since tissue elastic modulus is proportional to *P* (Nilsson et al., 1958), and compliance (to which elastic strain is proportional) is the inverse of modulus. However, while such a *P* decline might be the same phenomenon as that responsible for the rapid initial decline in NF in our experiments, comparison of the upper and lower panels in their Fig. 7 (in the latter, but not the former, of which values are specified on the ordinate) suggests instead that these plots simply lack the first ca. 20 post-loading seconds of the creep time course, during which retarded elastic components with short retardation times were being extended (their existence in this material being shown by the post-unloading recovery curve comprising the right hand part of the upper panel of Fig. 6). This makes it difficult to understand how a retarded elasticity component with a retardation time that is given as 25.7 s (their Table 1), or for that matter

a Newtonian component as noted above, could have been extracted validly from their creep data.

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