PROJECT REPORT TO

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THE VITICULTURE CONSORTIUM (VC)

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Project Title: GROWTH AND SUGAR TRANSPORT IN GRAPE:
EARLY EVENTS IN RIPENING

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Viticulture

Objectives:

The long term objective of this project is to ultimately permit the modification or control of grape ripening behavior in order to benefit grape growers, vintners and consumers. For this proposal the primary objective is to test the hypothesis that early events in veraison are an increase in apoplastic solute and decrease in mesocarp turgor. In order to test this hypothesis several specific objectives will be met:

1. To quantify the progression of cell turgor in and across the developing berry.

2. To quantify the progression of cellular and extracellular solutes in the developing berry.

3. To evaluate the contribution of wall degradation to berry softening.

4. To determine the role, if any, of a general loss of compartmentation in berry softening and sugar uptake at veraison.

5. To explore the consequences of altered berry transpiration for growth and sugar transport.

Most of the effort on this project to date has been toward successfully developing two techniques required to meet the experimental objectives: pressure microprobe analysis of intact berry cell turgor; and, collection of apoplastic sap from berry mesocarp tissue.

Turgor:
In order to begin experimentation on the central issue of this project, i.e. the hypothesis that turgor loss is an early event in veraison, it was necessary to develop both hardware and a protocol for measuring berry cell turgor with the pressure microprobe. For some tissues, this is next to impossible. For example, we designed the pressure probe in Matthews' lab for the study of leaf growth in grape (Shackel et al. 1987), but the difficulty in making those measurements forced us to shift from grape to Begonia leaves as a model system (Serpe and Matthews 1992, 1994). Our pressure probe experiments were carried out in Dr. Shackel's lab where there is his $30,000 custom instrument, his $5,000 tip puller, and experience making measurements, on fruit cells. We designed and fabricated a berry holder that allows us to rotate the berry sample on its axis in the pressure probe system. This worked well to test our initial hypothesis that turgor decreased early in ripening, but this arrangement cannot continue because of high demand for time on his instrument. Now that is established, must get our own system modified for berries.

For cell turgor measurements, the common problems are cells that leak upon impaling and tip plugging. For both problems, one can sometimes design custom tips that allow successful measurements. For grape berries, we tested several new pressure probe tip geometries before identifying a tip-pulling protocol that avoids plugging satisfactorily. We can make turgor measurements in outer mesocarp cells, but we still have difficulty with leaky cells. Besides the tip design, another factor in getting good seals of the cell and the tip is the kinetics of the impalement. That is the angle, speed, and distance the tip travels affect the frequency of successful sealing. The limited amount of time and berries near veraison precluded much experimentation with the protocol. We had one shot at veraison in field and very limited fruitful potted vines for additional work. Both the lack of plant material and further work to improve the technique are addressed in the proposed work.

Our most important observation is that there is a significant loss of cell turgor early in the onset of ripening in the grape berry. Berry cell turgor was evaluated using two developmental time scales. Figure 1 shows the pattern of turgor during berry development as indicated by Brix. The turgor data are for cells in the outer mesocarp below the hypodermis and dorsal vasculature. The Brix data are for the juice of the same berry after turgor measurements were completed.

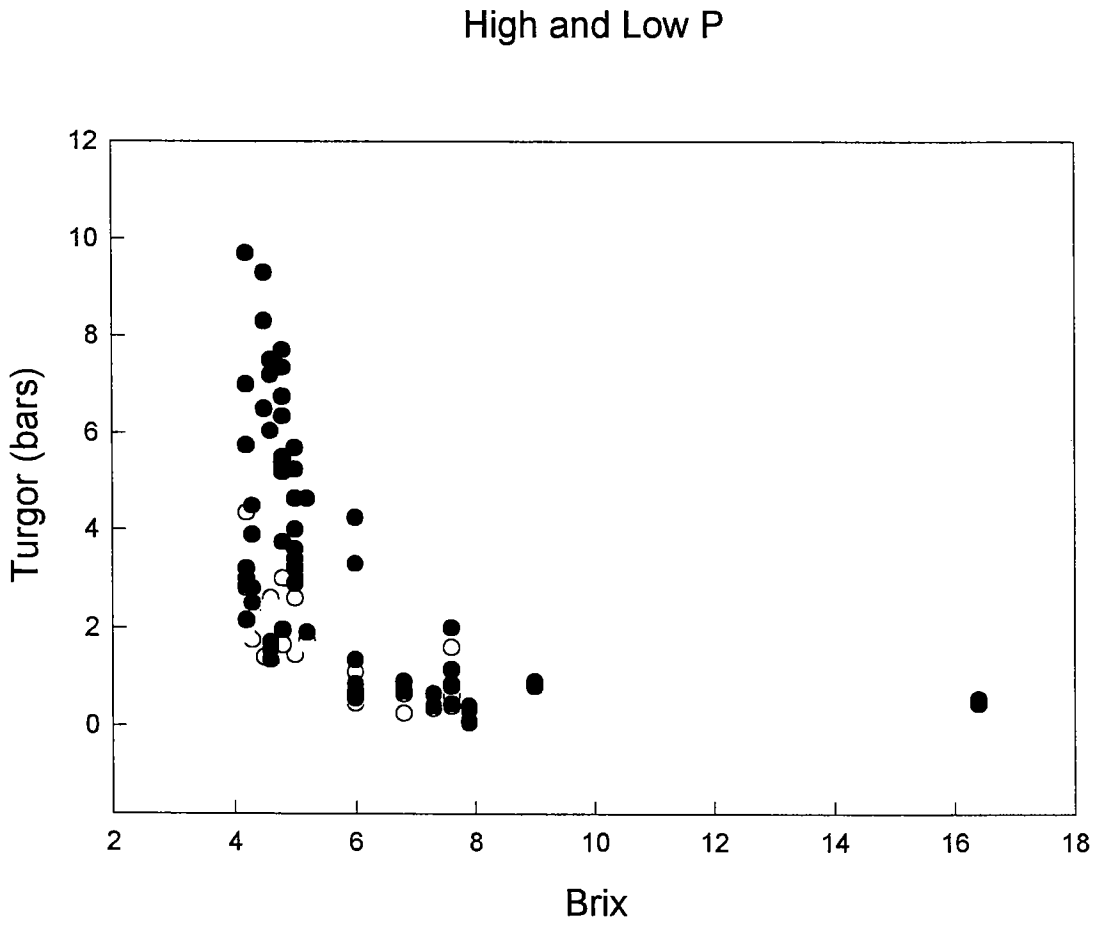
The data show that turgor declines several bars to a stable turgor of about 1 bar or less during ripening. For intact clusters, the highest cell turgor occurred early in development and was less than 4 bars. Values of turgor shown that are greater than 4 bars are from cells of clusters that were excised and hydrated with water. Thus, turgor could be increased 2 to 4 bars by supplying water prior to veraison. For any stage of development beyond 6 Brix, turgor was about 1 bar. The hydration experiments failed to increase turgor in these berries to as much as 2 bars except in one case at 7.5 Brix.

Turgor was also evaluated using "deformability" as a developmental scale (Fig. 2). With the limited data collected, a similarly precipitous decline in turgor was observed during development, in this case at deformability values of 10 -12%. This is the deformability that has been associated with veraison.

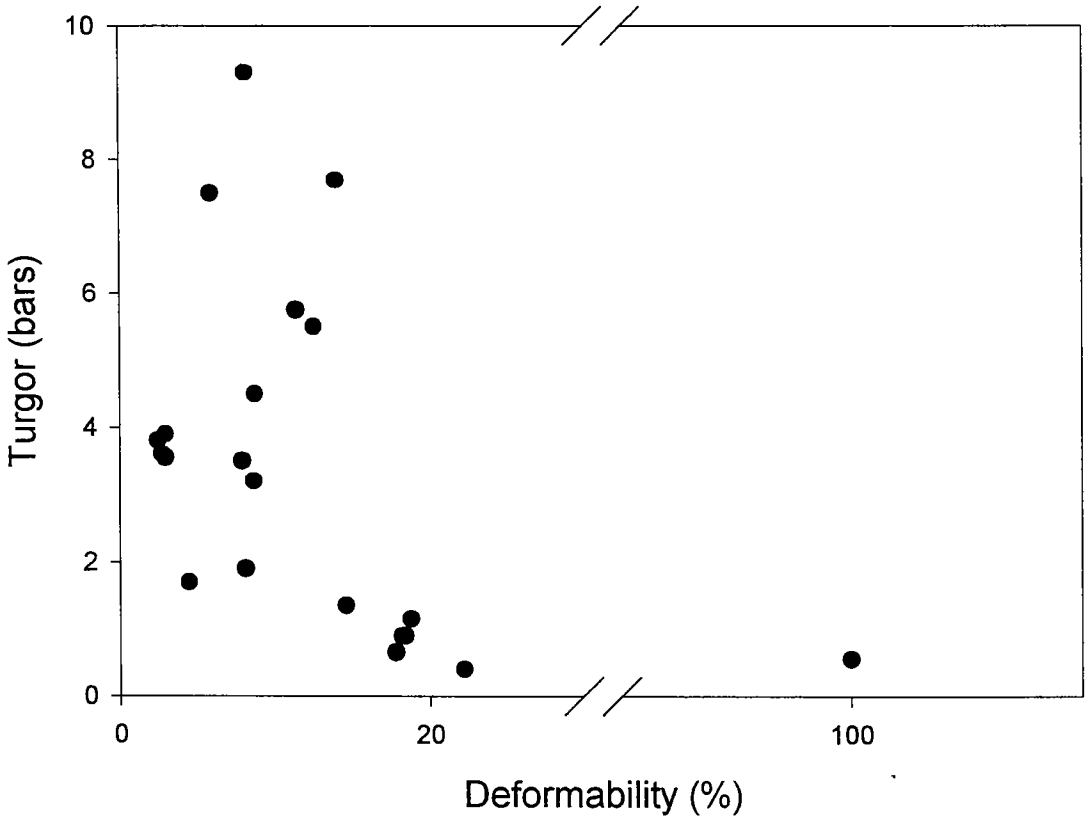
Veraison and developmental time

Recognition of the many physiological phenomena associated with "veraison" calls into question the definition of veraison and the selection of an appropriate scale for determining fruit age in developmental studies. Understanding the sequence of events and their interrelationships is probably key to developing modified genotypes or cultural practices to improve fruit composition, because later phenomena are likely to be at least in part a consequence of earlier phenomena. Historically and in the field veraison has often been determined by color change, but this apparently occurs after sugar accumulation and deformability increase, and after the xylem/phloem transition and turgor loss (see below). According to Coombe, the earliest observable change is a significant increase in deformability. Cell rigidity is primarily a consequence of turgor. Hence, although we may yet find that there is some contribution of altered cell wall elasticity to the change in deformability, it appears that the loss of turgor is the change in deformability. Therefore, our proposed work on cell wall metabolism and loss of pectins has moved lower in priority. The breakdown of cell wall may yet be found to play a role in the resumption of berry growth if the changes in cell wall lead to increased "plasticity". We have already shown that such an increase in plasticity occurs (Matthews et al. 1987). We hope to return to this area in a future proposal.

We observed a "rapid" loss of turgor prior to or coincident with "veraison" on both scales. On a Brix scale, veraison is usually about 7 Brix; on the deformability scale, it is usually somewhere above 10%. However, both scales are problematic in testing for earlier events in the onset of ripening. Brix is of limited use as a developmental scale before "veraison" - Brix for all of our green berries was between 4 and 5 regardless of "age". In addition, Brix determination generally requires destructive sampling of the berry, and it is not possible to know the "age" of the berry before sampling it. It may be possible to measure deformability nondestructively, i.e. on the vine, but it is not clear whether the measurement itself disrupts cell turgor and water relations. More importantly, deformability creates a "noisy" time line as shown in Figure 3. Coombe


High and Low P
Turgor (bars)
12
10
8
6
4
2
0
2
4
6
8
10
12
14
16
18
Brix

Graph of Turgor x Deformability



(1992) has interpreted this noise as arising from the asynchronous flowering dates of individual berries. Therefore, we propose to study berry development on the basis of days since bloom (cap fall) for each experimental berry.

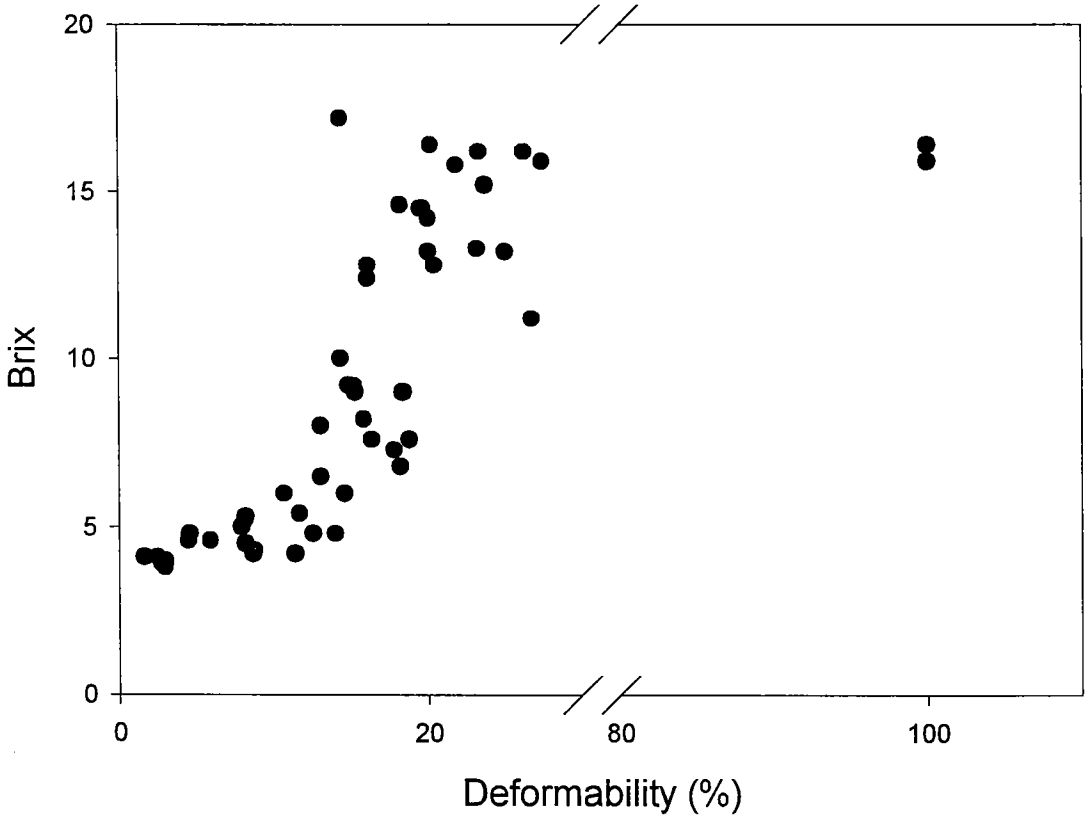
Lang and During (1991) hypothesized that the gradual increase in deformability observed in a population of berries (as in Fig. 3) is due to an increasing fraction of the cells suffering "a loss of membrane integrity". This loss would be expressed as a loss of turgor initially and as cell death eventually. We have observed the loss of turgor (at least to low values near 0.1 MPa or 1 bar) of some cells. If the increase in deformability is due to turgor loss as we speculate, then turgor loss must be observed in most mesocarp cells, not just the few hundred microns depth that we have been able to sample so far. If the progression of cell death is the cause as Lang and During have suggested, then there should be an origin of that progression. It is important to learn whether there is such a progression of turgor loss through the cells in the berry and whether that loss reflects cell death. We will address both in the proposed work.

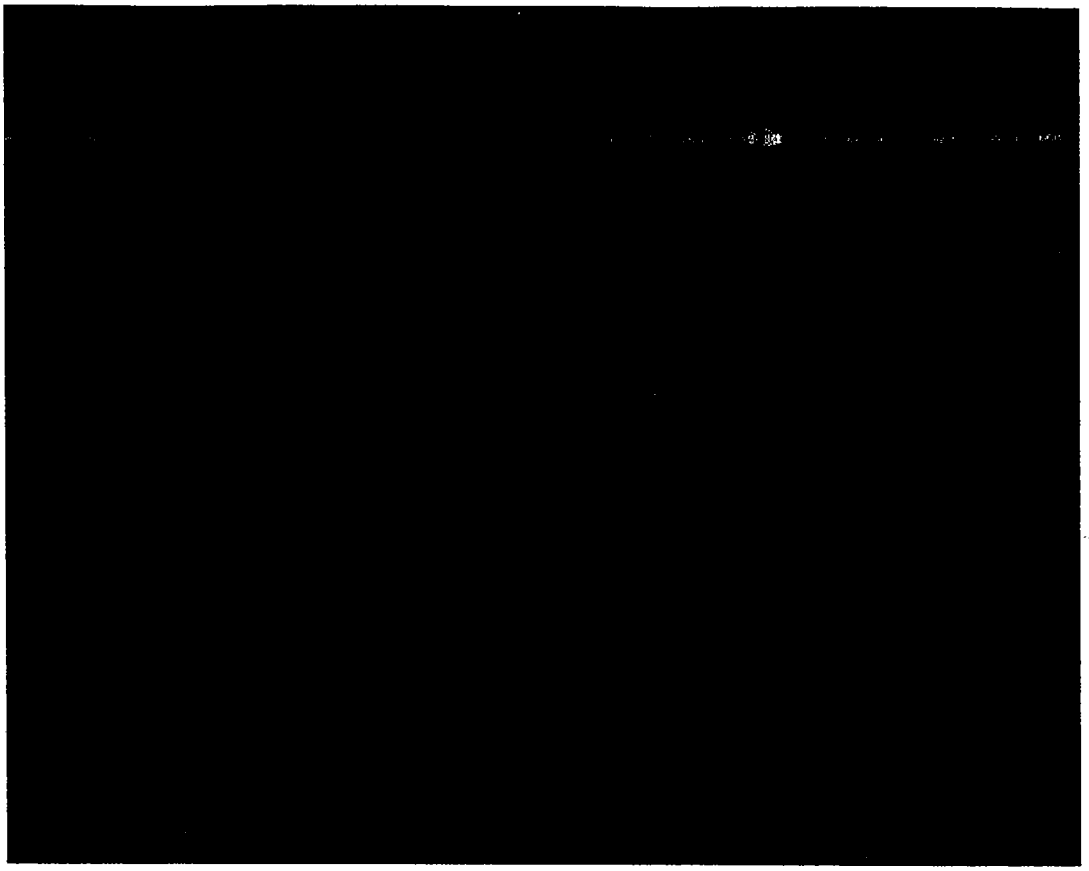
We have had limited success with a vital staining technique for cells of grape berries using fluorescein diacetate and ethidium bromide. Images of berry sections exposed to the dyes were visualized with a fluorescence microscope equipped with a digital camera and image processing system. The fluorescein stain has been used by Prof. Shackel to visualize impact injury in grape (both red and green varieties). Uptake of this stain has been used by Prof. Vito Polito (Dept. Pomology, UCD) as a measure of membrane integrity and cell vitality in pollen. The ethidium bromide has been used in bacterial and animal studies as a dead cell indicator. When we supplied fresh berry segments with the stains, getting sufficient uptake has been difficult. The behavior of the ethidium bromide as a dead cell stain has been sufficiently erratic for us to abandon it for now.

Examples of a preveraison berry (Fig. 4.) and a post veraison berry (Fig. 5) stained with fluoroscein diacetate are provided. In both segments there are cells near the center of the berry that do not activate the cell viability stain, but the area lacking indicator is considerably larger in the posveraison berry. Although this result suggests that there is an increasing number of dead cells originating near the center of the berry, we are concerned that the results could be an artifact of our staining technique or of differences in the "stainability" of different cell types. It is clear that cell types differ in the efficacy of the stain because cells around vascular bundles stain more clearly and consistently than ground mesocarp cells. We will continue to work with Prof. Vito Polito (an expert in microscopy) to increase

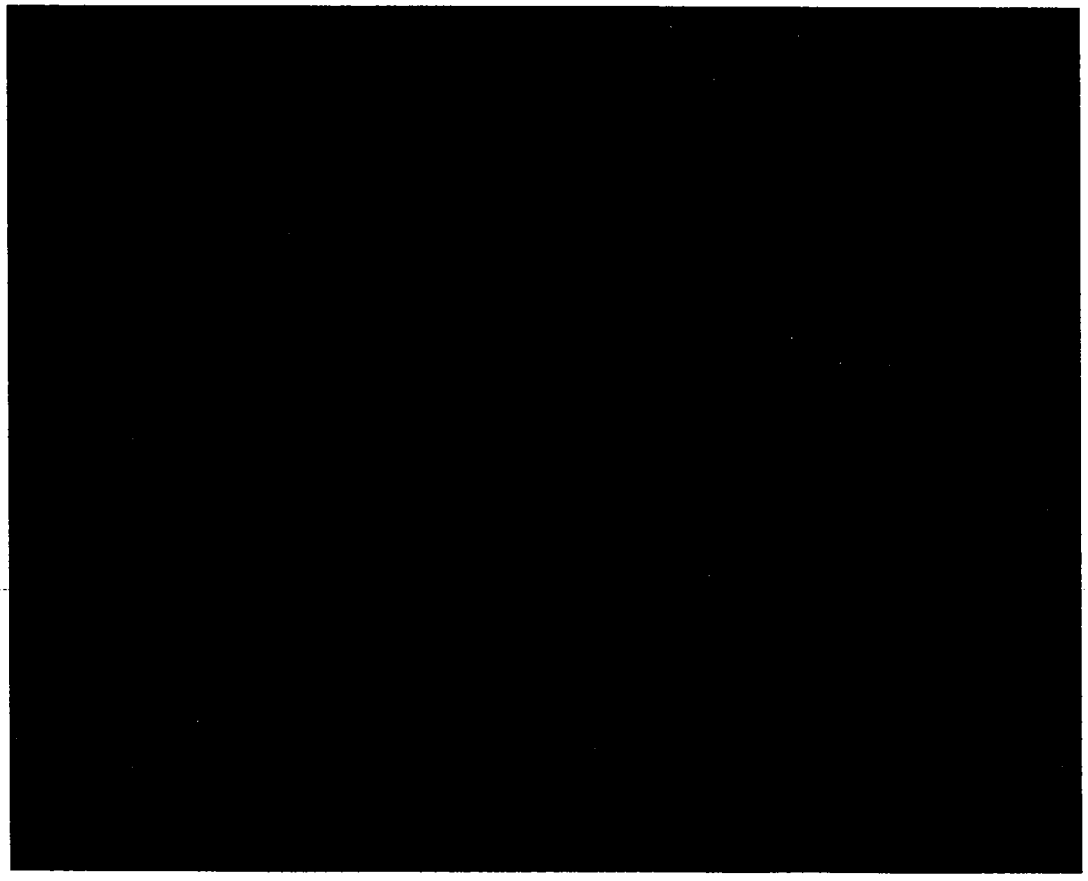
Another definition of veraison has been the resumption, or a rapid increase in the rate, of berry growth. Figure 6 (in paper copy of report) provides some detail about how that resumption comes about. The data show significant diurnal expansion and contraction of the berry for several days during Stage II (preveraison). There is little net growth at this time. At some point in berry development, the pattern changes such that significant net growth is realized. The source of the growth appears to be due as much or more to a loss of daytime contraction than to an increase in nighttime expansion. The transition to rapid growth was essentially complete in two days and several days before any change in color was detected. Thus, this transition from large to small daily contractions occurs early in veraison, similar to turgor loss and softening.

Graph of Brix x Deformability





Preveraison



Postveraison

The implications of these details is not yet clear, but the decrease in daytime contraction could reflect the loss of a direct connection through xylem vessels from the berry to the transpiration stream of the leaves, and the transition from xylem to phloemic water supply. The preveraison contraction could be due in part to xylem backflow to the parent plant, and this path is largely eliminated at "veraison". Also, the phloem transport is presumably less sensitive to transpirational demand in the daytime, and the phloem flow rate may be higher in the day than at night (Greenspan et al. 1994). We propose to conduct diurnal studies with intact fruit on the vine to see how turgor changes diurnally before and upon veraison. This has never been done before with any fruit as far as we know.

Apoplastic sap:

We found that collecting apoplastic sap from excised berries was much more difficult than we expected from the literature. We spent many scientist-days developing a system to seal a pedicel into the pressure chamber and collect the expressed sap volumetrically. This involved testing adhesives, tubing material and sizes, and protocols for excision, assembly prior to pressurization, and pressurization protocols. Despite our best efforts, we could not reproduce the volumes and flow rates of sap expressed from berries in a pressure chamber that were reported by Lang and During (1991). They reported a linear dependence of flow on applied pressure and steady flow rates of about 20 μL/hr for pressures of about 8 bars. We could get something close to that only from green berries. From postveraison berries, essentially no flow was observed at pressures below 10 bars. At pressures above 10 bars, flow was less than 5 μL/hr. Excising an apical portion of the skin prior to pressurization increased flow but only to 8-14 μL/hr. Thus, for us to collect a 20μL sample of apoplastic sap from post veraison berries usually required 3 hours of diligent monitoring and adjusting the pressure applied to the sample berry.

There is at least one important conclusion to be drawn from the difficulty in expressing sap from postveraison berries. It must be similarly difficult to move sap from the berry to the parent plant postveraison. The dramatic resistance to water flow out of the excised postveraison berry supports our hypothesis that the dramatic decrease in daily contraction of the berry is at least in part due to the loss of the xylem conduit pathway for water movement back into the parent plant.

We tested whether exposing the mesocarp to the perfusing water in the pressure chamber (by excising a skin segment) allowed that water to contaminate our apoplastic samples by filling the pressure chamber with a fluoroscein dye solution prior to pressurization. After a 4 hour pressurization, transport of the perfusing solution into the berry was visualized under a fluorescence dissecting microscope. The stain did not appear in the expressed sap and moved into the mesocarp less than 1mm. Thus, the apoplastic sap sample is not contaminated with the perfusing solution used to drive the apoplastic sap out of the tissue.

We began investigation of the composition of the apoplastic sap and comparing that composition to the composition of cell sap (juice of the crushed berry) by testing for differences

in pH and relative abundance of malate using paper chromatography because this could be accomplished quickly on small sample volumes. Some results are presented in Table 1.

Table 1. pH and malate in the sap expressed from a berry pedicel in a pressure chamber and in the juice of that same berry after crushing.

Maturity (veraison)	pH sap	pH juice	*malate sap	*malate juice
pre	3.5-4.0	2.0	-	-
pre	3.0-3.5	2.0	-	-
pre	3.5	2.0	1	10
pre	2.0	2.0	2	10
post	4.0	2.0	-	-
post	3.5-4.0	3.0	-	-
post	3.5	3.5	9	9
post	3.5	3.0	9	9

*an arbitrary scale form 1 to 10 was used to evaluate the amount of malate

The data show that the pH of apoplastic sap was approximately 3.0-3.5 in green berries and that the pH was unchanged or increased slightly to about 3.5 in postveraison berries. The pH of the berry as a whole was 2.0 in green berries and increased to 3.0-3.5 in postveraison berries. Thus, there is some evidence for high acid concentrations in the vacuoles of intact cells preveraison and that that concentration diminished postveraison as expected. The pH of the apoplast did not decrease during development as one would expect if the acidic vacuolar contents were moving into the apoplast. However, the similarity in pH of the apoplast and berry juice in postveraison berries is surprising and could be interpreted as evidence for a loss of compartmentation.

Consistent with that interpretation is the observation that the apoplastic sap of green berries contained little or no malate in contrast to the cell sap, but the apoplast of postveraison berries had similar malate to the cells themselves.

Solute concentrations in the apoplast of postveraison berries were 630-785 mmol/kg. Although this is a high concentration of solute for the apoplast, it is much less than the approximately 1600-1800 mmol/kg measured in the corresponding berry juice. Thus, the osmotic gradient in postveraison berries (taken from clusters undergoing veraison) pulls water into the cells and not into the apoplast.

The data describing apoplastic sap composition are provocative but inadequate. The time required to collect one sample was nearly prohibitive. Therefore, another approach was developed in which apoplastic sap is expressed from slices (approximately 2 mm thick) of berry tissue in a closed system using pneumatic pressure. The expressed sap is collected after passing a filtering membrane on which the sample rests. With this technique, samples of about 10 μL were obtained in 10 min. Analysis of sap pH, solute concentration, and malate were similar to apoplastic sap samples obtained from entire berries over 3-4 hours.

We determined that there is a loss of several bars of turgor in the outer mesocarp near or at the beginning of veraison. We determined that there is an accumulation of apoplastic solutes in ripening berries, and that the concentration of malate in the apoplast becomes similar to that in the berry as a whole as ripening progresses. We need to learn more about the geography of turgor and water transport in the berry in order to discover the site and timing of the origin of veraison. We need to increase our resolution of berry age and of the changes in apoplastic solutes that occur near veraison in order to draw conclusions about the source of apoplastic solutes and the cause of turgor loss at veraison.

Fig. 6. Diurnal pattern of berry contraction during veraison.