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Title: Substitution of Germanium for Boron in Suspension-Cultured Carrot Cells

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Dr. W. David Loomis

Boron has been recognized since 1923 as an essential micronutrient for vascular plants, but its function is still not known. Many diverse roles have been proposed, but none of these has been definitively shown. Problems that have made the study of the function of boron difficult include, no useful radioisotope, low level requirement (and toxicity at higher concentrations), and the wide spectrum of biochemical and physiological responses elicited.

Germanium forms chemical complexes similar to those formed by boron, suggesting the possibility of using germanium as a tracer for boron. Previous studies (McIlrath and Skok, 1966) with whole plants suggested that germanium could delay boron deficiency symptoms in sunflower seedlings for a few days, but not longer. To reevaluate this question, we have used suspension-cultured carrot cells and found that germanium can substitute for borate in this system. Germanate cultures were maintained by subculturing for more than two years. Cell growth was studied as a function of borate or germanate concentration of the medium. In

addition, elemental analyses of B, Ge, Ca, Mg, Fe, Mn, Na, K, P, and Zn by Induction Coupled Plasma Argon Emission Spectroscopy were performed on these cells.

To further characterize the role of boron, cells were fractionated into cell wall and protoplasmic fractions to determine the distribution of boron (or germanium) within the cell. Protoplasts were formed by enzymatic digestion of the cell wall. Alternatively, cells were fractionated by grinding in liquid nitrogen or passing through a French press, and centrifuging. These methods all yielded similar results, which showed that boron (or germanium) was concentrated in the cell wall fraction. At least 95% of the boron was present in the cell walls of cells grown in low boron concentrations. Germanium, was present in comparable concentrations in the cell wall, but was also found in significant amounts in the protoplasm. This suggests less than perfect control of the non-physiological element.

One of the proposed roles for boron is as a structural member of the cell wall. The ability of germanium to substitute for boron in our system, and the similarity of chemical complexes formed by the two elements suggest a rather non-specific interaction with polyols. This, along with our finding that B (Ge) is localized in the cell wall are consistent with a role as a structural component in the cell wall.

Substitution of Germanium for Boron in Suspension-Cultured Carrot Cells

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INTRODUCTION

Boron, as a nutrient element, is unique in that it is required only by certain specific groups of organisms. Diatoms (especially marine diatoms) and vascular plants require boron, while bacteria, fungi, green algae, and animals apparently do not (Lewis, 1980; Lovatt, 1985; Lovatt and Dugger, 1984). Recently, Mateo and coworkers (Mateo et al., 1986) reported that the cyanobacterium Anabena sp PCC 7119 required boron for dinitrogen fixation, but did not require it for growth on fixed nitrogen, while Anacystis nidulans, a cyanobacterium which is incapable of nitrogen fixation, does not require boron (Martinez et al., 1986). Recent reports of Nielsen and coworkers (Nielsen, et al., 1987) suggest that boron may have beneficial effects in animal nutrition relative to bone development and calcium retention. There is no evidence, however, that boron is an absolute nutritional requirement in animals as it is in plants (Underwood 1977).

The first unequivocal demonstration of a borate requirement for higher plants was published by Elizabeth Warington 65 years ago (Warington, 1923). She showed a clear-cut requirement for boron by several leguminous species, notably broad bean and crimson clover, but could not at that time demonstrate a requirement by winter vetch, or by rye. These studies were soon extended to other species, including non-legumes, by Warington and a number of other investigators. Boron, as boric acid or borate, is now recognized as an essential micronutrient for vascular plants in general as well as for diatoms

(Gauch and Dugger, 1954; Lewis, 1980; Lovatt, 1985; Lovatt and Dugger, 1984). However, there are wide variations in the levels of boron required, or tolerated, by various species of plants. Apparently correlated with these differences in boron requirement are differences in the content of or requirement for calcium and silicon. An important generalization is that graminaceous monocots have low requirements for both boron and calcium (Miller, 1938, Chapman, 1966). Graminaceous species also have high silicon content, as well as low contents of calcium (Richardson, 1920, cited in Miller, 1938, p.285) and of pectic polymers (Kertesz, 1951, Ishii, 1984, Bishop et al., 1958; Carpita, 1987). Richardson's analyses of Equisetum (horsetail) were very similar to analyses of Andropogon (a grass) and contrasted strongly with analyses of dicots grown on the same site (Miller, 1938, p.285). A silicon requirement for Equisetum has been established (Chen and Lewin, 1969). Asparagus and onion (both liliaceous monocots) have high tissue boron content (Chapman, 1966), and onion has been shown to have cell walls similar to those of dicots (e.g., high content of pectic substances) (Mankarios et al., 1980). Carpita (1987) has emphasized the uniqueness of the Graminaceae and presented contrasting models of primary cell walls of dicots and graminaceous monocots.

Since 1923, the plant nutrition and physiology of boron have been investigated extensively, and the literature has been reviewed frequently. Key reviews are those of Gauch and Dugger, 1954; Lewis, 1980; Parr and Loughman, 1983; Lovatt and Dugger, 1984; and Lovatt, 1985). Based on research observations, numerous hypotheses have been proposed, but the unique function of boron in plants has remained a mystery. Gauch and Dugger (1954) presented and evaluated "fifteen more or less

distinct, postulated roles of boron which have appeared in the literature". They favored a role in carbohydrate transport. Parr and Loughman (1983) cited a slightly shorter list of postulated roles:

1. Sugar transport
2. Cell wall synthesis
3. Lignification
4. Cell wall structure
5. Carbohydrate metabolism
6. RNA metabolism
7. Respiration
8. IAA metabolism
9. Phenol metabolism
10. Membranes

Based on their data, Parr and Loughman proposed that boron has a unique membrane function. Cohen and Albert (1974) proposed a role in DNA synthesis. Recently, Lewis (1980) proposed that the primary role of boron is in lignin biosynthesis. Lovatt and Dugger (1984) proposed that "The wide array of observed plant responses to boron deficiency indicates that the element is probably involved in a number of metabolic pathways or a cascade effect; therefore regulating metabolic processes somewhat as has been proposed for plant hormones", while Lovatt (1985) has proposed that the boron requirement is related to the evolution of xylem, and possibly to the production of pyrimidine nucleotides in apical meristems.

Boron, as a plant nutrient, shares with calcium the unique property that deficiency symptoms first appear at growing points rather than in mature tissues, and there is very little remobilization of these elements from mature tissues. Boron deficiency symptoms appear within

6 hours in root tips (Cohen and Lepper, 1977), but less quickly in shoot tips, because transpiration concentrates nutrients in the shoots. Initial boron deficiency symptoms in either roots or shoots consist of cessation of cell division, hypertrophy of the cells of the growing points, and eventual death of these abnormally enlarged cells (Reed, 1947). Boron deficient diatoms also have abnormally swollen cells (Lewin, 1966).

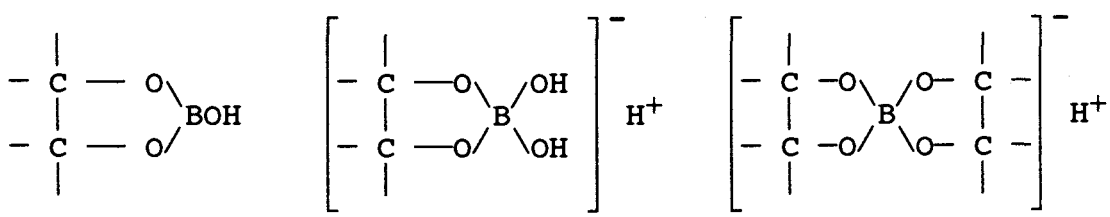
A classic symptom of boron deficiency in plants is "rosetting" and "brittleness" of shoot tip tissues. From experiences in our research group with boron-deficient peppermint (Mentha piperita L., cv. Black Mitcham) grown in a greenhouse, (A. Darussamin and W. D. Loomis, unpublished) we would say that "brittle" is an inadequate term; fragile or crumbly would be better. This suggests the presence of abnormally weak cell walls. At the same time, these abnormal shoot tips did not die if they were not broken off. This is in contrast to Ca deficiency, in which shoot tips appeared normal, but died, presumably due to membrane-Ca deficiency.

Lorenz (1942) reported that boron deficient beet roots showed abnormal cell wall breakdown, but that the cells nevertheless plasmolyzed normally when exposed to hypertonic solutions. This indicated that the cell membranes were normal. Germinating Nymphaea pollen may show boron deficiency symptoms (bursting of the germinating pollen grain or swelling and bursting of the pollen tube tip) within minutes or seconds (Schmucker, 1933, 1934). Schmucker suggested from his observations of pollen germination that boron has a "membrane" function (by "membrane" he clearly meant cell wall, a common usage in the older literature). Later work, (Dickinson, 1978) on pollen tube growth, has shown growth

related effects of boron and calcium. He reported that there was no growth if borate was absent, and that lack of calcium caused extensive bursting and leakage of pollen sugars into the medium.

Spurr (1957) and Kouchi and Kumazawa (1976), using tissue fixation and embedding methods that avoid shrinking of gels (Spurr, Carbowax; Kouchi and Kumazawa, permanganate or osmium tetroxide-glutaraldehyde fixation followed by embedding in epoxy), observed that the walls of parenchyma cells of boron-deficient plants were irregular, and thicker and less dense than normal. In contrast, collenchyma cell walls (Spurr, 1957) were thinner than normal. Spurr also observed that petioles of celery grown on toxic levels of boron were "much more flexible than normal". Darussamin and Loomis (unpublished) made a similar observation on leaves of peppermint grown on nutrient solution containing 5 ppm B. These leaves showed common boron toxicity symptoms: downward cupping, and yellowing at the edges. At the same time they were unusually resilient and springy.

Most discussion of the biochemical role of boron has centered on the ability of boric acid and borate to form cyclic diesters with cis-diol groupings of carbohydrates and phenolics. The principal types of esters are:



(BD) $(BD)^-$ $(BD_2)^-$

(B = boric acid or borate; D = diol)

These borate esters form and break spontaneously and with rapid kinetics (Friedman et al., 1974). Boron has been difficult to study due to its very low concentration in the plant (typically 1-100 ppm dry wt.), and because there is no useful radioisotope (half lives of 0.02-0.77 sec.).

Of the list of proposed functions for borate in vascular plants, those relating to cell wall structure or synthesis could best explain the observed distribution only in the plant kingdom. Cell walls of vascular plants have a unique structure, and chemical composition, made up of long chained and branched carbohydrates. Boron, with its ability to form cyclic esters with polyhydroxy compounds is a solid candidate for a role in the crosslinking of the cell wall. Deficiency symptoms occur first in very young cells, at the stage of cell expansion, not later at the time of lignification. If the primary cell walls are abnormal, allowing uncontrolled cell expansion, nothing will function normally. The mechanisms of cell wall extension and its control have not yet been identified. Our review of the literature supports a role for borate as a cross link in the primary cell wall.

To investigate the role of boron in the plant, we tried substituting germanium for boron. Both elements form anionic ester complexes with appropriate cis-diols, although their sizes are quite different (covalent radii, Ge 1.22 Å vs B 0.88 Å (Dean, 1979)). In these complexes, boron is tetrahedral while germanium is octahedral (Antikainen, 1959). Ge might prove useful as a tracer for B, in much the same way that Rb serves as a tracer for K. Skok (1957), and McIlrath and Skok (1966) tested for the ability of several complex-forming elements (Sr,

Ge, Al) to alleviate boron deficiency symptoms in sunflower seedlings. Of these, only germanium forms the same type of complexes as boron. Their tests indicated that treatment with germanic acid delayed the appearance of boron deficiency symptoms for several days, but not longer. They concluded that germanate enhanced the mobility of borate in the plant, thus delaying deficiency symptoms, rather than replacing borate. Careful reading of their papers suggested to us that their germanium treated plants might have suffered from germanate toxicity. Since the range between deficiency and toxicity with boron is narrow, and we know that transpiration can concentrate boron in the shoots, one might have healthy shoots, or even toxified shoots, on deficient roots. This is consistent with the observation that toxicity symptoms first show up in the shoots, while deficiency appears first in the root tips. We reasoned that a non-transpiring system might eliminate this problem and that growth with germanium in place of boron might be possible.

To test this, we elected to try substitution work with suspension cultured carrot cells. This system is non-transpiring, and also can be carefully chemically defined. Previous workers have used tissue culture techniques to select cell lines which had altered mineral utilization. Much of this work has been on selecting salt tolerant cell lines (Dix and Street, 1975; Nabors, et al., 1975; Rains et al., 1980), although Al resistance (Meredith, 1978) and increased iron efficiency (Sain and Johnson, 1984) have also been reported.

Carrots (whole plants) have a high boron requirement, which might be expected of the cultured cells as well, making low level detection and

quantification somewhat easier. Carrot is also one of the easiest suspension cultures to grow. Germanium, like boron, has no useful radioisotopes (half lives from 1.5 min-11 hrs, and X-ray producers). Both of these elements can be detected by Induction Coupled Plasma Argon Emission Spectroscopy (ICP), at the levels found in extracts or redissolved dry ash from plant materials.

MATERIALS AND METHODS

Cell material

Cell suspension culture of Daucus carota L. was obtained from Prof. Howard Bonnett (Univ. of Oregon). This culture was originally established by Eriksson (see Wallin and Eriksson 1973). The cell suspension was grown on modified Murashige-Skoog's medium (Eriksson 1965) as listed in Table 1. All water used was from a Milli-Q purification system (Millipore Corp. Milford, MA).

Culturing was carried out in 250 ml polycarbonate screw top Erlenmeyer flasks. 100 ml medium and the appropriate amount of boric acid (or germanic acid) was added to each flask. Polyurethane foam plugs were inserted (cotton contains boron) and lids screwed on loosely. Flasks were then autoclaved for 15-20 min. at 250°F.

Cultures were maintained on a rotary shaker at 25°C in the dark. Subculturing took place weekly by transferring 1 ml packed cell volume (PCV) with 10 ml of old medium to 100 ml of fresh medium. For B and Ge concentration, and time course experiments, cells were washed 3-5 times with fresh 0B (and 0Ge) medium prior to inoculating the test cultures with 1 ml PCV in 5 ml fresh medium (no old medium). Stock cultures were maintained at 0.01 mM B, 0.1 mM B, 1.0 mM B and 0.43 mM Ge. A single culture will yield ca. 10 ml packed cells, (ca. 400 mg) after 7 days growth.

Cell Measurement

Cell growth was assessed in two ways, packed cell volume (PCV), and dry weight. PCV was measured by transferring cells to sterile, graduated, 15 ml polycarbonate conical tubes, centrifuging gently (slowest speed, IEC clinical centrifuge) and recording the volume. PCV measurement could be done without harming, or contaminating the cells. Dry weight was measured by collecting the cells on tared filter paper (Whatman #1) by gentle vacuum filtration, followed by washing with 20-30 ml of water. The cells were then oven dried (50°C, 24-72 hrs) and weighed.

Chemicals

Reagent grade chemicals were used for preparation of cell culture medium. Ethylenediaminetetraacetic acid, mono zinc salt was obtained from Eastman Kodak Co., Rochester, NY. Germanium dioxide (99.999%) soluble form, and atomic absorption standards, were obtained from Aldrich Chemical Co., Milwaukee WI. Cellulase used for protoplast formation was commercially available Driselase (lots K13115 and K48026) from Koywa Hakko Kogyo Co., Ltd. Japan. Calcofluor white (C.I. 40622) was obtained from Sigma Chemical Co., St. Louis, MO.

Ashing

Dried tissue samples were ashed in covered porcelain crucibles, at 550°C for 4 hrs, in an electric muffle furnace and allowed to cool for 4 hrs. Ash was dissolved

with 3-10 ml 0.2N HCl. Samples sizes ranged from 50-1000 mg.

Spectrophotometric Elemental Analyses

Elemental analyses were performed on an Applied Research Laboratories, Div. of Bausch and Lomb Model 3580 induction coupled plasma argon emission spectrophotometer (ICP).

Plasma Operating Conditions:

RF generator frequency	27 MHz
Forward power	1200 Watts
Reflected power	<5 Watts
Observation height	15 mm
Coolant gas (argon)	12 l/min
Plasma gas (argon)	0.8 l/min
Carrier gas (argon)	1 l/min
Snout gas (argon)	1.75 l/min
Argon supply pressure	80 psig

Nebulizer:

Concentric glass pneumatic, non-adjustable, TR-30-C3, from Meinhard Associates, Santa Ana, CA. Uptake rate, 2.8 ml/min.

The preprogrammed simultaneous channels were used for analysis of Ca (317.933 nm), Mg (279.080 nm), B (249.678 nm), Fe (259.940 nm), Zn (213.856 nm), Mn (257.610 nm), K (766.49 nm), Na (589.59 nm), and P (178.29 nm) at the indicated wavelengths (Winge *et al.* 1985) The sequential channel was used for Ge analyses while parked at 265.118 nm, order 2. The only interferences are a 0.1% contribution from Fe to B, and a

0.07% contribution from Fe to Ge. Neither of these interferences was corrected for since the Fe levels were low enough that its contribution was not significant. Table 2 lists the detection limits of the instrument for the various elements.

Protoplast Isolation and Growth

Protoplasts were isolated according to the technique of Wallin and Eriksson (1973). Using a solution of 2% Driselase in 0.4 M α-methyl-D-glucoside, the removal of cell walls was complete in 2 hrs when the incubation was done on a rotary shaker at 25°C in the dark. The protoplasts were recovered by gentle centrifugation (5 min. slowest speed) on an IEC clinical centrifuge. To remove the enzyme solution, 2 washes were made with a solution containing 0.3 M α-methyl-D-glucoside and 0.05 M $CaCl_2$, with centrifugation each time. α-Methyl-D-glucoside was chosen as the osmoticum since mannitol (the usual choice) forms strong complexes with borate.

Protoplasts were grown on modified Murashige-Skoog's medium as listed in Table 3. Protoplast were transferred to the growth medium and the concentrations were measured with a hemocytometer, and adjusted to the desired value. 2 ml was then placed in 35 x 10 mm plastic petri dishes. Growth was carried out in the dark without shaking. Feeding of protoplasts involved 2-5 drops (25 drops/ml) growth medium added every 2-3 days.

Standard reference material

A sample of National Bureau of Standards, Standard Reference Material #1571, orchard leaves was obtained from Jerry Wagner of the Corvallis Environmental Research

Laboratory of the U. S. Environmental Protection Agency. This was the only plant material certified for boron content (in addition to Ca, Fe, Mg, Mn, P, K, Na, and Zn). It was analyzed to insure that our methodology was yielding good results.

Cell wall isolation

Two physical methods were employed to separate cell wall and protoplast fractions. Fresh cells were frozen in liquid nitrogen (LN_2), and ground to a fine powder with a precooled (LN_2) mortar and pestle. The frozen powder was transferred to a centrifuge tube, and 10 ml H_2O added. After thawing this was centrifuged at 10,000xg for ten minutes (Sorval RC-2). The resulting pellet was washed 2 times with 5 ml H_2O, resuspended and recentrifuged. The pellet was resuspended in 5 ml H_2O, transferred to a porcelain crucible, dried and ashed. The supernatant was transferred to a porcelain crucible, dried and ashed. The washes were analyzed directly.

Fresh cells were ruptured in a French press. Cells (typically 5 ml PCV) were washed with water and transferred with 10 ml water to the French press. Cells were the forced through at 15,000 psi, and the exudate centrifuged 10 min. at 10,000xg. The pellet was resuspended in water to facilitate transfer and ashed as above. The supernatant was analyzed directly. Because of dead volume within the press, ca. half of the cells were not forced out of the press.

RESULTS AND DISCUSSION

Growth vs Boron Concentration

Carrot suspension cell culture was used to study the effect of varying the borate concentration of the medium on cell growth and mineral composition. Concentration was varied from deficient to toxic concentrations, and the packed cell volume, dry weight, and mineral composition by induction coupled plasma argon emission spectroscopy of the cells were measured. Three different cell lines were maintained by repeated subculturing at 0.01 mM, 0.1 mM, and 1.0 mM borate (ca. 0.1 ppm, 1.0 ppm, and 10 ppm respectively). Each of these cell lines was used as inoculum for growth experiments. All of the inoculum cells were extensively washed with 0B medium prior to inoculation to prevent any external carryover of borate from the old medium.

The growth curves showed three distinct regions as borate concentration was increased (figure 1). At very low concentrations (up to ca. 0.001 mM) there was very little growth. Above this there was a sharp increase in growth to ca. 0.003 mM, followed by a flat region of maximal growth (80-100%), centering at about 0.1 mM. Between 3 mM and 30 mM there was a dramatic decrease in growth, with no growth at all above ca. 30 mM. These three regions will be referred to as the deficient, sufficient and toxic regions of growth. Both measures of growth (PCV and dry wt.) showed parallel results.

In the deficient region there was some growth, which increased with increasing boron concentration of the inoculum culture. Figure 1 shows that the growth in the deficient region was ca. 15% , 25%, and 40% of the

maximum growth for the 0.01 mM, 0.1 mM, and 1.0 mM cell lines, respectively. The growth that occurred in this region was essentially independent of the borate concentration of the medium, with all three cell lines showing constant amounts until the medium concentration reached ca. 0.001 mM. This was no doubt due to a residual intercellular pool of available borate from the inoculum. See later discussion on the growth and survival of cells grown at deficient concentrations.

The concentration range through which there was good growth (50% of maximum) was from ca. 0.001 mM-10 mM, and the curve is very steep on both the deficient and toxic ends of the range. The sufficient region for all three cell lines was considered to be somewhat more restricted (ca. 80% of maximum). The maximum growth was variable for the different cell lines, but did not seem to be related to the borate concentration at which the inoculating cells had been grown. This variation was probably due to inherent variability in the growth of cell cultures, and was seen throughout the study.

The toxic concentration was the same for all three cell lines, above ca. 10 mM. Cells at the highest concentration tested (100 mM) showed no growth at all, and some even shrank slightly (to 0.9 ml) compared to the inoculating PCV (1 ml). (See later discussion on growth and survival of cells grown at toxic concentrations.)

Dry weight measurement showed the same three-part bell shaped growth curve as packed cell volume. Subtle differences in the two measurements lead to the cell density (dry wt/PCV) results shown in figure 2. The high cell density that occurs within the deficient region may be due to accumulation of metabolic products. These cells

were viable but unable to grow or divide normally, due to lack of boron. Within the sufficient region, these products would be distributed to daughter cells as growth and division occurred. The increase that again occurs at the end of the sufficient and into the toxic region may be due to the same inability to grow and divide normally. When high borate concentrations were reached, cell death occurred. See later discussion on growth and survival in toxic medium.

Another interesting observation was made regarding the color of the cultured cells as a function of borate concentration. Normally, these cultured carrot cells are a light straw-yellow color. At deficient concentrations however, the color was distinctly brownish, and at toxic concentrations they were light yellow-brown to white in color. Lee and Aronoff (1967) reported that boron deficiency produced brown color due to an increase in phenolic compounds.

Growth and Survival in Deficient or Toxic Boron

Figure 1 showed some growth due apparently, as discussed above, to boron carried over in the inoculum cells. Experiments to test this and also to assess cell viability after treatment with deficient or toxic concentrations were carried out. Cells were transferred to deficient (0 mM) or toxic (29 mM) borate medium, and repeatedly subcultured to deficient and toxic medium respectively. Growth and cell viability were monitored. Viability was checked by transferring cells back to normal borate medium, and monitoring growth.

Cells in deficient borate medium (0 mM) grew slightly (1.3-2.4 ml final PCV from a 1 ml inoculum) with each subculturing (figure 3). It is possible that this slight growth was due to very low levels of boron present as contaminants in the other chemicals used to make the medium, from recycling from dead cells, or from the air. Cells returned to normal borate proved to be capable of recovery even after 5 subcultures at 0 mM borate. Cells transferred to 0 mM borate and allowed to grow (without subculturing) for long periods (25 days) also were quite viable when returned to normal borate medium. Recovery was never immediate, but showed nearly normal growth after an initial lag phase.

Cells grown at toxic borate concentrations (29 mM) grew briefly and only slightly, and then died (figure 4). Cells returned to normal borate after the first culturing at toxic levels, did grow normally, but after subsequent subculturing at toxic concentrations and return to normal borate they showed essentially no growth.

Growth and Survival in Deficient Calcium

Calcium has long been known to have a role in the structure of the cell wall (Jarvis, 1982, 1984; Demarty et al., 1984). Calcium chelators, such as EDTA, Ionophore A23187, and the antibiotic chlortetracycline, have been known to cause tissue to be dispersed into smaller clumps or even individual cells (Ginzburg, 1961; Herth, 1978). Therefore, we tested the effect of 0 calcium medium on the growth and viability of cultured cells. Figure 5 shows the growth and viability of cells after transfer to 0 calcium medium. The cells grew slightly, immediately after transfer (probably due to

residual Ca in the cells, in spite of extensive 0 Ca washing). After 15 days, there was some growth measured, which continued to increase until the end of the period tested (30 days). These cells were unusual in that they were in very small clumps. A possible explanation is that initially the cells contained considerable calcium tied up in the pectic substances of the cell walls and that this store of calcium was later released and became available for crucial membrane and messenger functions. This would account for both observations. Release of calcium from the walls would cause the cell clumps to break up, and the released calcium could allow additional growth to occur.

At the times shown in figure 5, 1 ml PCV was transferred back to normal Ca medium (3.0 mM). These cells did grow, but the recovery showed a lag of a few days prior to the start of growth. Subsequent experiments were performed to determine the minimal level of Ca at which some growth could be observed. It was found that at least 0.02 mM Ca was required to obtain minimal growth (the normal level in the medium was ca. 3.0 mM), and that at these low concentrations there was a lag before growth began. This is consistent with the unusual classification of calcium as a macronutrient due to its usual concentration, whereas it is only critically required at micronutrient levels (Loneragan *et al.*, 1968; Loneragan and Snowball, 1969).

Growth *vs* Time

The growth and mineral composition of the borate cell lines were measured as a function of growth time. Measurements were taken each day for up to 15 days. The

0.01 mM and 0.1 mM borate cell lines were used. The two cell lines showed nearly identical results.

Figure 6 shows the growth, measured by packed cell volume. There was a short lag phase from day 1 to day 3, followed by a log phase from days 4-10, and finally a roughly linear phase from days 10-15. The cells were still growing vigorously at the end of the period tested. Dry weight (figure 7) of both cell lines shows comparable results, but subtle differences between the dry weight and volume lead to small but consistent differences in cell density (dry wt/PCV). Figure 8 shows these differences. Density increased slightly, from 0.040-0.045 g/ml during days 1 and 2, followed by a gradual drop to 0.031 g/ml by day 8 or 9. A sharp jump back to 0.040 g/ml was then seen at day 9 or 10, followed by a constant value until the end of the study. This curve may be more indicative of the lag, log, and stationary phases than is readily seen in the growth curves themselves. The Ge cell line remained constant throughout the time period studied, and was considerably less dense, at ca. 0.028 g/ml. More work is needed to understand the reason for these differences.

Establishment of a "Germanium" Cell Line

A principal goal of this research was to determine whether germanate could replace borate in our suspension cultured carrot cell system. Cells which had been grown in 0.01 mM borate medium were carefully washed with 0B medium and transferred to various Ge concentrations. Initially, we had assumed that Ge, as an unnatural nutrient element, would probably be more toxic than B. Our first experiments using low levels of Ge

(0.0001-0.01 mM) were unsuccessful at producing growth. When higher levels (0.05 mM) were tried, there was slight growth. Finally, when Ge concentrations considerably higher (0.3 mM) than normal borate medium (0.01 mM) were used, we were successful at obtaining significant growth. Figure 9 shows the course of acclimation of a culture to growth on germanate medium through a series of subcultures. Cells showed fairly good growth during the first subculture period. This was partially due to residual borate contained within the inoculum cells, but was considerably greater than would be expected from any residual boron. Refer to figure 3 and the previous discussion about 0B growth, for additional discussion of the initial growth. This was followed by a very slow growth rate in the second and third subcultures and a subsequent slow increase in the growth rate in successive subcultures. The cells took considerable time (7 subcultures, 117 days), to begin growing adequately in the +Ge medium. It was found that growing cultures to an approximately equal packed cell volume (5-8 ml) by increasing the time between subculturing helped to establish the Ge line. This is similar to the conditions that Ojima and Ohira (1983) found necessary when they established Al and Mn tolerant cell lines. Routine subculturing of the +Ge cell line after the times shown in figure 9 was at 10 day intervals rather than the 7 day interval of the B cell lines.

Differences between the growth rate of the Ge cell line and the B cell lines were observed, with the Ge line growing slower than the B cell lines. While there is considerable variation in the growth rate of tissue culture cells, as they are repeatedly subcultured, results from all experiments were comparable. If cells were subcultured at either too long or too short an

interval, we observed that the rate of growth could change considerably. It is possible that this could explain some of the observed differences between the cell lines from one experiment to another. The acclimation of cells to a germanate medium was done numerous times and one of those cell lines grew at ca. 80% of the rate of the borate line. Other germanate lines grew as slowly as ca. 30% of the rate of the borate line. Results from all of the Ge cell lines were comparable. The concentration, time course and mineral composition data presented here are all from a single fast growing cell line that had been subcultured for at least 6 months. All of the Ge cells went through a regime of long time between subculturing (10-20 days, vs the typical 7 days for B the cell lines) while the cell line was being established. This is probably due to the typical requirement of suspension culture cells that there be a certain amount of growth or conditioning of the culture medium to maintain healthy cells. Long subculture time also caused the B cell line to grow at a somewhat slower rate, but never as slowly as the Ge cell line. After a Ge line had begun to grow it was then subcultured at 10 day intervals, and eventually at 7 day intervals. The longer a Ge cell line was subcultured at 10 or 7 day intervals, the closer it came to growing at the same rate as the B line. Germanate cell lines were maintained by repeated subculturing at 0.43 mM for more than two years.

Growth vs Germanium Concentration

The germanate cell line showed a similar three-part bell shaped growth vs Ge concentration curve as was seen for the B cell lines. The range between sufficiency and toxicity was considerably narrower for the Ge line than

for the B cell lines (figure 1). In the B cell lines the range was from 0.001 mM to 10 mM, which is ca. four orders of magnitude. This is considerably greater than the boron sufficient range observed in whole plants, which is ca. two orders of magnitude. In the Ge cell line the range was from ca. 0.015 mM to 5.5 mM, only slightly over two orders of magnitude. There was slight growth in the deficiency region, as there was with the borate cell lines, again probably due to slight residual intercellular Ge present in the inoculating cells. The toxic concentrations are very similar for both Ge and B, at ca. 5 mM and ca. 9 mM respectively. There is considerable difference in the upper limit of the deficient range with growth starting at ca. 0.001 mM B but not until ca. 0.015 mM Ge. The much higher concentration of Ge required for growth accounts for nearly all of the decrease in the sufficient range. The narrower sufficient range may explain why Skok (1957) found it impossible to grow whole plants on Ge.

Boron is required principally at regions of rapid growth, notably the root and shoot apices. Since borate is taken up in the roots and then utilized there without further transport, it must be available from the soil at a concentration that is sufficient for root growth. Subsequent transport via the xylem to the shoot followed by the 100-500 fold concentration due to transpiration (Noggle & Fritz, 1976) leaves a much more concentrated solution available in the shoot. If transpiration concentration causes the borate concentration to increase into the toxic range then these growing points will not grow normally. Likewise, deficiency symptoms will initially appear in the root if the available soil supply is too low. With the four orders of magnitude range observed for the sufficiency range with borate, this can

accommodate the concentration by transpiration and still leave a 100-fold range (typical of whole plants) of borate concentrations that still allow for growth at both the root and shoot. The much narrower sufficient range observed for Ge would leave little allowance for the concentration due to transpiration. It would be interesting to attempt to grow whole plants at a number of very closely spaced concentrations of Ge (and -B), under conditions that minimized transpiration to see if in fact they could be grown.

The concentration producing optimum growth is slightly higher for Ge (ca. 0.14 mM) than for B (ca. 0.09 mM). Early attempts in this study to establish a Ge cell line used concentrations at or below the concentration of borate that the B cell line was routinely subcultured at (0.01 mM). This was based on the assumption that the non-physiological Ge would be more toxic than B. Review of the data leads to the conclusion that there may instead be a one-one substitution of Ge for B in a critical function. Our initial trials were below the sufficiency threshold. The higher concentration of Ge required for growth may be due to non-specific binding. This is supported by the fact that the Ge content (1.4-2.8 μmoles/g dry wt) is higher than the B content (0.9-1.8 μmoles/g dry wt) of their respective cell lines. See figures 11 and 15, and also later discussion on the distribution of Ge in the cell. McIlrath and Skok (1966) speculated that the application of Ge in their system could be delaying deficiency symptoms by displacing boron from polyhydroxy compounds, thus remobilizing boron. This is certainly not the case here, as Ge cell lines were subcultured for more than two years and their B content was below the detection limit.

Reacclimation to Boron Medium

Cells from the Ge cell line were converted back to growth in borate medium. This was to show that the process was reversible. Figure 10 shows the growth as reacclimation proceeded. In the first two subcultures there was a slight reduction in the amount of growth followed by steady growth over the balance of time period tested. The cells grew adequately right from the start of the experiment, without the long subculture times used for the B to Ge acclimation. The cells never reached a growth rate as high as the original B cell line, but they were not maintained for an extended period.

Elemental Analyses

Standards and Recoveries

Induction coupled plasma argon emission spectroscopy (ICP) was used to study the mineral composition of cultured cells. The ICP is capable of simultaneously analyzing 10 elements, from a minimal amount of sample, at very low levels and with excellent accuracy. Data on boron, germanium, calcium, magnesium, iron, zinc, manganese, potassium, sodium, and phosphorus were gathered, with detection limits as listed in Table 2. Studies of the mineral composition of cultured cells from the previously discussed concentration, time and survival experiments were performed. Cells from those studies were dry ashed and the ash dissolved and analyzed for the previously listed elements. A large quantity of suspension cultured carrot cells was grown, and a homogeneous sample of this material (labeled Standard Carrot Material) was included along with each dry ashing

and ICP sample set to insure stable and consistent results. National Bureau of Standards, Standard Reference Material #1571 (orchard leaves) was also dry ashed and assayed by ICP. This is the only NBS standard plant material that is certified for boron. Table 2 lists the values we determined and the certified values for NBS #1571 and also the values of our own SCM. It can be seen that the methods used in this study yield excellent results except for iron and sodium. The listed NBS values are the total content, and were individually analyzed by techniques that insured complete solubilization of only one element at a time, with no regard to losses or incomplete solubilization of other elements. The dry ash method used in this study is known to not fully solubilize all of the elements in a sample (Pritchard and Lee, 1984), but was used since it gave excellent results for all of the elements analyzed in this study. While iron showed a low value, compared to the published value, it was probably incompletely solubilized since the % recovery from spiked samples was excellent. Values for sodium were extremely variable for both the NBS and SCM materials, which was probably due to volatilization by the dry ashing technique (Williams and Vlamis, 1961), which is not a good method for analysis of sodium. Samples of our Standard Carrot Material were spiked with element mixes used to standardize the ICP. Recoveries are listed in Table 2. All elements (except K, Na, and P which were not run) showed excellent recoveries, in the range of 97-112%, confirming that our technique was acceptable.

Cellular Boron Content vs Medium Boron Concentration

As the boron concentration of the medium was varied through more than 6 orders of magnitude (<0.0001 mM-100 mM), there was remarkably little change in the cell boron content. There were no differences observed between the three borate cell lines with regard to the boron content of the cells. Figure 11 shows the cellular boron concentration (in μmoles boron/g dry wt), and a representative growth curve for reference. In the deficient range the concentration was from 0.09-0.3 μmoles/g, rising slightly in the sufficient range to 0.3-1.8 μmoles/g, and then increasing rather quickly in the toxic region to a maximum of 18-28 μmoles/g. The cellular boron concentration then dropped to 4.6-9.2 μmoles/g at the highest levels of culture medium borate. Calculation of the concentration (as μmoles boron/ml PCV) showed that throughout the entire range tested, boron is accumulating within the cells. At low culture medium concentrations, this accumulation is ca. 100,000 fold, greater than the concentration of the medium, and decreases steadily until it is less than 100 fold in the toxic region. At the highest culture medium concentration tested (100 mM), the cell concentration equaled the medium concentration, ie. there was no accumulation. At this toxic level, it has been shown that the cells are dead. The reason for the drop in boron at the highest medium concentrations may be explained by this cell death. Since the cellular boron concentration, expressed as μmoles/g dry wt, remains so constant through the bulk of the growth range, this indicates boron is well regulated. The total accumulation of boron is independent of the rate of growth, and depends instead on the cell mass. The boron concentrations are less than that reported in carrot

leaves (Chapman, 1966), presumably due to the absence of transpiration in our cell cultures.

Cellular Boron Content *vs* Growth Time

The ICP analyses of the time course growth of the cell lines is shown in figure 12. These show a sharp drop in borate concentration of 50-83% right after transfer to the fresh medium (day 1), and a sharp rise on day 2, nearly back to the starting value. Another lesser drop at the same time that the cell density is increasing (day 8-10) is also seen. Ca, Mg, Zn and Mn all show a slight decrease during the early lag phase, followed by a slightly greater increase after the lag phase. Phosphorus showed a steady drop from 13 to 6 mg/g dry wt during the monitored time. Sodium showed an initial decrease of 85% (from 1.4 mg/g dry wt to 0.2 mg/g) in the first 3 days and then remained constant during the rest of the growth period. The low level (0.2 mg/g dry wt) observed through the majority of the study is more typical of the concentration routinely seen in these cell lines. It should be noted though that these routine measurements would have been on cells that were 5-7 days old. The cause of the increase in sodium at the start of this time course experiment was not investigated. It is known (M. Johnson Western Oregon State Univ., personal communications) that suspension cultured cells 'condition' the medium. Although the exact nature of this conditioning has not been investigated, pH adjustment, extracellular proteins and hormone release are frequently implicated (M. Johnson). It could be that the cells are initially accumulating Na as a means of balancing membrane potentials or ion gradients upon initial contact with fresh medium, and that a few days

later, after conditioning (pH adjustment), the potentials are maintained by proton gradients.

Germanium and Boron Content as Germanium Cell Line Established

Figure 13 shows the mineral composition as the acclimation to +Ge medium took place. Refer back to figure 9 for the growth of these cells. As would be expected, boron concentration dropped immediately from the typical level of 0.8 μmoles/g dry wt to 0.2 μmoles/g dry wt with the first subculture, and decreased with each subsequent subculture until it dropped below the detection limit. Concomitantly, Ge rose to ca 1.4 μmoles/g, (a level later seen as typical of the Ge cell line) with the first subculture, but was then rather variable throughout the period monitored. This may be due to the variable times for subculturing. See later discussion about germanium concentration vs time.

Figure 14 shows the B and Ge concentrations as the reacclimation of a Ge cell line back to B was studied. Refer back to figure 10 for the growth of these cells. The germanium concentration during the first two subcultures dropped from 1.65 μmoles/g dry wt. to below the detection limit (ca. 0.010 μmoles/g), while during the same period the boron concentration increased from below detectability to 0.5 μmoles/g dry wt. Boron concentration remained at this level, which was typical of the borate cell lines, throughout the period tested.

Cellular Germanium Content vs Medium Germanium Concentration

Cellular germanium concentration, in those experiments where the Ge concentration of the medium was the only variable, showed results similar to those of boron. Cellular Ge concentration rose very slowly over most of the tested range (figure 15), with a steep increase near the high end of the sufficient and into the toxic range. Through the sufficient range the cellular Ge concentration was found to be somewhat higher (0.2-4.1 μmoles/g dry wt) than the corresponding B concentration (0.3-1.8 μmoles/g dry wt) in the B cell lines. The rise in cellular Ge content that began in the sufficient range started in the middle of the range and rose more slowly, as compared to the rapid rise just before the toxic range in the boron cell lines.

Other Elements vs Medium Concentration

A summary of the observations of the concentration of each of the other elements analyzed by ICP follows. These data are from experiments where boron (or germanium) concentration was the only variable.

Calcium

Ca showed a very interesting inverse relationship to cell growth (Table 4). For all three B cell lines, the cellular Ca concentration was high in the deficiency range, dropped sharply at the start of growth, remained constant through the sufficient range and then increased again in the toxic range. The levels were ca. 6 mg/g in the deficiency range, dropping to ca. 1.5-2.0 mg/g in the sufficient range and then rising as high as 5-6 mg/g in

the toxic range. The high (1.0 mM) B cell line started out at a lower Ca level (4 mg/g) in the deficient range than did the other two cell lines. There has been some evidence for an increase in Ca content at low growth rates (Loneragan et al., 1968 and Loneragan and Snowball, 1969). These studies were on the growth and subsequent Ca content of shoots, due to varying Ca concentration in the nutrient solution.

In the Ge cell line there was not the dramatic inverse relationship as seen with the B cell lines (Table 4). Ca was high in the deficient range and dropped in the sufficient range, but did not rise in the toxic range. Ca concentration was slightly higher in the sufficient range (3 mg/g) than it was in the B cell lines.

Magnesium

In all three B cell lines the Mg concentration remained constant throughout the deficient and sufficient range, and dropped within the toxic range (Table 4). The concentration ranged from 1.2-1.5 mg/g, dropping below 1 mg/g in the toxic range.

Mg in the Ge cell line was quite different from the B cell line (Table 4). The concentration of Mg mimicked the growth curve, until the toxic range, when Mg only dropped 30% while growth dropped 90%. The Mg concentration was also higher in the Ge cell line (1.6-2.0 mg/g) than it was in the B lines.

Iron

The B cell lines showed an inverse growth vs Fe concentration relationship (Table 4). The Fe concentration was low or moderate (300-600 μg/g) at deficient B levels. Through the sufficient B level the Fe concentration dropped to 150-300 μg/g. Fe concentrations at the toxic B levels rose rapidly as growth dropped and ranged from 1400-4700 μg/g.

Fe in the Ge cell line was quite different from what was observed in the B cell line (Table 4). The concentration of Fe mimicked the growth curve, until the toxic range, when Fe only dropped ca 10% while growth dropped 90%.

Zinc

Zn showed a concentration relationship that was inverted from the growth curve (Table 4). In the deficient region, the Zn concentration was high (400-1000 μg/g), and constant. In the sufficient range, the Zn concentration dropped to 150-250 μg/g, again remaining constant. In the toxic B range, the Zn concentration first again rose to 400-500 μg/g and then showed slight decreases at the highest B levels.

The Zn composition of the Ge cell line showed the same inverse relationship that was seen in the B cell lines (Table 4).

Manganese

Mn showed a concentration relationship that was inverted from the growth curve (Table 4). In the deficient region the Mn concentration was high

(25-30 μg/g), and constant. In the sufficient range the Mn concentration dropped to 9-15 μg/g, again remaining constant. In the toxic B range the Mn concentration rose to 20-40 μg/g.

The Mn composition of the Ge cell line showed the same inverse relationship that was seen in the B cell lines (Table 4). The high concentrations seen at the deficient and toxic B concentrations were somewhat lower (ca 23 and 18 μg/g respectively) than the B cell lines.

Potassium

The K concentration followed the growth curve of all three B cell lines and the Ge cell line almost exactly (Table 4). The level of K varied from 10-15 mg/g in the deficient range, to 32-38 mg/g in the sufficient range and back down to 7-12 mg/g in the toxic region. In the Ge cell line the concentration in the sufficient range was slightly lower, at 18-24 mg/g.

Sodium

Na was highly variable and uncorrelated to the B concentration or to growth. Values ranged from 30 to over 1500 μg/g, although most values were in the 200-300 μg/g range.

Phosphorus

The P concentration paralleled the growth curve almost exactly (Table 4). The level of P varied from 8-10 mg/g in the deficient range, to 10-14.5 mg/g in the sufficient range and back down to 7-10 mg/g in the toxic region. While these differences are small, they were

consistent in all studies. This parallel behavior of P and growth was shown in all of the B cell lines and in the Ge cell line. This result is consistent with the findings of Odhnoff (1957) (and Reed 1947). She found that root and leaf phosphorus levels in bean seedlings under boron deficient conditions were lower than under +B conditions.

In summary, the elements may be grouped according to their observed cellular content as the concentration of B or Ge in the medium is varied, as follows. Ca, Fe, Zn, and Mn all have concentration curves which are inversely related to the amount of growth. K and P concentrations parallel the growth curve. The differences between the minimum and maximum concentrations were ca. 2-4 fold. B and Ge rise very slowly as the B or Ge concentration increases, and are always concentrated within the cell (relative to the concentration of the medium). Mg remained constant and Na was highly variable.

Subcellular Localization of Boron and Germanium

Three methods of separating cells into a cell wall fraction and a cytoplasmic fraction were used to determine the mineral balance, especially B and Ge, of the two fractions produced by the techniques. After each of the treatments the fractions were analyzed by ICP to determine the elemental distribution between the two fractions.

Protoplasts were produced from the cultured cells by enzymatic hydrolysis of the cell wall and separation of protoplasts from the solubilized wall was accomplished by centrifugation.

Alternatively, cells were ruptured by passing them through a French press, or cells were frozen in liquid nitrogen and ground with a mortar and pestle. The fractions were then separated by centrifugation.

Protoplasts

Protoplasts were produced by treatment with Driselase, a mixture of cell wall hydrolysing enzymes, which leaves intact and viable protoplasts. The protoplasts were stained with calcofluor white, a fluorescent cell wall stain (Herth and Schnepf, 1980; Hahne et al., 1983), and found to be free of stainable material. Each of the fractions recovered from the Driselase treatment, the spent enzyme, the washes, and the protoplasts were analyzed by ICP. The spent enzyme and washes were analyzed directly, while the protoplast fraction was ashed prior to analysis. A sample of cells used to generate the protoplasts was also ashed and analyzed as was the starting enzyme, to determine the input of B to the experiment. Table 5 lists the distribution in the various fractions. It was found that the majority (96-99%) of the B recovered was present in the solubilized wall fraction (spent enzyme plus protoplast washes). Very little boron (0.7-4.1%) was present in the protoplasts themselves. Cells from the various boron cell lines were all used as starting material for protoplast formation. As the boron concentration of the cell line changed from 0.01 mM, to 0.1 mM, to 1.0 mM, the total quantities of boron recovered increased (4.6, 13.8, 46.6 μg respectively). These values are comparable to the cellular concentration seen in the growth experiments except for the 1.0 mM cell

line, where the total quantity of boron seen here is considerably greater than previously measured. There was a rapid increase in the cellular boron concentration as the medium concentration increased. This, along with the general variability of the cell lines, may be the cause of the higher values seen here. At the same time, the total boron present in the protoplasts also increased, but only very slightly (0.1, 0.15, & 0.33 μg respectively). This contrasts with the distribution of the other elements analyzed. Calcium was found to be distributed ca. 54% in the protoplasts, and 46% in the solubilized wall fraction. The total quantity of calcium was quite variable, due to its presence as a component of the Driselase mixture. In some experiments the enzyme was dialyzed prior to starting the protoplast formation, but the enzyme doesn't function as well in the absence of calcium. Manganese distribution was also low in the protoplasts (11-15%). Potassium showed a somewhat high % in the solubilized wall fraction (65-70%), which may be explained by leakage out of the protoplasts. Phosphorus, and possibly iron also appeared to leak out of the protoplasts. Leakage was assumed when the quantity recovered in the first wash was greater than what was present in the spent enzyme. It was also noted that the second wash still contained detectable amounts of most of the elements.

Protoplasts were formed from a germanium cell line in the same manner as from boron cell lines. The most significant difference was that the solubilized wall fraction contained a significantly lower proportion of Ge than was observed in the case of B distribution. Approximately 60% of the germanium was found in the solubilized wall fraction, as opposed to >95% in the boron cell lines. Although the percent distribution is

lower for germanate than for borate, the total quantity of germanate in the wall fraction was slightly higher (ca. 1.0 μmoles/g Ge vs 0.8 μmoles/g B).

Protoplasts were produces and then transferred to protoplast growth medium, at two boron levels (9 μM and 18 μM), and at three protoplast concentrations (0.7, 1.8, & 3.3 X 10^5 cells/ml). The initial protoplasts were stained with calcofluor white and found to be free of stainable material. Some cell division and growth had begun within 24 hrs. After 4 days there was considerable growth, cells were in 4-20 cell clumps and there was strong staining by calcofluor. Many of the cells, however were shriveled or plasmolyzed and didn't stain. Initial growth was better at the higher inoculum levels and at the lower boron level. By 6 days there was considerable growth and a few drops of fresh growth medium were added to each culture. As time progressed (8-15 days), there was less discernable difference between the boron levels and inoculum levels, as all of the treatments showed significant growth. No further experiments were performed that looked at the suspension culturing of the regenerated protoplasts.

French Pressure Cell

Cells were passed through a French press to rupture them, and the exudate was separated by centrifugation and analyzed by ICP to determine the distribution. Observation of the exudate under a low power microscope (100X) showed that all of the cells were ruptured. The exudate was fractionated by high speed centrifugation for 10 min. into a 10,000xg pellet and the associated supernatant. At this speed only the wall fragments (and

any remaining whole cells) would be expected to sediment out. After sitting for 1-2 days the supernatant had acquired a visible precipitate, which was removed by low speed centrifugation and analyzed separately. Table 6 shows the recovery and the distribution of material between the supernatant, and the two pellets. The total recovery is low due to whole cells that remained within the press and tubing. It is consistent for most of the elements. The results presented here are very similar to those found in the protoplast experiments. Boron was found in the pellet at 84-96%. This is somewhat lower than the 95-100% distribution in the protoplast experiments, but still much higher than most of the other elements analyzed. As observed in the protoplast work, the Ge distribution was lower in the pellet fraction (74%) than B. In contrast to the protoplast data, most of the other elements were distributed at fairly high percentages in the pellet. Exceptions were K and P which were at 15-20% and 35-50% respectively. Mn was present in high proportion in the pellet (66-90%), as was observed in the protoplast analyses. Mg is approximately evenly distributed between the two fractions, ranging from 40-55% in the pellet. The others (Ca, Fe, Zn, and Na) all are present in high proportion in the pellet, ranging from 60-93%.

Mortar and Pestle Grinding

Grinding with liquid nitrogen in a mortar and pestle, breaks cells and yields fractions very similar to the French press technique discussed above. The results (Table 7) show that this is true. Recovery of material was much higher with this method than was possible with the French press. Boron is present in the pellet at a

higher percentage (88-98%) than any of the other elements. The boron levels present in the supernatant and the washes were very close to or below the detection limits for B. Potassium and phosphate are present at low percentages in the pellet (6-8% and 26-32% respectively), as was seen in the French press data. Mg was somewhat lower in the pellet (38-44%) than seen in the French press data. The others (Ca, Fe, and Zn) all are similar to the French press, ranging from 53-75% in the pellet. Na showed a much lower level in the pellet (10-22%) than was seen in the French press data.

All of these techniques show that the distribution of boron is considerably higher in the wall fraction than in the cytoplasmic fraction.

CONCLUSIONS

The use of cell culture as a model system to study the effect of boron concentrations on growth has been shown. Suspension cultured carrot cells show boron deficiency and toxicity effects comparable to those of whole plants. Studies of growth as a function of boron concentration in the medium, showed good growth through a range of ca. four orders of magnitude. Studies of the survival of cells cultured at very low and very high (deficient and toxic) concentrations showed that while cells don't grow at low concentrations, they do remain viable. High concentrations are toxic and kill the cells. Cells were also shown to be viable after culturing in deficient calcium.

Substitution of germanium for boron proved to be possible. Cell culture lines were established in which 0.43 mM germanium substituted for the boron requirement (typically 0.01 mM). The Ge content of the Ge cell lines was ca. twice the B content of the B cell lines on a molar basis. The Ge cell lines were maintained for over two years by continuous subculturing. Studies of growth as a function of Ge concentration showed comparable results to those seen for B concentration. One distinct difference was that the concentration range through which good growth was observed was considerably narrower for Ge (ca. two orders of magnitude) than for B.

Our finding that germanium can replace boron, suggests that the cellular function of boron may involve rather non-specific interactions. To further elucidate the role of boron, we fractionated cells into cell wall fragments and protoplasm, and analyzed for boron (or germanium). Cell walls were solubilized by enzymatic

digestion, which left an intact and viable protoplast. These two fractions were then analyzed by ICP to determine their boron contents. It was found that ca. 96% of the boron was present in the solubilized wall fraction. Ge showed a lower percentage distribution (ca. 74%) in the wall fraction. However, the molar Ge content of the wall fraction was nearly identical to the B content of the B cell lines (ca. 1.0 μmoles/g vs 0.8 μmoles/g respectively), due to the higher total Ge content.

Two other methods of fractionation were also used to corroborate the findings from the protoplast experiments. Grinding cells in a mortar and pestle under liquid nitrogen or rupturing cells in a French press, followed by centrifugation were used to separate the cells into protoplasmic and wall fractions. The ICP was then used to determine which fraction contained the B (Ge). These methods yielded the same high percentages in the wall fraction as enzymatic treatment.

Our finding that both B and Ge are localized primarily in the cell wall is consistent with the cell wall cross-link hypothesis.

Carrot suspension culture was also used to study the effect of varying the B (Ge) concentration of the medium on the mineral composition (B, Ge, Ca, Mg, Fe, Zn, Mn, K, Na, and P) of the cells. Boron and germanium (as μmoles/g dry wt) both showed remarkably little change (ca. 50 fold) even though the concentration of the medium varied over six orders of magnitude. Throughout the entire range tested, boron and germanium are accumulating within the cell. Other elements showed interesting relationships, which are reported, but were not further

investigated. Cellular concentrations which were correlated to growth were observed. Strong inverse relations for Ca and Zn, and weakly by Fe and Mn were seen. Strong parallel relations for K and P, and possibly Mg were also observed.

Based on the results reported here and in combination with the reports in the literature, we feel that a much stronger case for boron as a structural component of the cell wall can now be made.

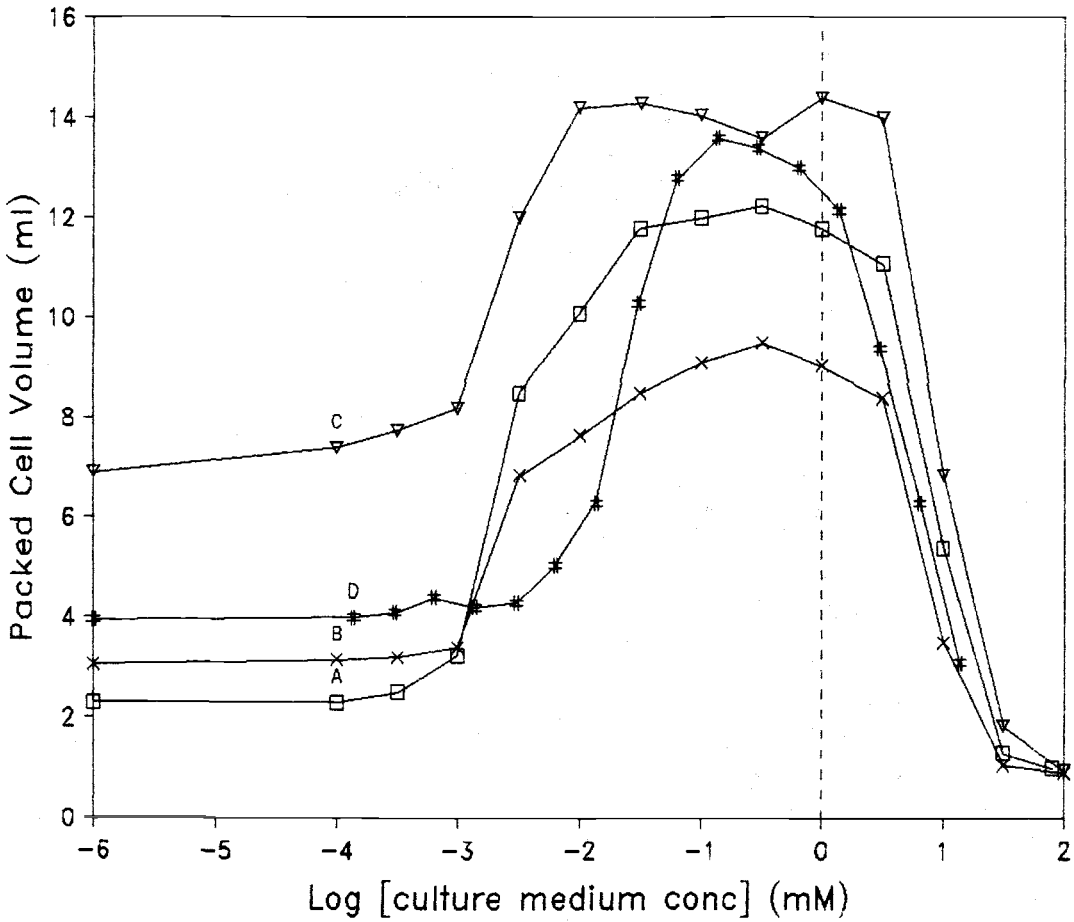


Figure 1 Growth vs Boron or Germanium Concentration of the Culture Medium

Packed cell volume as a function of borate (germanate) concentration of the culture medium. Three borate cell lines grown at A) 0.01 mM, B) 0.1 mM, and C) 1.0 mM borate, and D) a germanate cell line grown at 0.43 mM germanate are shown. 1 ml packed cells from the indicated cell line was well washed with 0B (0Ge) medium and transferred to the indicated concentration. Borate cell lines were harvested at 7 days, and the germanate cell line was harvested at 10 days.

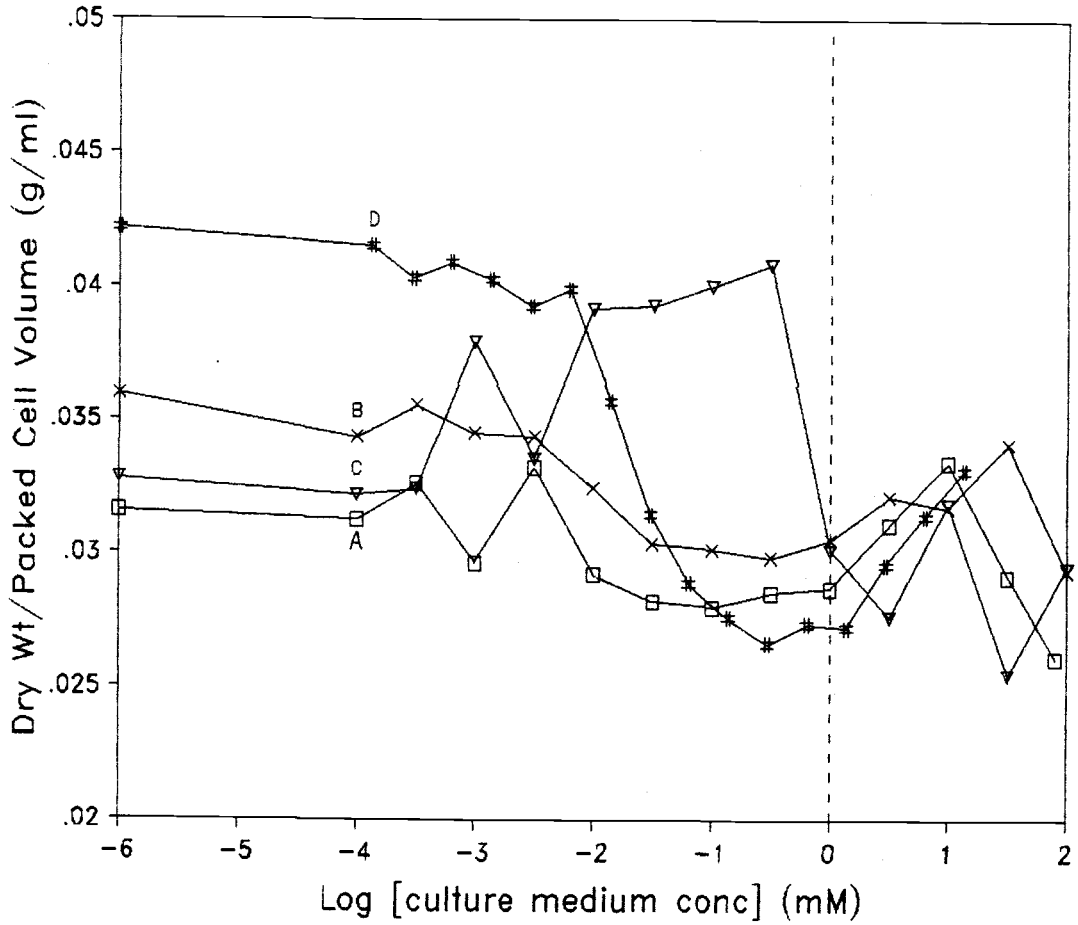


Figure 2 Density (Dry wt/PCV) vs Boron or Germanium Concentration of the Culture Medium

Density (in g dry wt/ml PCV) of cells as the concentration of borate or germanate in the medium was varied. Three borate cell lines, grown at A) 0.01 mM, B) 0.1 mM, and C) 1.0mM borate, and D) a germanate cell line grown at 0.43 mM germanate are shown. Conditions the same as in figure 1.

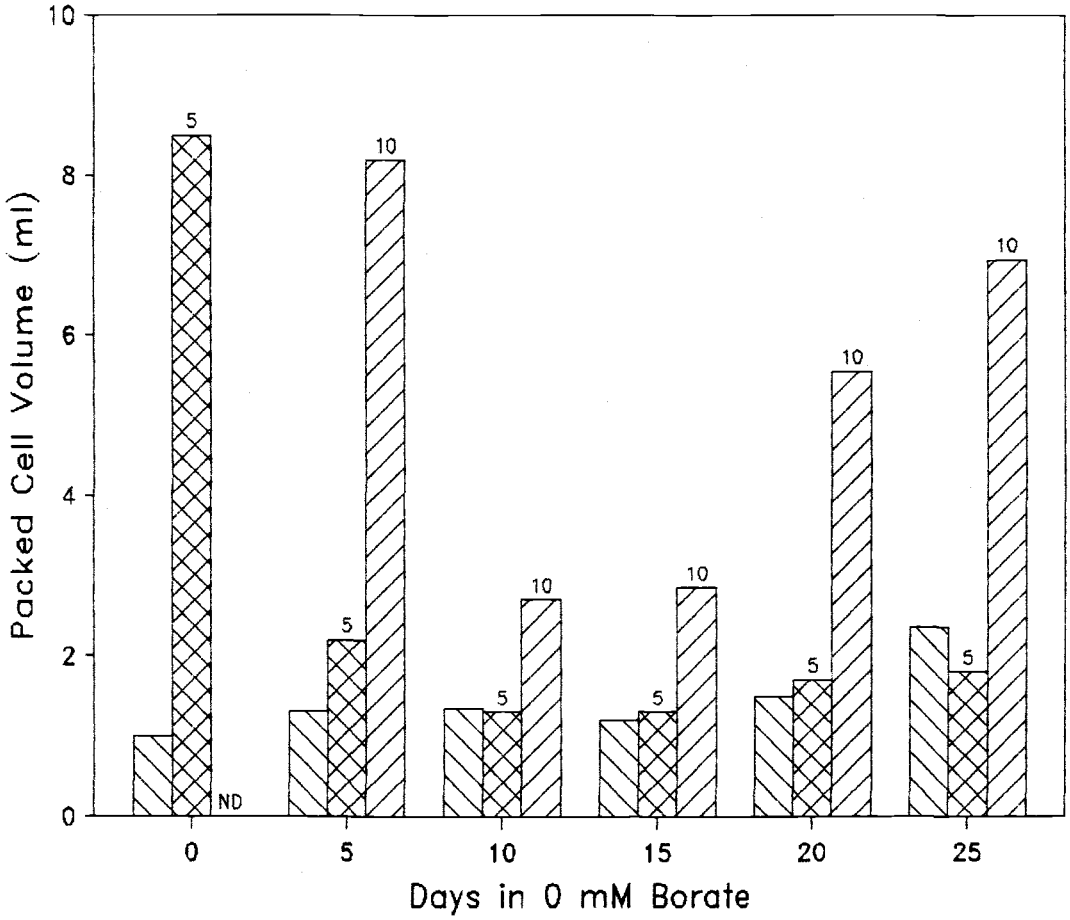


Figure 3 Cell Growth and Survival in Deficient Boron Medium

Cells were grown for the indicated times in 0 mM borate medium, and growth measured ( ). 1 ml packed cells was then transferred to normal (0.01 mM) borate. Growth (as packed cell volume) was then measured after 5 ( ) and 10 ( ) days. ND-not determined.

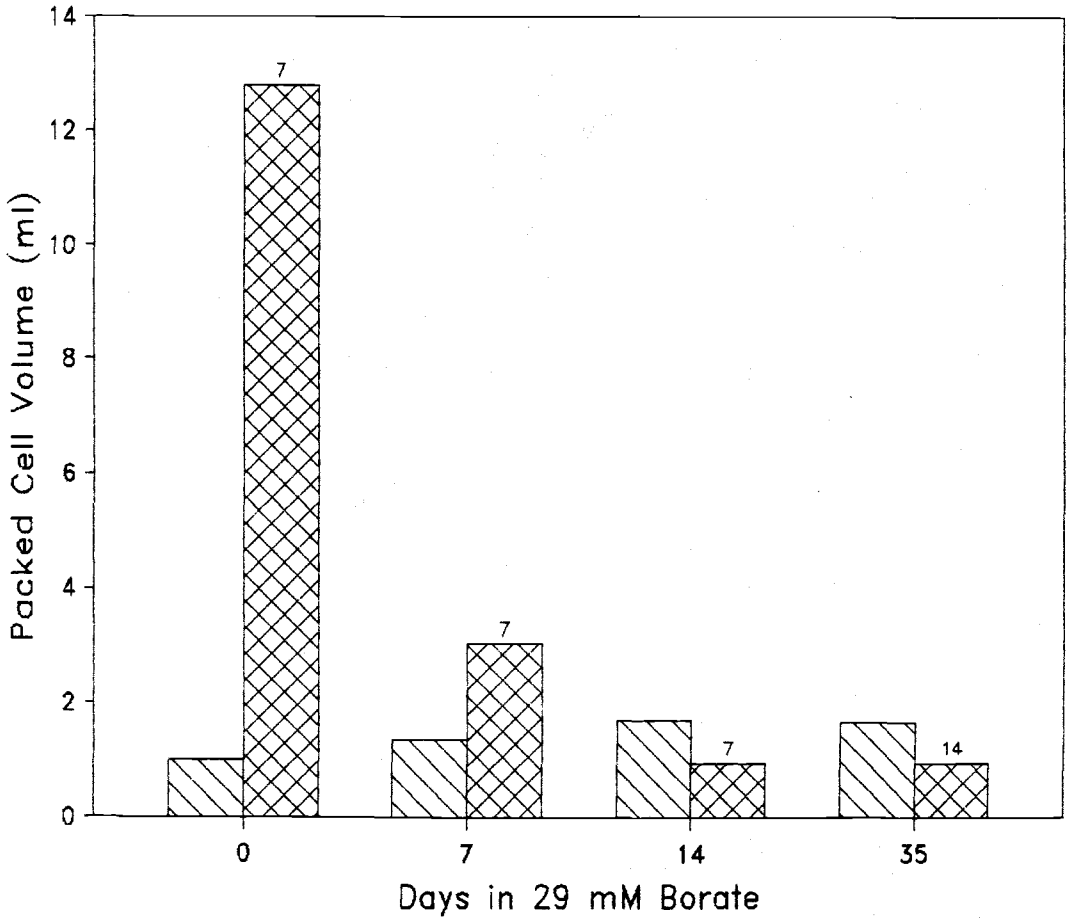


Figure 4 Cell Growth and Survival in Toxic Boron Medium

Cells were grown for the indicated times in 29 mM borate medium, and growth measured (▨). 1 ml of packed cells was then transferred to normal (0.01 mM) borate. Growth (as packed cell volume) was then measured after 7 or 14 (▩) days.

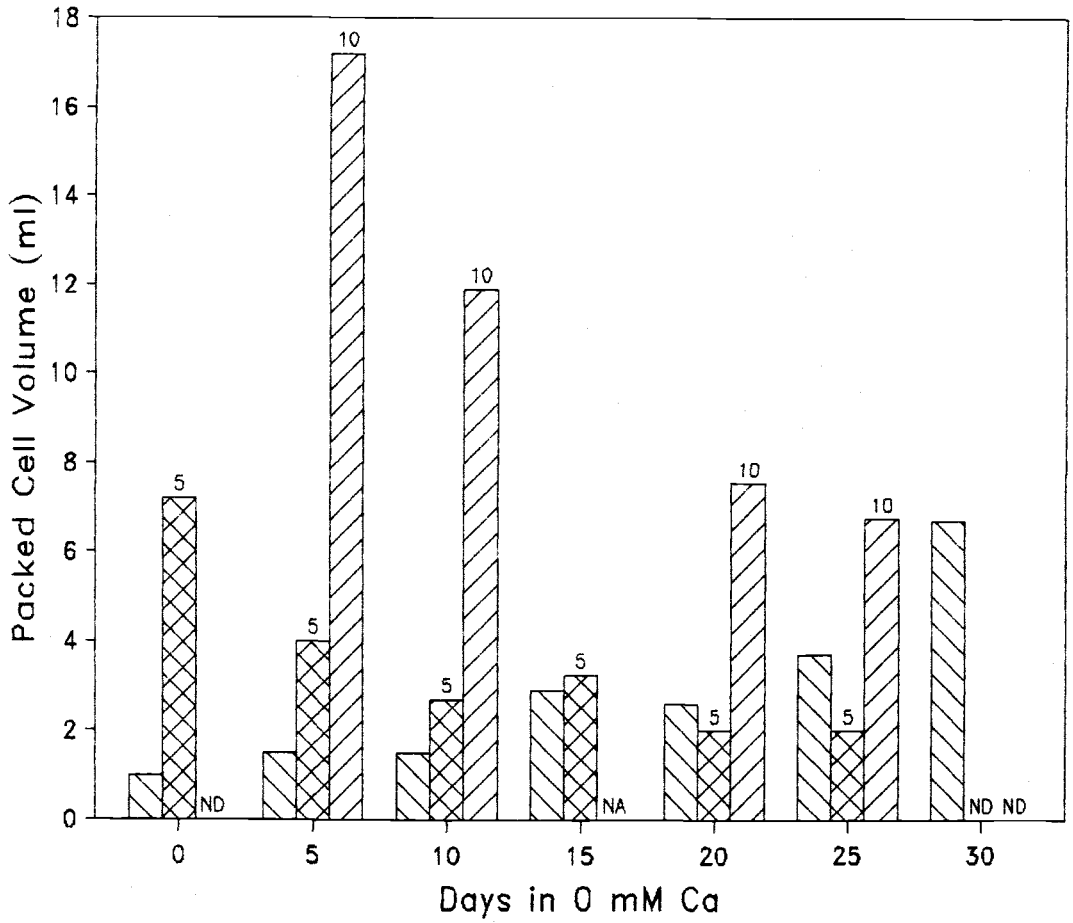


Figure 5 Effect of Deficient Calcium on Cell Growth and Survival.

Cells were grown for the indicated times in 0 mM Ca medium, and growth measured (▨) 1 ml packed cells was then transferred to normal (3.0 mM) Ca. Growth (as packed cell volume) was then measured after 5 (▩) and 10 (▨) days. ND-not determined.

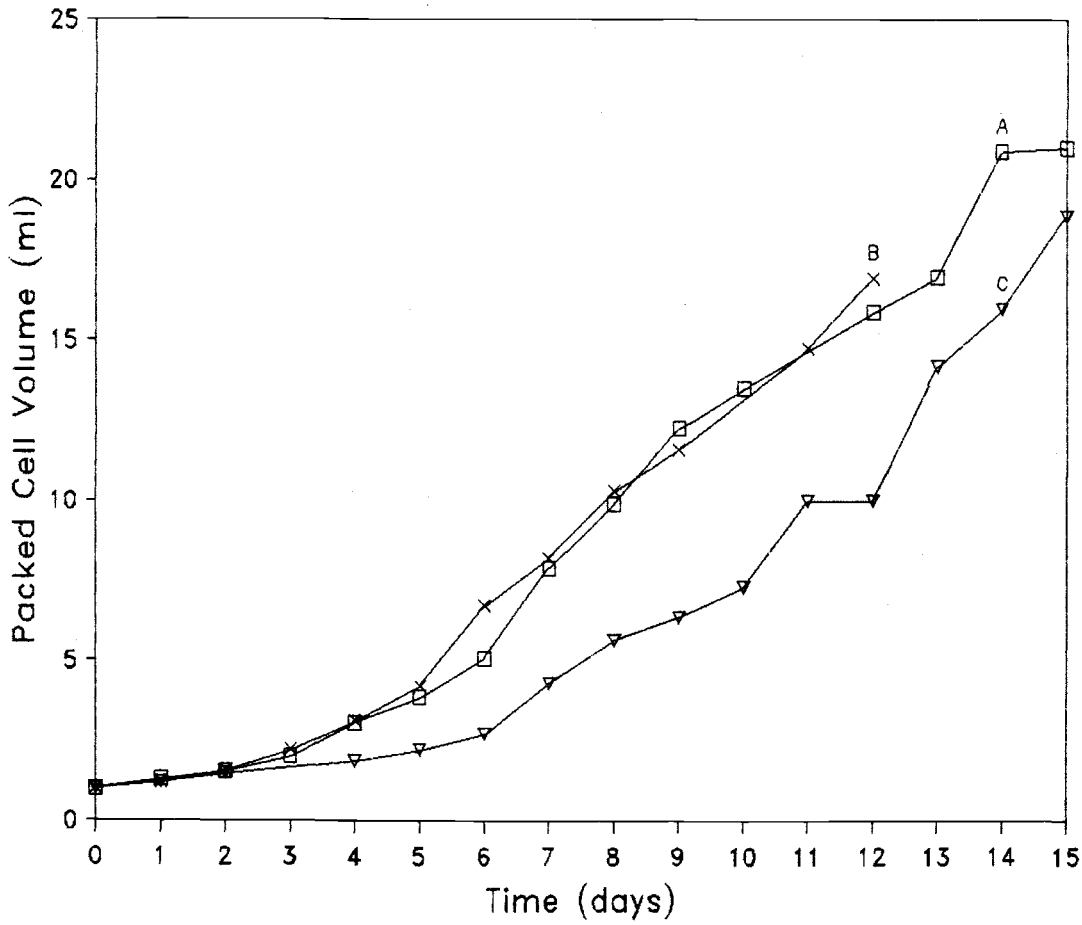


Figure 6 Growth (Packed Cell Volume) of Cells as a Function of Time

Growth (PCV) for two borate cell lines, A) 0.01 mM, and B) 0.1 mM, and C) a 0.43 mM germanate cell line are shown. 1 ml packed cells transferred to each of 15 flasks of fresh medium and total cell growth measured at the indicated times.

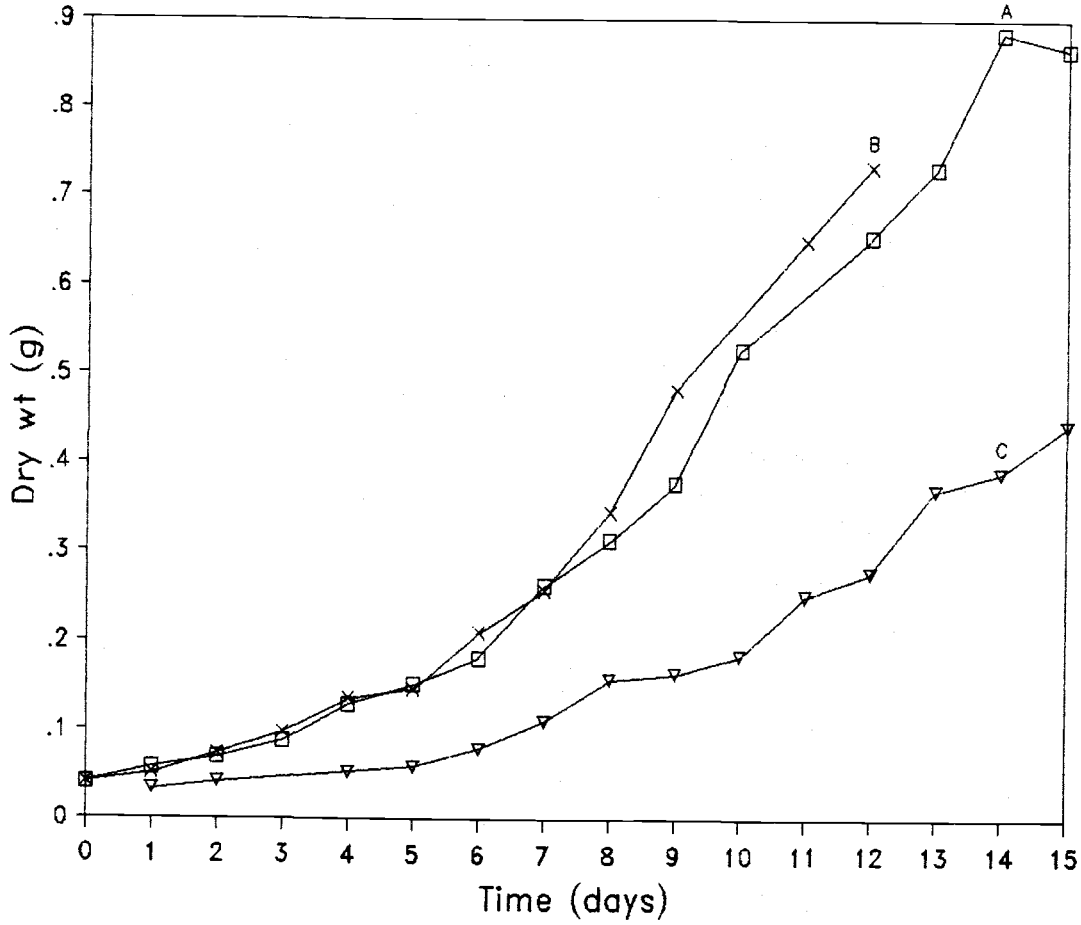


Figure 7 Growth (Dry wt) of Cells as a Function of Time

Growth (Dry wt) for two borate cell lines, A) 0.01 mM, and B) 0.1 mM, and C) a 0.43 mM germanate cell line are shown. Same cultures as figure 6.

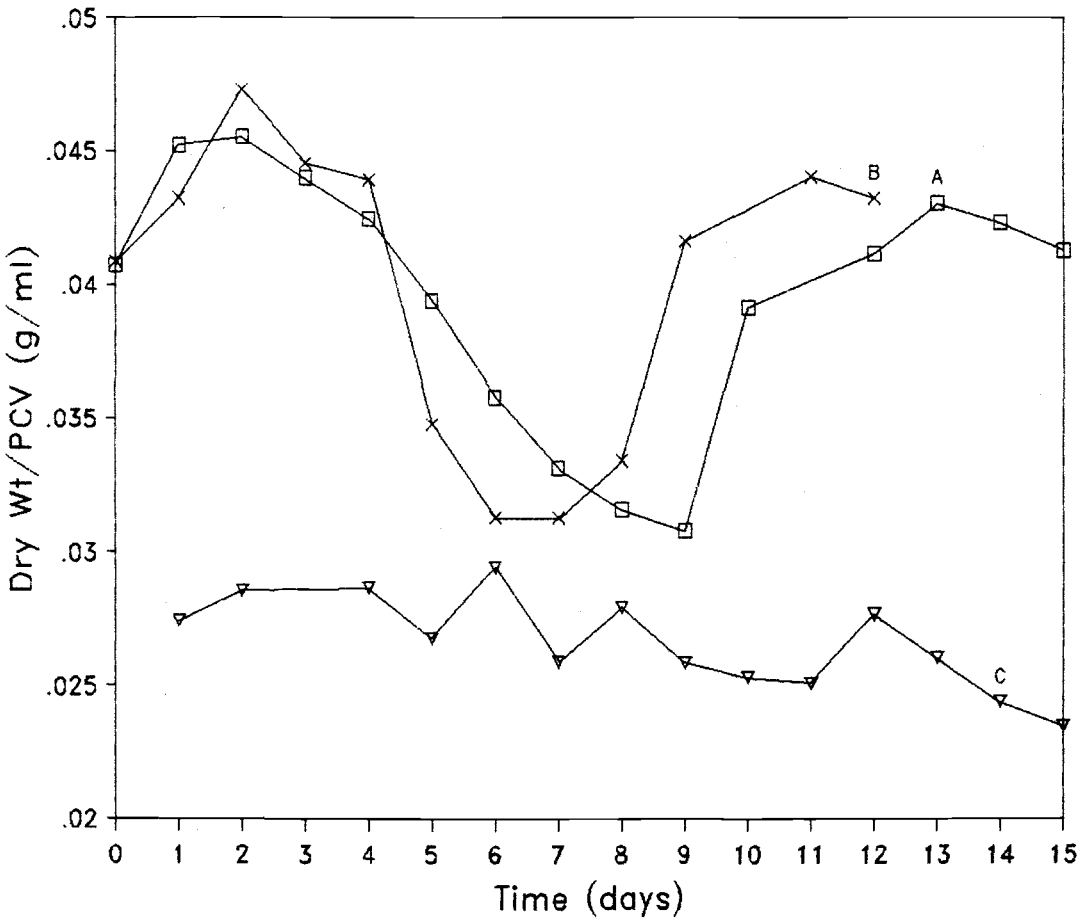


Figure 8 Density (Dry wt/packed cell volume) as a Function of Time

Density (dry wt/PCV) for two borate cell lines, A) 0.01 mM, and B) 0.1 mM, and C) 0.43 mM germanate cell line are shown. Same cultures as figure 6 and 7.

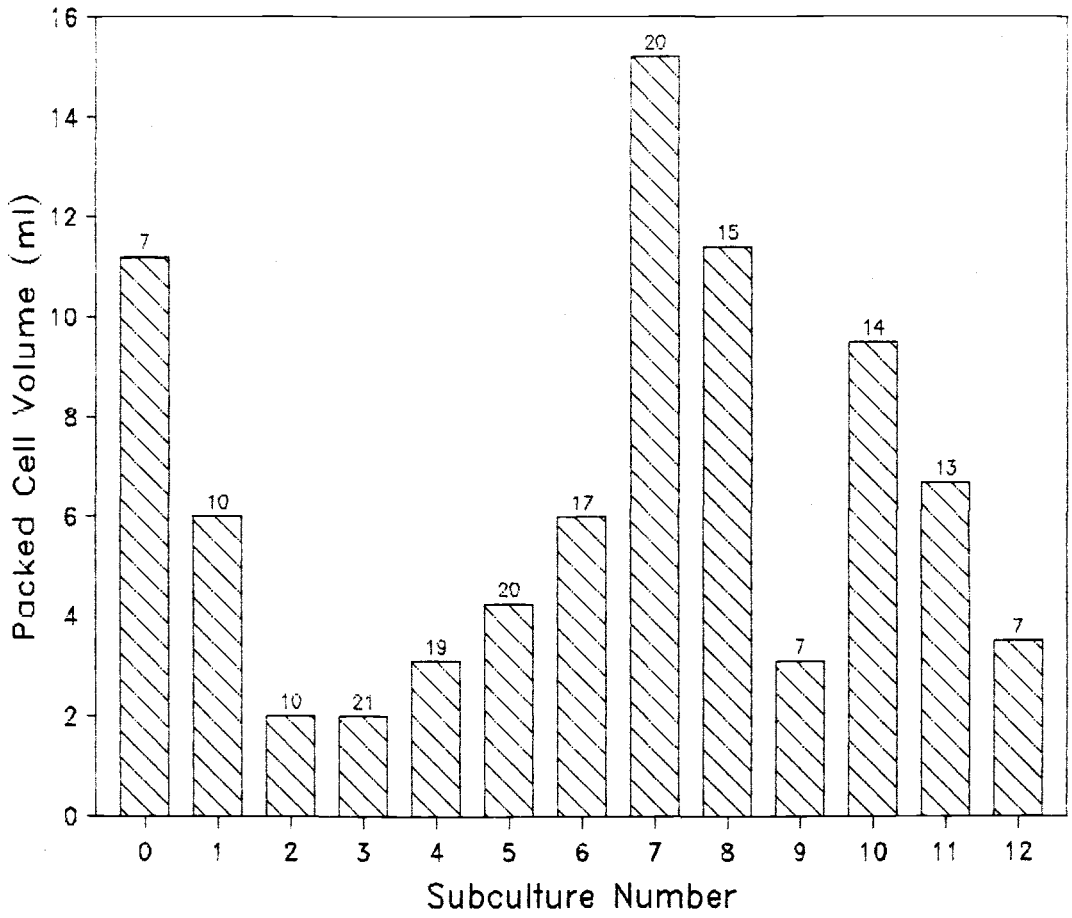


Figure 9 Acclimation of Cultured Carrot Cells to Growth in Germanium

Cell growth, expressed as packed cell volume per culture, as cells were transferred from 0.01 mM B to 0.43 mM Ge. Subculture 0 was the original starting culture grown in 0.01 mM borate. Numbers above the bars are time (in days) that each culture was grown prior to measurement and subculturing. Each subculture involved the transfer of 1 ml of packed cells from the previous culture to fresh medium.

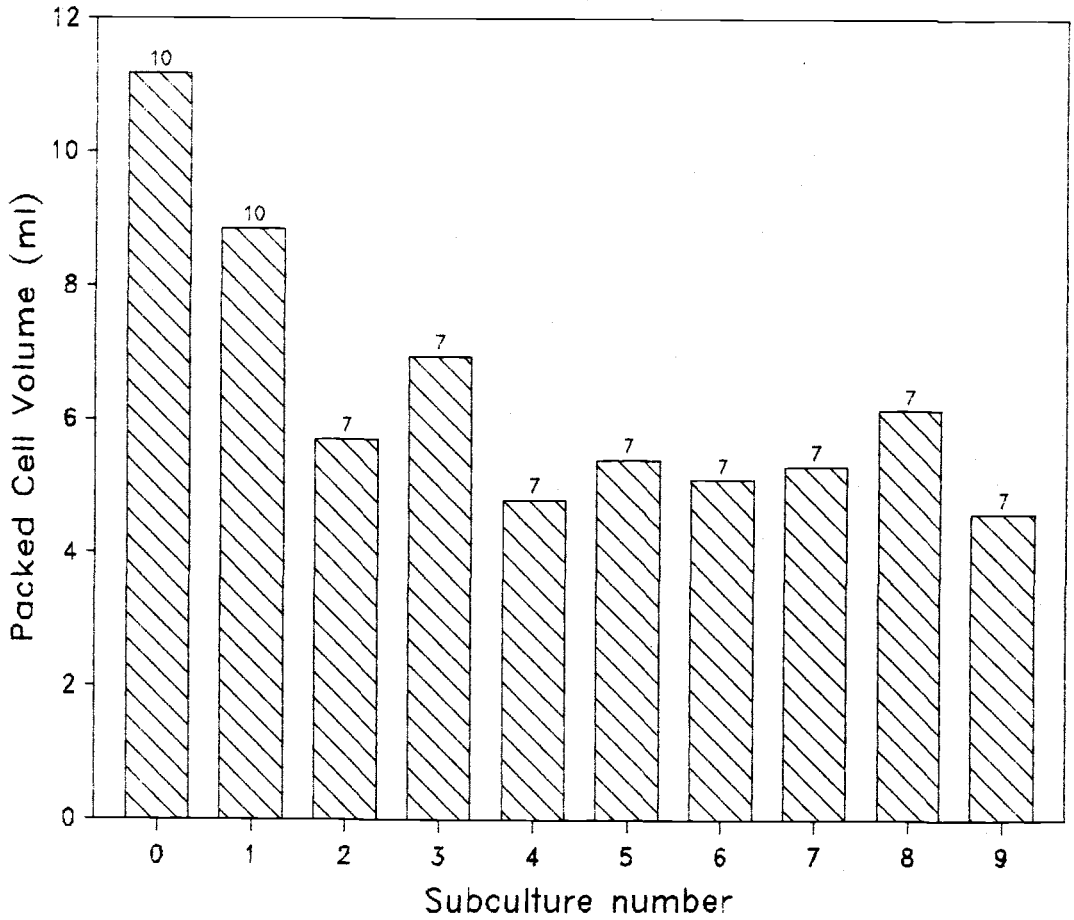


Figure 10 Reacclimation of a Germanium Cell Line to Growth in Boron

Cell growth, expressed as packed cell volume per culture, as cells from the Ge cell line were transferred back to 0.01 mM borate medium. Subculture 0 was the original starting culture grown in 0.43 mM germanate. Numbers above the bars are time (in days) that each culture was grown prior to measurement and subculturing. Each subculture involved the transfer of 1 ml of packed cells to fresh medium.

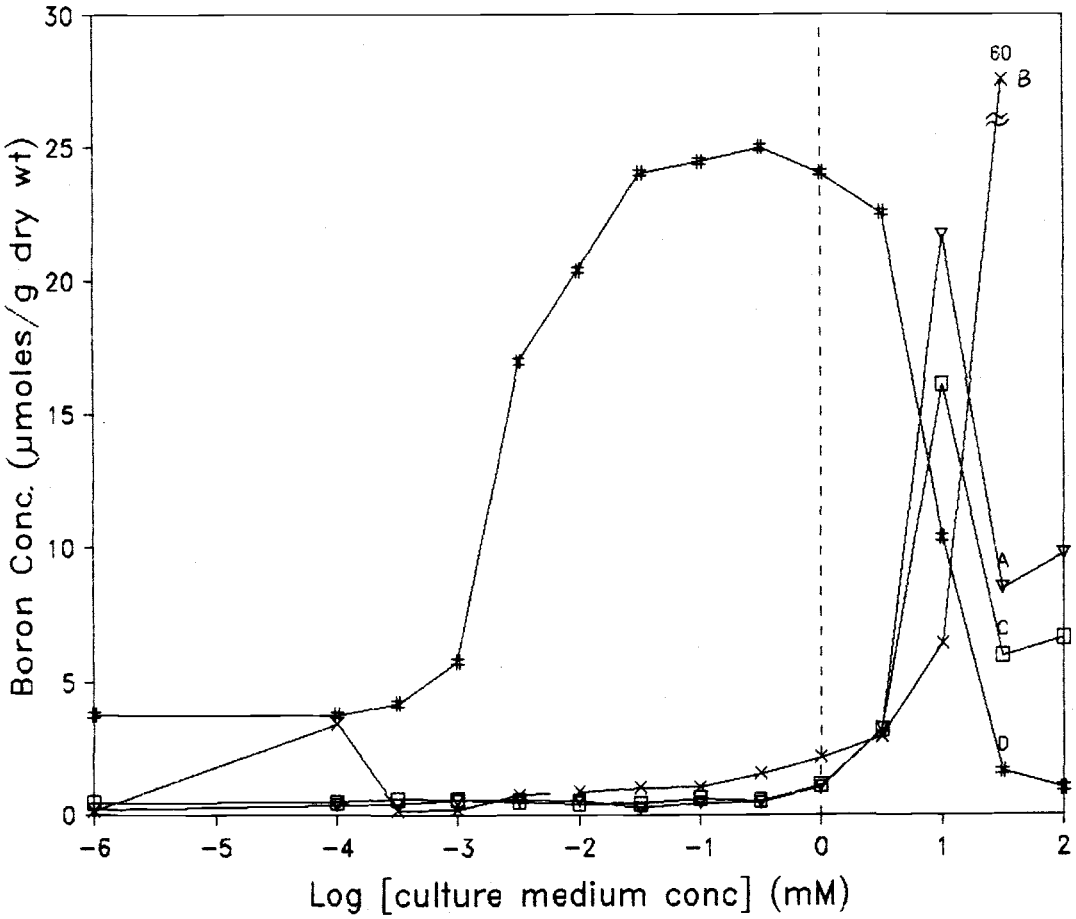


Figure 11 Boron Content of Cells vs the Boron Concentration of the Culture Medium

Boron content in µmoles/g dry wt of the cells. Results from all three boron cell lines A) 0.01 mM, B) 0.1 mM and C) 1.0 mM are shown. A single representative growth curve D) (same curve as A) in figure 1) is shown for reference. Same cells as shown in figures 1 and 2.

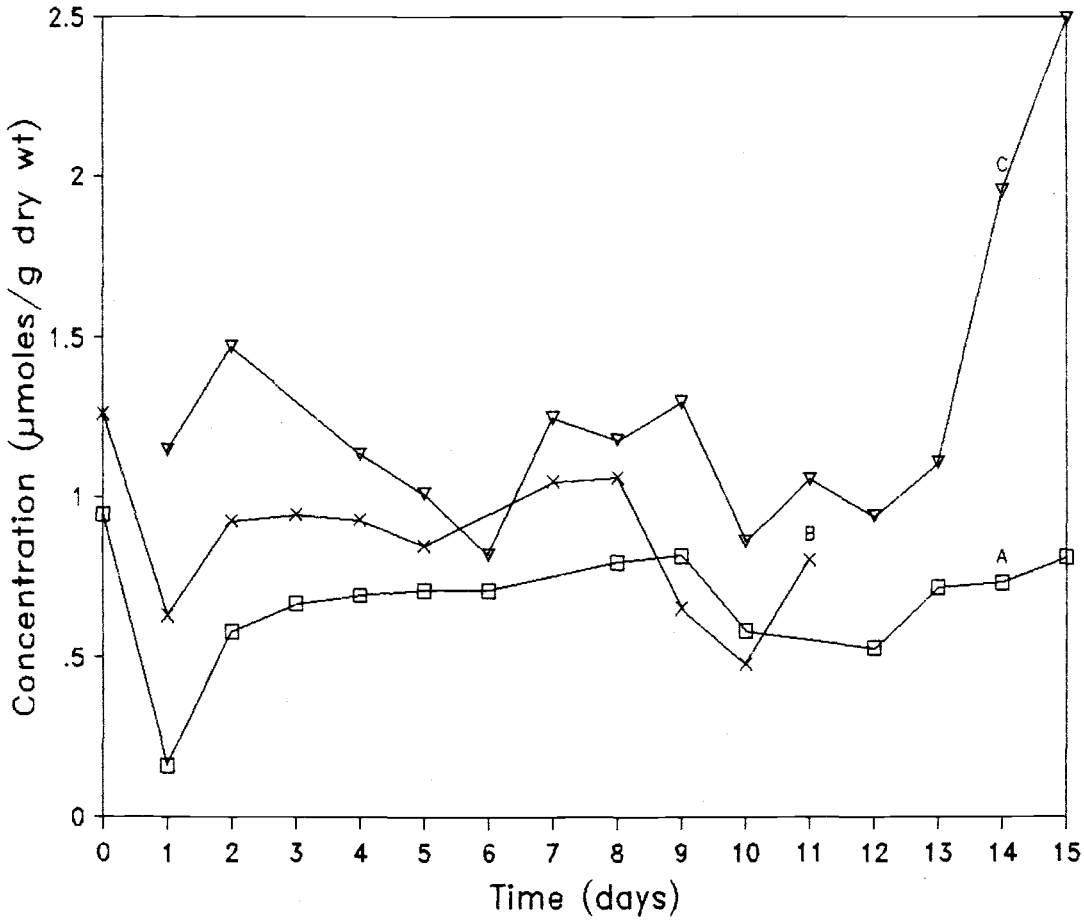


Figure 12 Boron and Germanium Content of Cells vs Time

Boron and germanium content in µmoles/g dry wt of the cells. Results from two boron cell lines, A) 0.01 mM, and B) 0.1 mM and C) a 0.43 mM germanate cell line are shown. Same cells as shown in figures 6, 7, & 8.

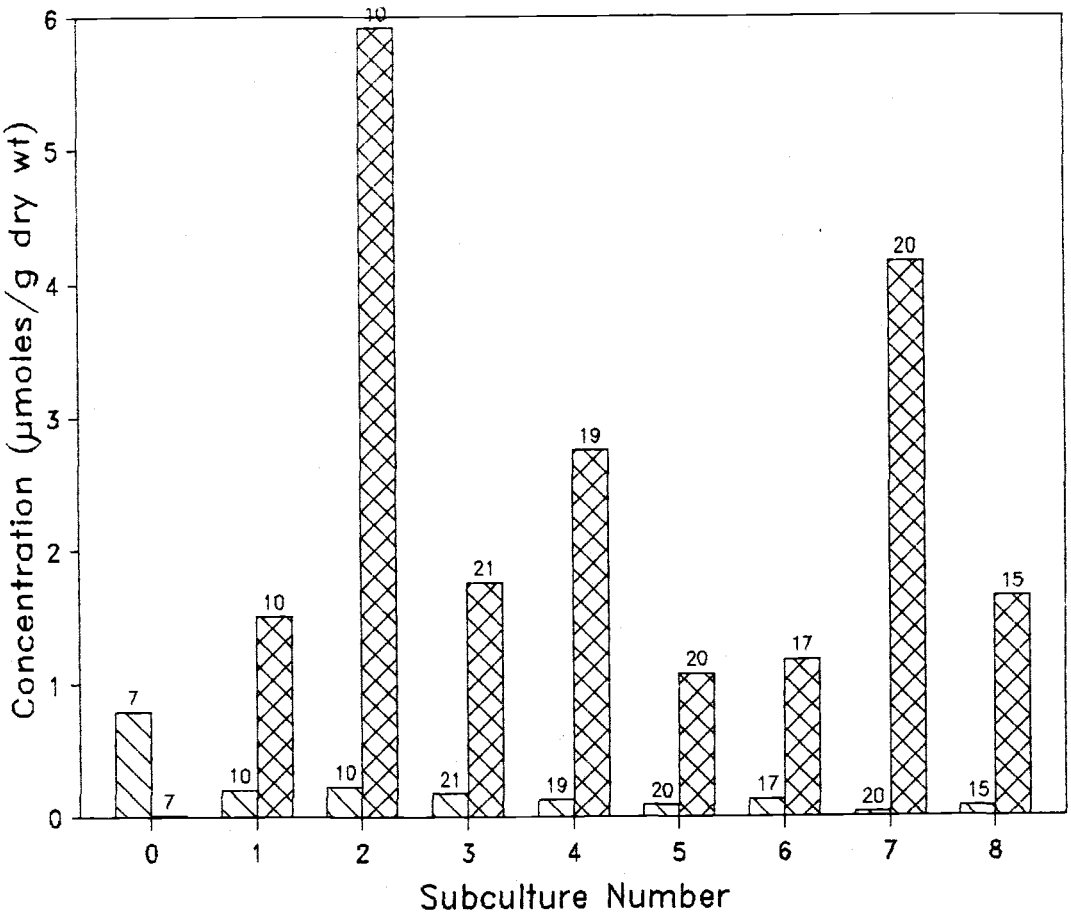


Figure 13 Cellular Content of Germanium and Boron as Cells are Transferred from Boron to Germanium Culture Medium

Germanium (▩) and boron (▨) content in µmoles/g dry wt as cells are acclimated to germanium medium. Numbers above the bars are the time (in days) that each culture was grown prior to measurement. Refer to figure 9 for subculturing information.

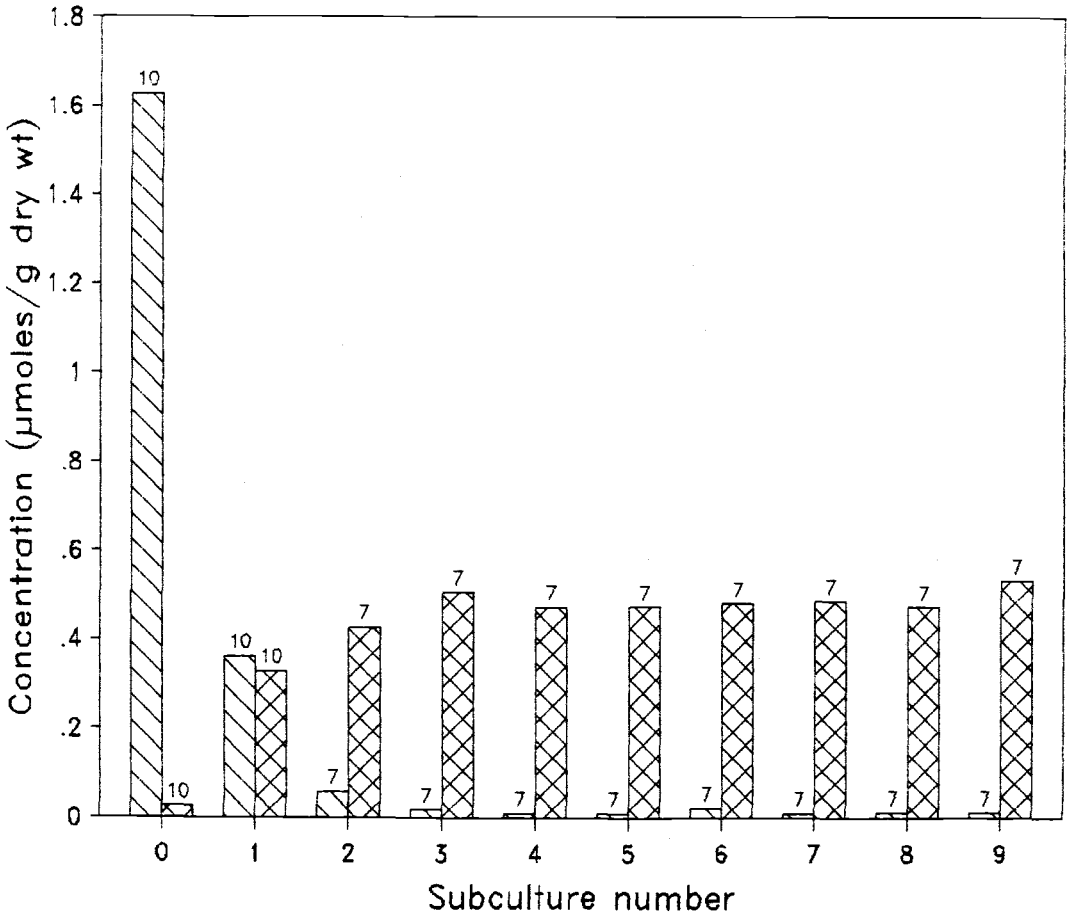


Figure 14 Cellular Content of Germanium and Boron as Cells are Reacclimated from Germanium to Boron Culture Medium

Germanium (▨) and boron (▩) content in µmoles/g dry wt as cells are reacclimated to boron medium. Numbers above the bars are the time (in days) that each culture was grown prior to measurement. Refer to figure 10 for subculturing information.

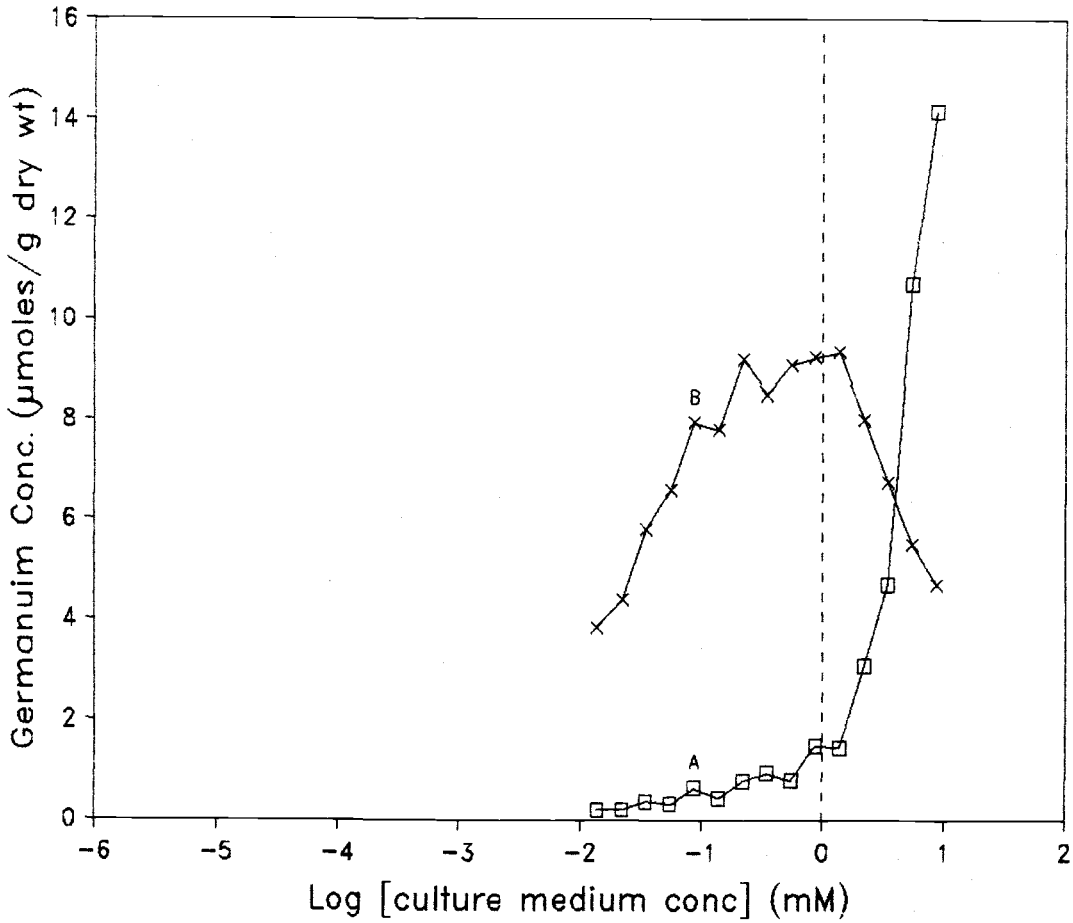


Figure 15 Germanium Content of Cells vs the Germanium Concentration of the Culture Medium

A) Germanium content in µmoles/g dry wt of the cells. A representative growth curve B) is shown for reference.

Table 1 Nutrient medium for culture of carrot cells.

The following stock solutions were added to ca. 900 ml water, the pH adjusted (with 2N KOH) to 5.8-6.0. 40 g sucrose added and the final volume adjusted to 1000 ml.

Nutrient Added	Concentrated Stock Solutions	Final Nutrient Solution (ml stock/L)
Macro Nutrient 1.		10
KNO_3	19. g/100 ml	
NH_4NO_3	12. "	
$MgSO_4 \cdot 7H_2O$	3.7 "	
KH_2PO_4	3.4 "	
Macro Nutrient 2.		10
$CaCl_2$	4.4 g/100 ml	
Minor Elements - without Boron.		.1
$MnSO_4 \cdot H_2O$	169. mg/100 ml	
MoO_3	1.82 "	
$CuSO_4 \cdot 5H_2O$	.25 "	
$CoCl_2 \cdot 6H_2O$	.25 "	
Boric Acid.		(varied)[a]
H_3BO_3	62. mg/100 ml	
Germanium dioxide		(varied)[b]
GeO_2 (soluble form)	450. mg/100 ml	
Organics:		
Naphthalene Acetic Acid	10. mg/100 ml	10
Kinetin	4. mg/100 ml	.0.5
Niacin (Nicotinic Acid)	25. mg/50 ml.	.1
Thiamine·HCl	25. mg/50 ml.	.1
Pyridoxine·HCl	25. mg/50 ml.	.1
Zn EDTA	375. mg/50 ml.	.2
Fe EDTA.		.5
$FeSO_4 \cdot 7H_2O$	557. mg/100 ml	
Na_2 EDTA	745. mg/100 ml	

[a] 0.1 ml of stock in 100 ml yields 0.01 mM
[b] 1.0 ml of stock in 100 ml yields 0.43 mM

Table 2 ICP Detection Limits, Recoveries, and Standards.

Instrument detection limits and minimum sample values for all of the elements are shown. Our analyzed values and the accepted values of NBS Standard Reference Material #1571 are listed, as are values of our own standard carrot suspension cells (SCM) that were analyzed with each ashing. Also listed is the % recovery for most elements from spiked samples of SCM.

			Concentration (μg/g dry wt)									
			Ca	Mg	B	Fe	Zn	Mn	K	Na	P	Ge
Detection Limits of the ICP (μg/ml)[a]			0.01	0.03	0.008	0.005	0.005	0.002	0.15	0.03	0.05	0.005
	Replicates											
Standard Carrot Material (SCM)	42	Min->	1718	1393	1.98	252	164	9.49	29961	191	10147	0.09
		Max->	2029	1582	7.08	289	197	11.12	33555	891	12023	2.16
		Ave->	1905	1490	3.99	266	180	10.44	31219	430	11164	0.51
Spiked SCM[b]		Recovery %->	108.6	103.7	98.2	112.3	98.3	107.8				97.4
SRM 1571[c]	6	Min->	18604	5543	28.01	152	21	59.18	11442	233	1819	0
		Max->	19988	5992	33.18	194	24	81.12	13636	369	2140	4
		Ave->	19131	5689	31.21	178	22	74.00	12130	313	1923	2
NBS published values			20900	6200	33	300	25	91	14700	82	2100	-
		Recovery %->	91.5	91.8	94.6	59.3	88.1	81.5	82.5	382.2	91.6	-

[a] Detection limit of the analyte
[b] SCM spiked with known concentration, analyzed and % recovery determined.
[c] National Bureau of Standards, Standard Reference Material #1571, orchard leaves.

Table 3 Protoplast Growth Medium

The following components were added to ca. 90 ml water, the pH adjusted to 5.7 with 2N KOH and the final volume adjusted to 100 ml.

D-sorbitol	4.55 g
sucrose	3.40 g
myo-inositol	10 mg
D-glucose	18 mg
D-xylose	15 mg
L-arabinose	15 mg
isopentenyladenine (IPA) (1 µl of 1 mg/ml IPA in dimethyl sulfoxide)	0.001 mg
macro nutrient 1 (see Table 1)	1 ml
macro nutrient 2 (see Table 1)	1 ml
minor elements (see Table 1)	100 µl
Fe EDTA (see Table 1)	0.5 ml
Zn EDTA (see Table 1)	200 µl
boric acid (see Table 1)	(varied)[a]
pyridoxine·HCl (see Table 1)	100 µl
niacin (see Table 1)	100 µl
thiamine·HCl (see Table 1)	100 µl

[a] 0.1 ml of stock in 100 ml yields 0.01 mM

Table 4 Elemental composition as a function of varying boron concentration in the growth medium. Growth (PCV) is shown for reference. Cells harvested at 7 days for B and 10 days for Ge.

[Boron] mM[a]	PVC ml	Concentration of Element in Cells (µg/g dry wt) Ca	Mg	B	Fe	Zn	Mn	K	Na	P	Ge
0.0000	2.3	6406	1338	1.58	1247	317	29.2	10392	218	8687	
0.0001	2.3	6567	1271	2.57	962	327	39.1	7944	205	7980	
0.0003	2.5	6264	1279	4.38	1439	365	27.7	11162	152	8524	
0.0010	3.3	6261	1551	3.31	1274	596	29.7	16270	883	9496	
0.0032	8.5	2488	1272	11.95	482	183	15.3	24794	196	11263	
0.0100	10.1	2707	1462	9.24	377	164	13.0	23848	186	11968	
0.0316	11.8	2589	1353	9.99	350	169	11.4	27513	247	11659	
0.1000	12.0	2719	1431	12.30	350	184	12.4	29892	232	12517	
0.3160	12.3	2732	1348	18.91	353	177	13.6	28840	233	11789	
1.00	11.8	2759	1352	38.86	350	184	15.3	29360	198	12082	
3.16	11.1	2411	1432	89.52	312	188	11.1	32101	368	11982	
10.00	5.4	2419	1400	282.55	623	355	16.5	31160	503	11476	
31.60	1.3	3196	885	213.36	163	320	13.8	7110	240	7688	
80.00	1.0										
[Germanium] mM[b]	PCV ml										
0.0000	4.0	3886	1579		88	335	22.6	9597	86	9654	3.01
0.0001	4.0	3882	1579		95	310	22.3	9686	237	9449	3.27
0.0003	4.1	4000	1598		85	328	22.9	9275	83	9858	3.47
0.0006	4.4	4071	1609		105	330	23.0	9445	247	9775	4.39
0.0014	4.2	4172	1666		102	332	23.5	11126	88	10206	5.35
0.0030	4.3	3987	1654		126	331	22.7	7261	66	9745	6.29
0.0063	5.1	3956	1684		96	321	22.8	10084	80	10446	7.59
0.0138	6.3	3419	1680		115	283	19.0	10993	51	10944	9.66
0.0303	10.3	3191	1784		236	246	16.2	17545	128	12224	21.25
0.0634	12.8	3441	1918		211	252	16.4	19285	292	12673	28.08
0.1377	13.6	3308	1987		332	242	15.9	24369	280	13359	78.08
0.2961	13.4	3356	1998		260	259	16.0	23183	188	13225	98.46
0.6391	13.0	3277	1996		262	264	15.6	22427	170	12969	155.50
1.3774	12.2	2837	1818		241	229	14.6	19112	99	12773	235.04
2.9614	9.4	2717	1827		276	244	14.3	17004	107	12493	370.49
6.3912	6.3	2876	1905		236	319	15.2	14644	271	11889	480.65
13.7741	3.1	3068	1891		294	397	18.3	10162	215	10527	669.45

[a] Boron concentration in the culture medium
[b] Germanium concentration in the culture medium

Table 5 Elemental composition of cell fractions from protoplast formation.

Protoplasts were formed by treatment with Driselase. The concentration of all of the elements in the protoplast and solubilized wall fractions from the 0.1 mM boron cell line are listed.

	Total μg recovered from 1.5 ml Cells[a]								
Sample	Ca	Mg	B	Fe	Zn	Mn	K	Na	P
Protoplasts	723	670	0.15	145.7	77.9	1.90	12710	4246	6788
Solubilized Wall	570	280	3.36	49.4	16.3	2.91	3745	19610	1781
Wash 1	31	24	nd[b]	nd	nd	nd	922	798	344
Wash 2	24	22	nd	nd	nd	nd	902	201	317
% in Protoplasts	53.7	67.2	4.3	74.7	82.7	39.5	69.5	17.1	73.5
% Solubilized[c]	46.3	32.8	95.7	25.3	17.3	60.5	30.5	82.9	26.5

[a] 1.5 ml packed cells is ca. 60 mg dry wt.
[b] none detected (B<0.08, Fe<0.05, Zn<0.05, Mn<0.02 μg)
[c] Solubilized wall fraction plus washes

Table 6 Elemental composition of fractions from French Press disruption of cells. The pellet contains the cell wall material.

Cell Line/ Fractions	Total µg recovered from 5 ml packed cells[a]									
	Ca	Mg	B	Fe	Zn	Mn	K	Na	P	Ge
0.01 mM Boron/										
Pellet	316	153	2.19	11.8	19.5	2.02	1217	61.7	1065	
Pellet 2[b]	12	12	nd[c]	nd	nd	nd	421	50.2	456	
Supernatant	73	146	0.10	3.5	10.8	0.31	7067	5.4	1571	
% in Pellet	81.8	53.1	95.6	77.1	64.4	86.3	18.8	95.4	49.2	
0.1 M Boron/										
Pellet	278	119	1.75	9.6	18.5	1.38	1110	20.9	839	
Pellet 2[b]	32	36	nd	4.0	2.5	0.71	890	80.5	787	
Supernatant	113	182	0.08	2.1	10.3	0.62	6263	7.6	1573	
% in Pellet	73.3	39.5	95.6	82.2	64.2	69.0	15.1	73.5	34.8	
1.0 mM Boron/										
Pellet	320	145	2.44	12.3	21.7	1.58	1578	33.2	966	
Pellet 2[b]	43	39	nd	3.5	3.7	0.63	728	184.2	869	
Supernatant	135	209	0.46	2.7	14.6	0.83	6577	7.3	1693	
% in Pellet	72.9	41.0	84.1	82.0	59.9	65.6	19.4	81.9	36.3	
0.43 mM Germanium/										
Pellet	411	187		13.3	32.3	2.66	431	38.3	1340	10.98
Supernatant	122	150		1.1	5.1	0.57	2169	2.6	760	3.82
% in Pellet	77.1	55.5		92.6	86.5	82.4	16.6	93.6	63.8	74.2

[a] ca. 50% extruded from the press
[b] Pellet 2 was a precipitate that formed in the supernatant ca. 24 hrs after the first pellet was separated.
[c] none detected (B<0.08, Fe<0.05, Zn<0.05, Mn<0.02 µg)

Table 7 Elemental composition of fractions from grinding cells in liquid nitrogen. The pellet contains the cell wall material.

Cell Line/ Fractions	Total μg recovered from 3 ml packed cells[a]								
	Ca	M	B	Fe	Zn	Mn	K	Na	P
0.01 mM Boron/ Starting Cells	599	382	2.20	33.5	37.2	2.23	9079	104	3264
Pellet	423	167	2.59	23.1	19.6	1.17	669	72	1050
Supernatant	143	168	nd[b]	8.4	13.8	0.78	6541	254	1551
Wash 1	3	28	nd	1.7	2.6	nd	1207	3	419
Wash 2	1	18	nd	1.7	2.4	nd	404	nd	319
% in Pellet	74.2	43.9	100	66.3	51.1	60.0	7.6	21.9	31.5
0.1 mM Boron/ Pellet	619	233	3.92	33.0	31.5	1.77	951	34	1482
Supernatant	254	322	0.32	10.4	19.4	1.11	9889	298	2676
Wash 1	5	43	nd	2.3	4.5	0.19	1538	3	565
Wash 2	2	22	nd	2.3	4.3	0.11	529	nd	369
% in Pellet	70.3	37.6	92.5	68.8	52.7	55.9	7.4	10.1	29.1
1.0 mM Boron/ Pellet	534	217	3.78	33.7	24.2	1.73	717	70	1261
Supernatant	230	305	0.41	10.5	19.1	1.05	9207	278	2681
Wash 1	2	31	nd	2.4	1.9	0.05	1346	3	590
Wash 2	4	21	nd	1.9	2.0	0.08	424	nd	319
% in Pellet	69.5	37.9	90.2	69.6	51.3	59.5	6.1	19.9	26.0

[a] 3 ml packed cells is ca. 120 mg dry wt.
[b] none detected (B<0.08, Mn<0.02, Na<0.3 μg)

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