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Regulation of Cytoplasmic and Vacuolar Volumes by Plant Cells in Suspension Culture[1]

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ABSTRACT

Quantitative microscopical measurements have been made of the proportion of cell volume occupied by cytoplasm in a cell suspension culture derived from cotyledons of bush bean (cv. Contender). On a 7-day culture cycle, the content of cytoplasm varies from 25% at the time of transfer to 45% at the start of the phase of rapid cell division. If the culture is continued beyond 7 days, the vacuole volume reaches 90% of cell volume by day 12.

Attempts to measure relative cytoplasmic volumes by compartmental analysis of nonelectrolyte efflux were unsuccessful. The proportion of cell volume occupied by cytoplasm is roughly correlated with protein content, but shows no correlation with cell size or with intracellular concentrations of K or Na. The most striking observation is that the growth of cytoplasmic volume for the culture as a whole appears to be constant throughout the culture cycle, despite changes in the rate of cell division, cell size, rate of increase in fresh or dry weight, amount of cytoplasm per cell, the cellular concentration or fluxes of Na or K, and the rate of vacuolation. It is suggested that cytoplasmic volume is under the control of its own regulatory mechanism, which operates to give a constant exponential increase in cytoplasmic volume independently of most other observed cellular properties.

This study has three objectives: (*a*) to investigate a plant cell suspension culture as a course of cytoplasmic cells for transport studies; (*b*) to assess alternative methods of estimating cytoplasmic and vacuolar volumes; and (*c*) to make a start toward understanding the process of vacuolation in plant cells, and the control of cytoplasmic volume.

Studies of the solute composition of the cytoplasm of plant cells, and of transport across the plasmalemma, have been greatly limited by the fact that the cytoplasm typically occupies such a small proportion of the cell volume. Although use has been made of the relatively cytoplasmic cells in root tips (*e.g.* 23) and in nematode-infected plants (10), these materials are difficult to prepare in quantity and lack homogeneity. On the other hand, cytoplasmic algal cells such as *Chlorella* (2) are complicated by the presence of large chloroplasts, and also may not be representative of plant cells in general. It is known that cells in culture may become quite cytoplasmic at certain stages in the culture cycle (28), but there has been apparently no quantitative estimate of cytoplasmic volume in such cells. The present study indicates that cells containing up to 45% cytoplasm may be reproducibly prepared in a suspension culture derived from cotyledons of bush bean, *Phaseolus vulgaris* L., cv. Contender, grown in defined medium.

Concerning the measurement of cytoplasmic volume. it has been suggested by Glinka (7) that compartmental analysis of efflux of a marker such as thiourea may provide a convenient measure of compartment volumes. We compare the results of such efflux measurements with quantitative microscopical volume measurements. As reported below, only the microscopical method appears to give a reliable measure of cytoplasmic volume.

Our final objective, the study of the mechanism of control of cytoplasmic volume during cell vacuolation, has received very little previous attention. Suspension cultures provide favorable material for this purpose. We report a quantitative study of cytoplasmic and vacuolar volumes throughout the culture cycle, and discuss possible regulatory mechanisms.

MATERIALS AND METHODS

Cultures. Cell suspension cultures derived from cotyledons of *P. vulgaris* L. cv. Contender (1, 14, 19) were obtained from Dr. W. G. Boll. These cells were considered suitable for the present study because they grow rapidly in suspension in a defined medium, and show conspicuous changes in the relative volumes of cytoplasm and vacuole during the culture cycle. Figure 1 shows cells at 2 days after transfer, at which point the ratio of cytoplasmic to vacuolar volume is close to its maximal value. The medium (Table I) is a modification of Gamborg's B5 medium (4). The cells were grown in 250-ml Erlenmeyer flasks, each containing 50 ml of culture medium. The flasks were plugged with cotton and shaken at 2 cycles/s on a rotary shaker at 30 C in the dark. Every 7 days a volume containing about 0.7 g fresh weight or about 5×10^6 cells was transferred by serological pipette to a flask of fresh medium, all sterile operations being carried out in a laminar flow hood. Although some of the experiments reported below continued for more than 7 days without transfer of cells to new medium, in all cases the cultures had been maintained on a 7-day cycle up to the time of the experiment.

Cell Number. Cells were separated by treatment with chromic acid (27). One volume of cells (usually 1 ml) was added to 2 volumes of CrO_3 (10%, w/v) and left overnight at room temperature. The suspension was then heated to 70 to 80 C for about 30 min, then shaken vigorously and passed through a Pasteur pipette. Cells were counted in a hemacytometer in which the depth was increased to 1 mm by supporting the coverslip with two pieces of glass slide. At least 500 cells were counted for each sample, which should give a counting error of less than 5%.

Fresh and Dry Weight. Cells were collected by filtration on Whatman No. 1. filter paper and weighed to give the fresh weight. The cells were then transferred to preweighed aluminum trays, dried at 80 C for 24 h, cooled in a desiccator, and reweighed for dry weight.

Extracellular Volume for Filtered Cells. In some experiments, [^{14}C]inulin (0.5%) or [^{14}C]sorbitol (0.5%) or [^{36}Cl]HCl (2 meq/liter Cl in medium) was added to the culture flask 5 to 30 min before

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filtration. A sample of filtered cells was then weighed, transferred to a scintillation vial, and counted in Aquasol (New England Nuclear). These counts, corrected for quenching, measured the volume of culture medium retained on the filter. This amounted to 0.46 ± 0.05 ml/g fresh weight, and was found to be independent of the age of the culture, weight of cells filtered, time of exposure to isotope, or the choice of extracellular marker. Corrections for this extracellular volume have been made where necessary as indicated in the figure legends. For the compartmental analysis experiments (see below), the medium was also labeled with ^3H_2O prior to filtration, and the exchangeable intracellular water space was calculated as the volume corresponding to the ^3H counts retained on the filter minus the volume corresponding to the ^{14}C counts retained.

Protein Content. Protein was extracted as described by Street (27). Cells were collected by filtration and weighed. A portion weighing 0.7 to 1 g was then returned to the filter assembly and washed four times with 70% (v/v) ethanol at 80 C. The filter pad was placed in 10 ml 1.0 N NaOH and heated to 80 C for 100 min. After centrifugation, 0.5 ml of the supernatant was diluted to 10 ml, and 1.0 ml of this was assayed for protein by the method of Lowry *et al.* (17).

Ion Content. Cells were collected by filtration, weighed, then heated with 10 ml H_2SO_4 (0.09 N) at 90 to 100 C for at least 10 min. Debris was removed by filtration. The filtrate was neutralized with 3 ml of 1 M Tris and made up to 50 ml with water. Potassium concentration was measured with a potassium-sensitive electrode (Orion). Similar results were obtained by flame photometry. Sodium concentration was measured with the flame photometer.

Compartment Volumes by Microscopy. Over the period of the 7-day culture cycle it was possible to use the following standard fixation schedule, adapted from Sutton-Jones and Street (28). Cells were fixed overnight at 4 C in a solution containing 6% glutaraldehyde, 3% sucrose, and 0.1 M phosphate buffer (pH 7.2). After washing at room temperature in three changes of 0.2 M buffer for 15 min each, the cells were postfixed in 1% OsO_4 for 1 to 2 h, dehydrated in a graded ethanol series, infiltrated with Spurr (25) epoxy resin, and embedded in Beem capsules. For cultures which were continued for more than 7 days without transfer, it was necessary to reduce the osmolarity of the fixative progressively to avoid plasmolysis. Thus in Figure 3, cytoplasmic volumes for days 1 to 8, measured after the standard fixation, are joined by a line. A second line, connecting points for days 6 to 12, joins values measured after fixation in 3.5, 2.5, 1.5, and 1.5% glutaraldehyde, respectively, in each case with 0.1 M buffer but omitting the 3% sucrose. These samples were washed in 0.1 M buffer before postfixation.

In an effort to ensure random sampling, at least six blocks were made and sectioned for each batch of cells. Sections approximately 0.25 μm thick were cut on a Sorvall Porter-Blum 2 ultramicrotome. They were stained in 0.05% Toluidine blue, mounted in Permount (Fisher Scientific), and photographed under phase contrast optics. The photographs were printed on Kodak RC paper at a final linear magnification of 1150×.

Individual cell sections were cut from the photographs, and the paper sections were dried at 70 C for 30 min, cooled in a desiccator, and weighed to give a measure of section area. They were then cut with a razor blade into portions representing cytoplasm and vacuole and reweighed. The numerous small vacuoles seen in cytoplasmic cells (Fig. 1) were included in the vacuole fraction, down to the smallest vacuoles which could be separated by this method. All complete cell sections from each frame were used, and a minimum of 80 cells was measured for each day. It was

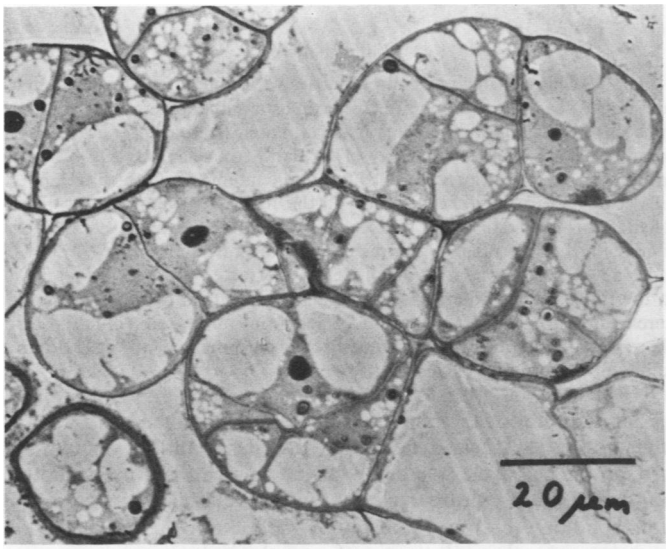

FIG. 1. Phase contrast micrograph of a section through cells of bush bean (*P. vulgaris* L. cv. Contender) 2 days after transfer to new medium.

Table I. *Composition of Growth Medium (pH adjusted to 5.4 with NaOH)*

The medium was modified from Gamborg's B5 medium (4) by the addition of amino acids, urea, adenine, riboflavin, and kinetin, and an increased concentration of sucrose and of ammonium sulfate. Some of the KNO_3 is replaced by $NaNO_3$ for purposes unrelated to this study.

Major Inorganics			Minor Inorganics		
Compound	mg/liter	mM	Compound	mg/liter	mM
$CaCl_2 \cdot 2H_2O$	147	1.0	$MnSO_4 \cdot H_2O$	10	0.06
KNO_3	1011	10.0	$ZnSO_4 \cdot 7H_2O$	2	0.006
$NaNO_3$	1275	15.0	H_3BO_3	3	0.05
$(NH_4)_2SO_4$	396	3.0	KI	0.75	0.05
$MgSO_4 \cdot 7H_2O$	370	1.5	$NaMoO_4 \cdot 2H_2O$	0.25	0.001
$NaH_2PO_4 \cdot H_2O$	138	1.0	$CoCl_2 \cdot 6H_2O$	0.025	0.0001
Organic					
Substance	mg/liter	mM	Substance	mg/liter	mM
2,4-D	2	0.009	*myo*-Inositol	100	0.5
Kinetin	0.64	0.003	Glycine	1.2	0.017
Sequestrene-NaFe	40.0	0.08	L-Glutamic acid	10	0.067
Adenine sulfate	1.0	0.02	L-Aspartic acid	10	0.08
Riboflavin	0.1	0.0002	L-Glutamine	20	0.15
Thiamine	1.0	0.02	Urea	20	0.3
Nicotinic acid	1.0	0.08	Asparagine	5	0.04
Pyridoxal	1.0	0.05	Sucrose	30,000	88

ascertained that this was sufficient to give a negligibly small sampling error (less than 2% of cell volume). The area of the cytoplasm sections divided by the area of cell sections gives the proportion of the cell volume occupied by cytoplasm. For the validity of this procedure, see reference 16.

Compartmental Analysis of Nonelectrolyte Efflux. Cells were loaded by the addition of 1 mM [^{35}S]thiourea or 1 mM [^{14}C]-acetamide to the culture flasks. Preliminary experiments with thiourea showed that cells loaded at 30 C, but not those loaded at 4 C, accumulated label to a higher concentration than that in the medium. The experiments were therefore conducted at 4 C. After loading for 12 h (see below), 5-ml samples of the culture were transferred to 50-ml Erlenmeyer flasks on a shaker. After removal of most of the labeled medium, a continuous flow of medium containing unlabeled nonelectrolyte was passed through the flasks by means of a peristaltic pump, at an initial rate of 100 ml/h. Medium was removed from the flasks through a glass fiber filter, collected in scintillation vials, and counted after the addition of Aquasol. At the end of the efflux period, cells were collected by filtration, weighed, and counted in Aquasol.

RESULTS AND DISCUSSION

Pattern of Growth. The material used in this study showed a pattern of growth similar to that previously observed in this (1, 14, 15, 19) and other (*e.g.* 6, 9) cell suspension cultures. Cell division (Fig. 2A) showed a lag period followed by a 3-day period of exponential growth with a doubling time of 24 h, and finally a "stationary" phase. Increase in fresh and dry weight (Fig. 2B) tends to lag behind the changes in cell number so that, as shown in Figure 3, the fresh weight per cell decreases from 8×10^{-8} g at time of transfer to a minimum of 4×10^{-8} g on days 3, 4, and 5, then rises again as cell division slows down on days 6 and 7. Also, the protein content per gram (Fig. 3) reaches its maximum during the cell division phase (*cf.* 6). The short culture cycle used here was intended to keep the cells in a more active state. Accordingly,

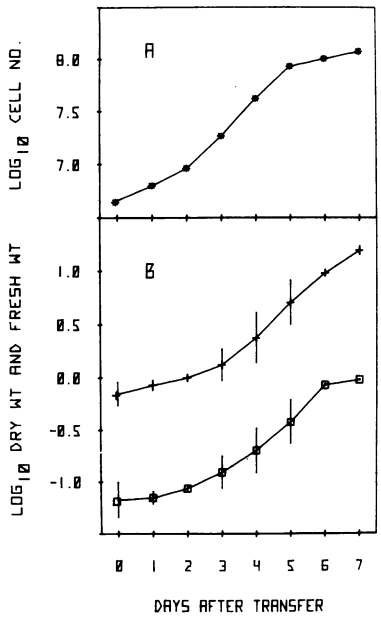

FIG. 2. Semilogarithmic plot of (A) cell number per culture flask and (B) fresh weight (+——+) and dry weight (□——□) *versus* time after transfer to new medium. Each point in B represents the mean of two measurements, indicated by vertical bars except where the difference is less than the size of the symbol. Duplicate measurements of fresh weight were made during two different culture cycles. Fresh weight values in this figure have not been corrected for the weight of medium retained on the filter.

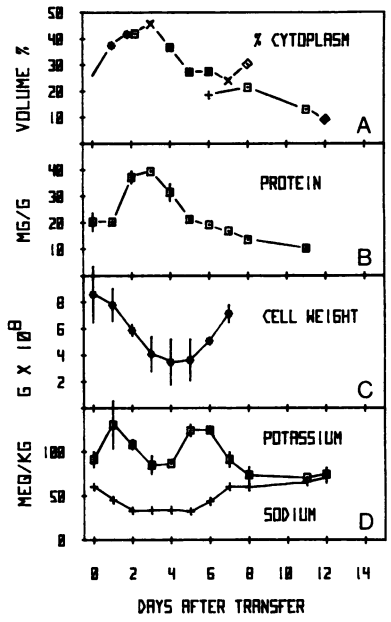

FIG. 3. Changes in some cell properties with time after transfer to new medium. A: average volume of cytoplasm as a percentage of cell volume. Each different symbol represents material taken from a different passage or culture cycle. Curves from days 1 to 8 and from days 6 to 12 indicate differences in fixation procedure. B: protein content (mg/g fresh weight of cells corrected for medium retained on the filter). C: average cell weight, calculated from fresh weight (corrected for medium retained on the filter) divided by cell number. D: intracellular concentrations of K (□——□) and Na (+——+) in meq/kg fresh weight (corrected for medium retained on the filter). Standard errors for the measurements of cytoplasm volume were less than 2% of the cell volume. Each point for protein content, K, or Na represents the mean of two measurements, indicated by vertical bars, made during different cycles.

the lag and stationary phases are not as pronounced as in some other studies (1, 15), and the change in cell size and in vacuolation through the cell cycle (see below) is not as great. It was also noted that on the short culture cycle the cells were much more uniform in morphology (*cf.* Fig. 1 and ref. 1). Cell clumps remained small enough to pass through the tip of a serological pipette.

Volume Measurements: Microscopy. Cytoplasmic volumes measured from photomicrographs are presented in Figure 3. Each different symbol for cytoplasmic volume in Figure 3 represents material taken from a different passage or culture cycle (*i.e.* a different week). The curves from day 1 to day 8 and from day 6 to day 12 indicate differences in fixation procedure. Comparison of data obtained by different fixations at days 6 and 8 suggests that the osmolarity of the fixative could have some influence on the apparent volume of cytoplasm in these moderately vacuolated cells. However, this appears not to be the case for the more cytoplasmic cells, since at 2 days after transfer, material fixed at lower osmolarity, indicated by the open square, gave a result identical to that from cells fixed by the standard procedure. It remains possible that undetected changes in cytoplasmic volume occur during fixation, regardless of the osmolarity of the fixative, and this possible qualification should be borne in mind in assessing the results.

By means of the various symbols in Figure 3 it is possible to compare the changes in cytoplasmic volume for different cycles: it will be seen that the general pattern is consistent. The curve for cytoplasmic volume is therefore drawn from zero time on the assumption that the volume on day 0 is equal to that on day 7.

Apparent Volumes by Compartmental Analysis. Glinka (7) found that [^{35}S]thiourea entered carrot tissue to reach the same concentration as in the external solution, and that, after removal of isotope from the extracellular space, the time course of efflux

corresponded to the sum of two first order components. It was suggested that these components of efflux gave a measure of cytoplasmic and vacuolar volumes, which on this basis represented 11 and 89% of the intracellular volume, respectively. In the present study, Figure 4 shows that the markers [^{35}S]thiourea and [^{14}C]-acetamide both entered the cells to approximately the same concentration as in the external solution at 4 C. Moreover, the loss of radioactivity after transfer to unlabeled medium in both cases appeared to show two first order components, with half-exchange times of about 15 min and about 2 h. The proportion of label appearing in the fast component of efflux (Fig. 5) showed no correlation with the proportion of cell volume occupied by cytoplasm based on microscopical measurements (Fig. 3). It is more difficult to separate cytoplasmic and vacuolar effluxes in cells where the volumes of these compartments are more equal. The fact that these cells nevertheless showed two apparent components of efflux warns against too facile an assignment of these components to cellular compartments.

Relative Volumes of Cytoplasm and Vacuole. The results of quantitative photomicroscopy (Fig. 3) show that the proportion of cell volume occupied by cytoplasm is about 25% at the time of transfer of the cultures to new medium. This increases to 45% by the start of the phase of rapid cell division, but falls during this "exponential phase" to a value of 27% by day 5. Sutton-Jones and Street (28) also showed that in cell suspensions of *Acer pseudoplatanus* the cytoplasm reaches its maximum thickness in the early stages of the cell division phase, but they gave no quantitative data. In the present culture, cells on a 7-day cycle never become highly vacuolated, but if the culture is continued for a longer time, the vacuole volume reached 90% of the cell volume by day 12 (Fig. 3).

There is little quantitative information of this type in the literature. In the unicellular algae *Chlamydomonas reinhardii* and *Chlorella pyrenoidosa* the vacuole has been shown to occupy only 8% and 8 to 13% of the cell volume, respectively (2, 24). Much of the cytoplasmic volume in these cells is occupied by chloroplasts. In the cytoplasmic cells of the first 1 mm of the mung bean root tip, cytoplasm was found to occupy an average of 77% of the intracellular volume (5). In vacuolate cells, there are a number of rough estimates of cytoplasmic volume based on the average thickness of the cytoplasmic layer. The cytoplasmic volume for oat coleoptile cells (20) and pea epicotyls (18) was estimated in both cases at 3.5%. For storage tissue of beet and for barley roots, values of 5 and 8.3%, respectively, of the tissue volume have been estimated (21, 22).

Significance of Changes in Cytoplasmic Volume. In addition to the proportion of cell volume occupied by cytoplasm, Figure 3 shows some other cell properties, namely protein content per gram, cell size (cell fresh weight), and the intracellular concentrations of Na^+ and K^+. An increase in protein content and a decrease

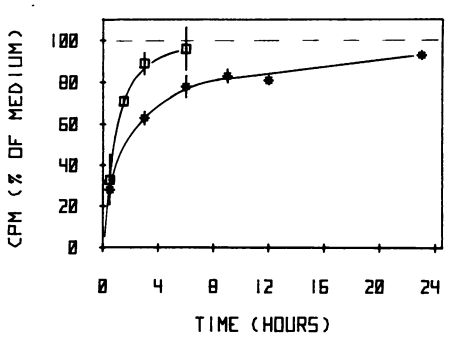

FIG. 4. Time course of loading of cells with [^{35}S]thiourea (*——*) or [^{14}C]acetamide (□——□) at 4 C. Concentration of isotope in the cells is corrected for medium retained on the filter and is calculated on the assumption that it occupies the exchangeable intracellular water space. Data are for cells at 1 day after transfer to new medium.

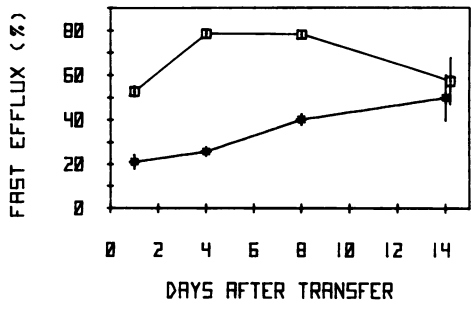

FIG. 5. Percentage of total cellular radioactivity appearing in the fast component of efflux. At various times after transfer, cells were loaded for 12 to 13 h at 4 C with [^{35}S]thiourea (*——*) or [^{14}C]acetamide (□——□) then transferred to unlabeled medium at 4 C. Points are means of duplicates, indicated by bars.

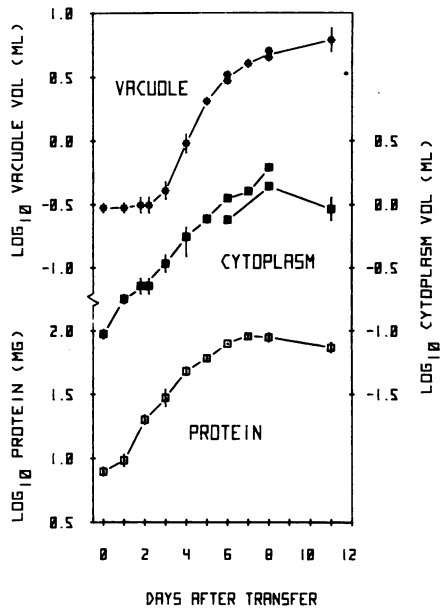

FIG. 6. Increase in vacuolar volume (*——*), cytoplasmic volume (■——■), and protein (□——□) for the whole culture with time after transfer to new medium. Estimates of total cytoplasmic and vacuolar volumes were obtained by multiplying the fresh weight of the culture (corrected for medium retained on filter) by the proportion of intracellular volume occupied by cytoplasm or vacuole. Each point for cytoplasmic or vacuolar volume is calculated from one measurement of percentage cytoplasm in Figure 3; bars indicate volumes calculated from duplicate measurements of fresh weight. Separate line connecting points for cytoplasmic volume at 6, 8, and 12 days indicates a different fixation procedure (see Fig. 3 and Materials and Methods). Curve for total protein is also calculated from the data of Figure 3 together with fresh weight measurements.

in cell volume have commonly been observed during the exponential division phase in cell cultures (*e.g.* 6, 15). In Figure 3, there is a fairly close correspondence between the time course of cytoplasmic volume and that of protein content. There is a discrepancy between these curves at day 1 and to a lesser extent at day 8. More data would be required to judge the significance of this. No relationship is apparent in Figure 3 between cytoplasmic volume and cell size.

The data on ion content (Fig. 3) show an increase in the ratio of K^+ to Na^+ at the beginning and a decrease at the end of the culture cycle. This is attributable to changes in the external concentration of K^+, which is in rather short supply in this medium, and becomes exhausted by the end of the cycle. The sudden increase in K^+ content between days 4 and 5 is of more interest, and may indicate an activation of transport during rapid

vacuolation of the cells. It may be calculated from the data that the rate of net uptake of K^+ increases from approximately 0.3 μeq/g·h between days 2 and 3 to approximately 3 μeq/g·h between days 4 and 5. This more than compensates for the dilution of cell contents resulting from expansion of the vacuole (Fig. 3). It may be concluded that cytoplasmic volume shows no correlation either with the cellular concentrations of K^+ and Na^+, or with their fluxes.

A more striking result is obtained if the data are plotted to show the increase in cytoplasmic and vacuolar volumes in the culture as a whole (Fig. 6). Although vacuolar growth shows the expected lag followed by a rapid increase, the curve for cytoplasmic volume is not significantly different from a straight line during the 7 days of the normal culture cycle. The rate of increase in cytoplasmic volume only declines at a later stage, by which time the osmolarity of the medium has fallen from an initial value of 168 mosmol to 15 mosmol (data not shown) and increase in fresh weight, dry weight, and protein content has ceased. The rate of increase in cytoplasmic volume is in fact the only parameter measured in these cultures which shows no detectable change for the duration of the culture cycle. It may be noted that the values for cytoplasmic volume (Fig. 6) are obtained by multiplying the fresh weight of the culture by the proportion of intracellular volume occupied by cytoplasm. The total volume will be in error to the extent that the density of the cytoplasm differs from unity, but the pattern of results will not be significantly affected.

Thus, at least under these particular experimental conditions, the growth of cytoplasmic volume is apparently unaffected by the rate of cell division, cell size, the rate of increase in fresh or dry weight, the amount of cytoplasm per cell, the cellular concentrations of Na^+ or K^+, the rate of K^+ uptake, or the rate of vacuolation. Of all of these factors, only cytoplasmic volume seems to show an uninterrupted exponential increase during the culture cycle.

The increase in total protein (Fig. 6) is also of interest in that it comes closer to a constant exponential curve than any other factor except cytoplasmic volume. As seen in Figure 3, there is a suggestion of a lag in synthesis at day 1 and an earlier decrease in growth compared with cytoplasmic volume. Nevertheless, like the cytoplasmic volume increase, net protein synthesis also appears to be independent of most of the variables of the culture cycle.

Regulation of Cytoplasmic Volume. Cytoplasmic volume control is a problem which may be considered more or less distinct from the question of cell turgor pressure regulation. These two features of plant water relations have been considered in a number of recent reviews (3, 8, 30). Regulation of cytoplasmic volume in vacuolate cells appears to be analogous to the regulation of cell volume in wall-less cells. There is evidence both from animal cells (*e.g.* 12, 13) and from wall-less algae (*e.g.* 11) that cell volume is not determined in a simple passive way, resulting from the combined effects of protein osmotic pressure and the independent activity of ion pumps, but rather that there must be a positive volume control involving a volume-sensing mechanism and feedback regulation of ion transport or organic solute synthesis. Similar homeostatic mechanisms have been proposed for the regulation of cytoplasmic volume in vacuolate cells (26, 29).

Our understanding of volume regulation is still extremely limited. For instance, it has been suggested that the volume of wall-less cells may be sensed by the tension of the plasma membrane (11, 12), but this concept is less simple in vacuolate cells, where the tension of the tonoplast presumably depends more on the volume of the vacuole than on the volume of cytoplasm. Moreover, current concepts of volume regulation focus on adaptation to environmental changes, but largely ignore the question of how the cytoplasmic volume keeps pace with other cellular growth processes. The results shown in Figure 6 suggest that cytoplasmic volume is under the control of its own regulatory mechanism, which operates not in a homeostatic mode, to give a constant volume, but rather in a servo mode (*cf.* 3) to give a constant exponential increase in volume.

It has been noted above that the net growth of protein is also remarkably constant over most of the culture cycle. However, it is not yet clear to what extent the control of cytoplasmic volume may be linked to protein content or to protein synthesis. Although this and other questions remain unanswered, the relative constancy of the rate of increase in cytoplasmic volume shown in this study may throw new light on the processes of growth and vacuolation in the plant cell.

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