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SUBCELLULAR LOCALIZATION OF DIFFUSIBLE IONS IN THE YEAST *SACCHAROMYCES CEREVISIAE*: QUANTITATIVE MICROPROBE ANALYSIS OF THIN FREEZE-DRIED SECTIONS

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SUMMARY

In yeast cells, of which the intracellular potassium had been partly replaced by rubidium or caesium, the intracellular ion distribution was studied by means of energy-dispersive X-ray microanalysis. The cells were rapidly frozen and thin sections were cut at low temperature on a cryo-ultramicrotome without the use of a trough liquid. By this dry cryosectioning procedure, complete retention of the diffusible ions in the cells was obtained. Unless the sections had been exposed to moisture, no signs of redistribution were apparent. For quantitative determinations a gelatin standard, containing known amounts of the elements of interest, was prepared in the same way as the cells. The concentrations of potassium, rubidium, caesium and chloride in the nucleus, the cytoplasm and the vacuole could be measured. The intracellular distributions of potassium, rubidium and caesium were very similar. The concentrations of these ions in the cytoplasm were about equal to those in the nucleus and twice those in the vacuole. The total concentration in the cytoplasm was 180–190 mmol/kg fresh weight, in the nucleus 190–200 mmol/kg fresh weight and in the vacuole 75–90 mmol/kg fresh weight. The permeability of the yeast cell for chloride is markedly lower than for the cations.

INTRODUCTION

A quantitative determination of ion concentrations at the subcellular level can be of interest in studies of the distribution of ions between nucleus and cytoplasm, in compartmental analysis of vacuolated cells, or in kinetic studies of ion fluxes. At present, methods for the chemical analysis of cell compartments include either direct analysis of mechanically separated compartments, or measurements of ion activities with ion-selective electrodes, which can be accomplished only in large cells, or indirect determinations by means of radioactive tracers. Electron microprobe analysis provides a direct method for determinations of ion concentrations at the subcellular level, which can also be applied to relatively small cells such as the yeast cell.

Preparation of biological tissue for microprobe analysis of diffusible elements has posed severe difficulties. Conventional fixation techniques for electron microscopy

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cannot be used, since they lead to extensive loss and redistribution of ions. To prevent displacement of motile ions, freeze substitution of the tissue (Pallaghy, 1973; Spurr, 1972) or cryosectioning of unfixed material (Appleton, 1972) have been applied.

The yeast cell presents in many respects a favourable system for the study of ion transport, and it has therefore been widely used in ion influx and efflux studies (Armstrong & Rothstein, 1967; Borst-Pauwels, Wolters & Henricks, 1971; Rothstein, 1974). Generally, yeast cells are grown in a medium which contains much potassium and little sodium. Consequently, the cells have a high internal potassium concentration of about 150–200 mmol/kg fresh weight. The intracellular potassium can be replaced by other monovalent cations by exposure of metabolizing cells to these ions in the absence of extracellular potassium (Azoulay & Borst-Pauwels, 1974; Rothstein, 1974).

In the present study, the intracellular potassium was partly replaced by rubidium or caesium, and the distribution of these ions over the main cell compartments was studied by energy-dispersive X-ray analysis of thin cryosections.

MATERIALS AND METHODS

Yeast cells

The yeast, *Saccharomyces cerevisiae*, strain Delft 2, was aerated for 1 day at room temperature in distilled water. These cells were loaded by incubating 10 g fresh weight per litre in an aerated solution of 100 mM CsCl or 50 mM RbCl in 45 mM Tris-succinate buffer pH 4·5, containing 50 g/l. glucose, for 8 h at room temperature (22 °C).

Preparation for electron microscopy

One volume (about 2 ml) of yeast suspension was diluted with an equa ivolume of ice-cold 10% bovine serum albumin and 10% sucrose in buffer and centrifuged at 0 °C. The albumin was then cross-linked by placing a drop of ice-cold 20% glutaraldehyde on the pellet and allowing it to remain there for 30 s (Kuhlmann & Viron, 1972). The pellet was then rapidly cut into small pieces, which were placed on to a silver pin and immediately frozen in a nitrogen slush bath. Thin sections (80–100 nm) were cut on an LKB Ultrotome III equipped with an LKB Cryokit at a specimen temperature of −140 to −145 °C and a knife temperature of −100 °C. The sections were cut and collected dry (Sevéus & Kindel, 1974; L. Sevéus & L. Kindel, in preparation) on carbon-coated Formvar films. The ice was sublimated and the sections were allowed to reach room temperature in an atmosphere of dry nitrogen gas. Unless analysis was performed immediately, the grids were stored under vacuum. To minimize specimen damage, the sections were sandwiched between 2 layers of carbon.

Microprobe analysis

Analysis was performed with a Kevex energy-dispersive spectrometer in combination with a JEOL 100C electron microscope provided with a JEOL ASID scanning attachment. Under standard operating conditions, the sections were viewed in the scanning-transmission mode at 80 kV, at a magnification of 10000 times and analysed using a take-off angle of 45°. The relative peak intensity R was calculated from

$$R = \frac{P-b}{W-W_b}$$

(Hall, Clarke Anderson & Appleton, 1973). $P-b$ is the number of specific counts (b is the background); W (the continuum radiation) was determined from 15 to 20 keV, W_b was

measured on a specimen-free part of the grid. To minimize the background, the grid was inserted in a carbon holder and measurements were carried out at a maximal distance from the grid bars.

Standards

For quantitative determinations, a single standard was used, containing 100 mmol/kg each of KCl, RbCl and CsCl in 20% gelatin, to which 5% glycerol was added. To compare concentrations in the cells with those in the standard, the following equation was used, which can easily be derived from the expression given by Hall *et al.* (1973):

$$C_{x,\,\mathrm{cell}} = C_{x,\,st} \cdot \frac{R_{x,\,\mathrm{cell}}}{R_{x,\,st}} \cdot \frac{\overline{Z^2/A}_{\mathrm{cell}}}{\overline{Z^2/A}_{st}},$$

where C_x is the concentration (mass fraction) and R_x the relative peak intensity of element x in the cell or standard (st), Z is the atomic number and A the atomic weight. $\overline{Z^2/A}$ is the average value of Z^2/A for all elements present in the specimen, weighted according to mass fraction.

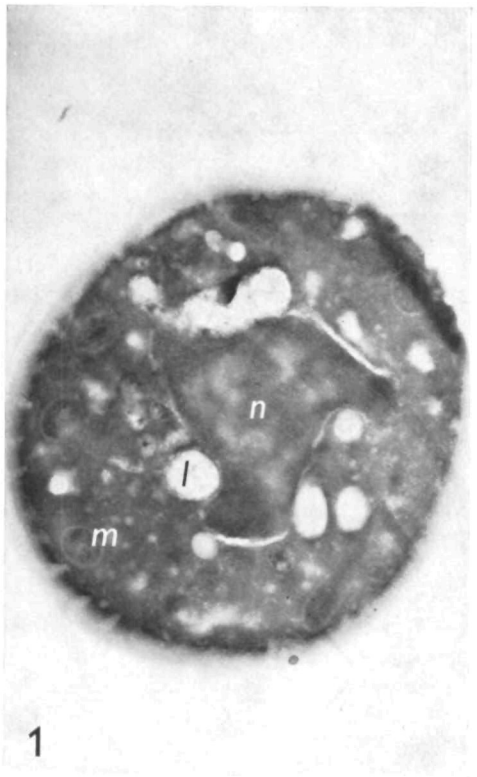
Morphology

For morphological studies on contrasted cryosections, the sections were collected on a drop of saturated sucrose and negatively stained with 4% silicotungstate. Alternatively, yeast cells were fixed for 15 min in 9% lithium permanganate, dehydrated in a graded ethanol series and embedded in Spurr's epoxy resin (Spurr, 1969). Silver to dark-grey sections were cut on an LKB Ultrotome III and stained with uranyl acetate and lead citrate.

RESULTS

The observed general morphology of the cryosectioned yeast is in good agreement with the findings of other investigators (Bauer & Sigarlakie, 1973). The ultrastructure is well preserved with the occasional exception of the lipid droplets (Bauer & Sigarlakie, 1975). The non-metabolizing cells have 2 main compartments, nucleus and cytoplasm, the latter containing mitochondria (Fig. 1). The cells which have been metabolizing in an aerated suspension for 8 h have formed a vacuole (Figs. 2, 3) and thus consist of 3 main compartments. In uncontrasted sections, few details could be observed and only the main compartments and structures identified (Fig. 3).

Analysis was performed in the nucleus, the cytoplasm and the vacuole. The cytoplasmic concentrations were measured in the outer layer of the cytoplasm, directly under the cell membrane, but outside the mitochondria. The analysed locus had an estimated radius of about 150 nm. To obtain quantitative data, the R_x-values of the elements in the cell were compared to those of the standard. Since absorption of X-rays and secondary fluorescence is negligible in thin sections (Russ, 1974), and specimen and standard behave identically under the electron beam with respect to mass loss (Hall & Gupta, 1974), only a correction for the atomic number of the matrix has to be made (Hall *et al.* 1973). The value of $\overline{Z^2/A}$ for the gelatin standard could be calculated. To calculate the atomic number correction for the cells, the procedure used by Hall *et al.* (1973) had to be modified. The value of $\overline{Z^2/A}$ for protein was used, but corrections were made for the content of K, Cl and Rb or Cs. This could be done by introducing an estimated value for $\overline{Z^2/A}$ and computing from this (on an IBM 360 computer) the concentrations by an iterative process until the value of $\overline{Z^2/A}$ and the ion concentrations matched.



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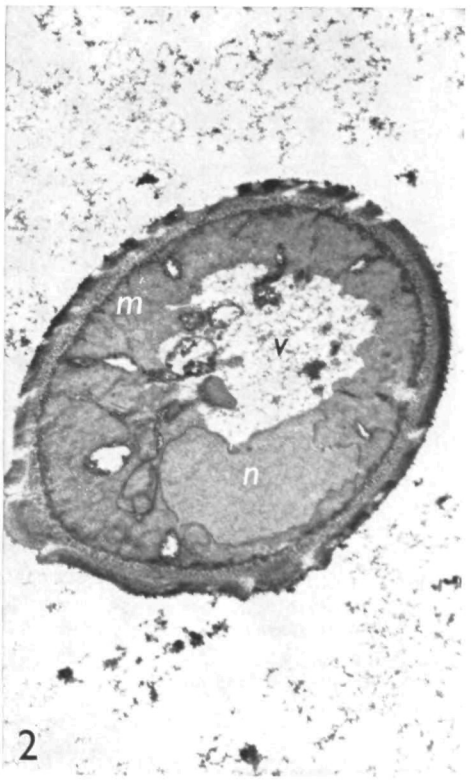

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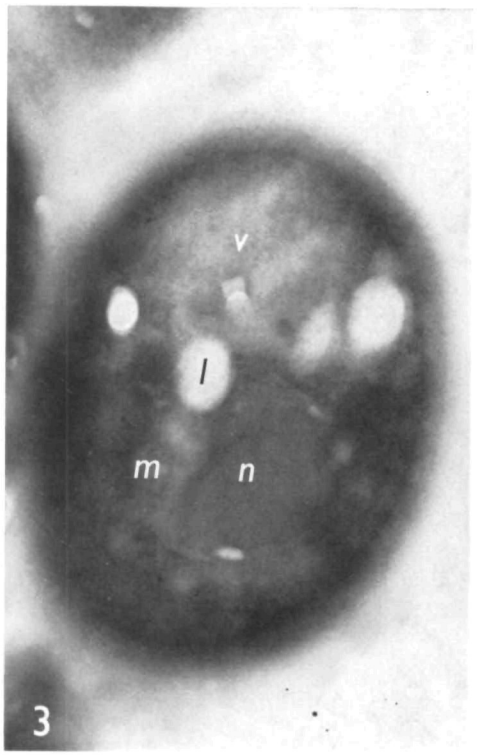

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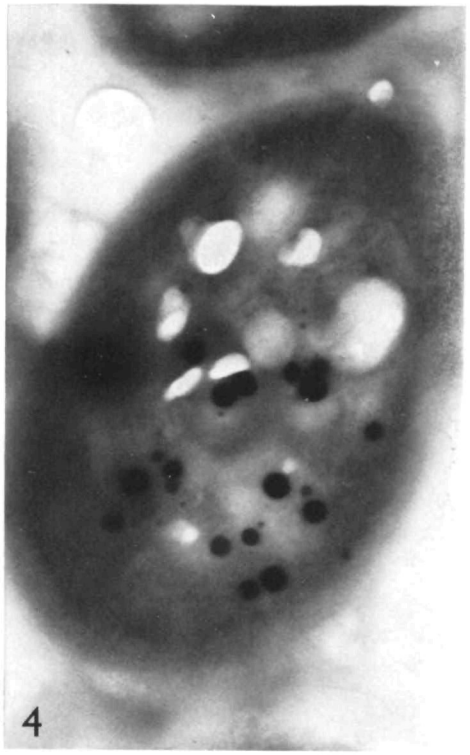

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Finally, to obtain the data expressed as mmol/kg fresh weight, corrections had to be made for the dry weight/fresh weight ratio for the cell compartments and the standard. A relative value for the dry weight could be obtained from a comparison between the continuum radiation originating from nucleus, cytoplasm and vacuole, respectively. The continuum radiation originating from the nucleus did not differ

Table 1. *Intracellular distribution of Rb, Cs, K and Cl in yeast cells*

	Rb or Cs*		K		Cl	
Compartment	*R*	*C*	*R*	*C*	*R*	*C*
Rb-loaded cells						
Cytoplasm	1·29 ± 0·09 (6)	165	0·34 ± 0·06 (6)	27	0·67 ± 0·07 (6)	44
Nucleus	1·31 ± 0·08 (3)	168	0·34 ± 0·10 (3)	27	0·67 ± 0·14 (3)	44
Vacuole	1·12 ± 0·15 (7)	79	0·29 ± 0·07 (7)	13	0·66 ± 0·07 (7)	24
Cs-loaded cells						
Cytoplasm	1·87 ± 0·27 (8)	162	0·28 ± 0·04 (8)	27	0·76 ± 0·07 (8)	60
Nucleus	1·95 ± 0·26 (7)	170	0·30 ± 0·03 (7)	29	0·72 ± 0·07 (7)	58
Vacuole	1·36 ± 0·20 (5)	60	0·24 ± 0·04 (5)	13	0·80 ± 0·09 (5)	33

* Rb for Rb-loaded cells, Cs for Cs-loaded cells.

R is the relative peak intensity. Mean, standard error and the number of compartments measured are given; every compartment was measured on 2 spots.

C is the concentration in mmol/kg fresh weight.

For the calculation of C_x, use was made of the following data:

Standard: $R_{Rb} = 0{\cdot}70$, $R_{Cs} = 1{\cdot}24$, $R_K = 1{\cdot}13$, $R_{Cl} = 1{\cdot}36$.

Dry weight/fresh weight ratio: standard, 0·286; cytoplasm, nucleus, 0·316; vacuole, 0·178.

Atomic number correction: The value of $\overline{Z^2/A}$ for the standard was 4·82. The computed values of $\overline{Z^2/A}$ for the cell compartments were, for Rb-loaded cells: cytoplasm, 3·90; nucleus, 3·91; vacuole, 3·81. And for Cs-loaded cells: cytoplasm, 4·67; nucleus, 4·75; and vacuole, 4·20.

significantly from that originating from the cytoplasm. Since non-metabolizing cells consist only of nucleus and cytoplasm as main compartments, the absolute value for the dry weight/fresh weight ratio could be taken from a determination of this ratio for non-metabolizing cells.

The results (Table 1) show that Rb, Cs and K have a very similar distribution within the cell. The relative peak intensity *R* of these elements in cytoplasm and

Fig. 1. Cryosection of a non-metabolizing yeast cell, contrasted with 4% silicotungstate. *l*, lipid droplets; *m*, mitochondria; *n*, nucleus. × 18000.

Fig. 2. Permanganate-fixed cell, which has been metabolizing for 8 h. The cell contains a nucleus (*n*) and a vacuole (*v*). *m*, mitochondria. × 10000.

Fig. 3. Uncontrasted dry-cut cryosection of a caesium-loaded cell. Nucleus (*n*), vacuole (*v*) and lipid droplets (*l*) are clearly, mitochondria (*m*) faintly, discernible. × 19000.

Fig. 4. Uncontrasted dry-cut cryosection of a rubidium-loaded cell which had been exposed to room atmosphere for several days. Extensive precipitation has occurred. × 20000.

nucleus exceeds that in the vacuole. Calculation of the concentration of these ions expressed as mmol/kg fresh weight shows a concentration ratio slightly greater than 2:1 between cytoplasm and vacuole. The intracellular distribution of chloride differs from that of the cations. The concentration ratio for chloride between cytoplasm and vacuole is less than that for the cations. The lower chloride content of the Rb-loaded cells can be explained by the fact that a lower external concentration was applied.

DISCUSSION

It is well known that in microprobe studies of the intracellular distribution of diffusible ions, the preparative methods are extremely critical. There is an appreciable risk of artifacts at most stages during preparation. Leakage and/or redistribution of the ions can occur during the encapsulation in albumin, or during freezing, cutting, drying and storage. To minimize the leakage during the encapsulation of the cells, this step was carried out at 0 °C, and both the time needed and the number of manipulations involved were kept to a minimum. In the short time needed for the cross-linking of the albumin (less than 30 s), no appreciable fixation of the cells by glutaraldehyde occurs (Kuhlmann & Viron, 1972). Glutaraldehyde permeates only very slowly through the yeast cell wall (Ghosh, 1971). To minimize freezing damage to the cells, 5% sucrose was present as a cryoprotective. Results obtained by freezing the cells in melting nitrogen were superior to those obtained with the use of boiling nitrogen, where a thin gas layer is formed around the specimen. The absence of this thermally insulating layer during freezing in melting nitrogen contributes more to the increased freezing rate than the temperature difference. During cutting, part of the section might thaw, which could cause redistribution of ions. To avoid this, the cutting temperature had to be lowered so that specimen and knife temperature during cutting were never higher than -100 °C. For the same reason the cutting speed was kept as low as possible. It is clear that no trough liquid could be used. The sections were therefore both cut dry and collected dry (Sevéus & Kindel, 1974). After sublimation of the ice, the sections are hygroscopic. Moisture from the air can cause floating of the ions on the sections, thereby causing severe redistribution. The effect of storing the sections for several days in room atmosphere is shown in Fig. 4. Strict precautions were therefore taken to avoid exposure to moisture. The sections were brought to room temperature in extra dry nitrogen gas and stored in vacuum until the moment of analysis.

Use of osmium vapour stain has been suggested as being helpful in the unequivocal identification of cellular structures without interfering with the ion distribution (Werner, Morgenstern & Neumann, 1973). We observed, however, a change in the ion distribution after osmium vapour staining, namely a markedly lower distribution ratio between cytoplasm and vacuole. A tentative explanation for this phenomenon is that water is transferred from the osmium tetroxide crystals to the hygroscopic sections, causing the ions to float on the sections.

The total concentration of potassium and rubidium or caesium in cytoplasm and nucleus, as calculated in Table 1, is about 190–200 mmol/kg fresh weight. Correcting for the lower cation content of the vacuole, the average intracellular concentration can

be estimated at about 160 mmol/kg fresh weight, which is in good agreement with values obtained by flame spectrophotometry by us and others (Rothstein, 1974). No appreciable leakage of ions has therefore occurred during the preparation of the specimen for microprobe analysis. By exposing the sections to moisture, gross redistribution and precipitation of salts can be obtained as shown in Fig. 4. This is most probably an artifact, and it seems justified to assume that the situation as depicted in Fig. 3 closely resembles the situation *in vivo*, and that precipitation has been successfully avoided.

Rubidium and caesium are readily taken up by the yeast cell (Armstrong & Rothstein, 1967) and replaced about 80% of the intracellular potassium (Azoulay & Borst-Pauwels, 1974). The amount of chloride ions taken up is substantially less than that of the cations. The permeability of the yeast cell membrane for chloride is indeed known to be relatively low compared to that for potassium (Conway & O'Malley, 1946). To maintain electroneutrality under these conditions, the influx of rubidium or caesium is balanced by an efflux of potassium and protons (Conway & O'Malley, 1946; Rothstein & Bruce, 1958). Within the cell, the difference between the concentration of chloride ions and the sum of cation concentrations is made up by succinate and carbonate ions and negatively charged macromolecules (Conway & Bradey, 1947). Potassium and rubidium behave similarly in their intracellular distribution. Rubidium is generally, for the sake of convenience, used in ion transport studies as a tracer for potassium. The present study seems to support the validity of this approach.

The distribution of diffusible ions between cytoplasm and vacuole has been studied in a number of plant cells, mainly in giant algae, where the vacuole can easily be separated from the surrounding cytoplasm. Also efflux studies and kinetic compartmental analysis, and the use of ion-specific electrodes, have given information on the intracellular ionic distribution. It is commonly found, both in giant algae and in cells of higher plants, that the concentration of potassium in the cytoplasm exceeds that in the vacuole (Higinbotham, 1973; Macklon & Higinbotham, 1970; MacRobbie, 1971). As rubidium and caesium have an intracellular distribution similar to that of potassium, the distribution of these ions in the yeast cell is found in this study to be consistent with data from other cells. It should be remembered, though, that the yeast vacuole is not completely comparable to the plant cell vacuole (Matile & Wiemken, 1967).

The distribution of monovalent cations between the cytoplasm and the nucleus has been measured in several animal cells with relatively large nuclei (Century, Fenichel & Horowitz, 1970; Palmer & Civan, 1975). Microprobe analysis also enables a study of this distribution in much smaller cells. In yeast cells the concentrations of potassium, rubidium and caesium in the nucleus are not or are only slightly higher than in the cytoplasm. This points to the possibility that the main part of these ions is freely soluble, and in electrochemical equilibrium across the nuclear membrane (assuming that the activity coefficients in nucleus and cytoplasm do not differ appreciably). The nuclear membrane thus would not present a permeability barrier for these ions, and there would only be a negligible resting potential between nucleus and cytoplasm, as is the case in some of the animal cells investigated (Loewenstein & Kanno, 1963; Palmer & Civan, 1975).

It is generally assumed that the uptake of monovalent cations in yeast takes place via a carrier system, which has at least two sites to which cations can bind (Borst-Pauwels *et al.* 1971). A theoretical, kinetic description of such a system has been given by Borst-Pauwels (1974). It can be derived from this model that the uptake of an ion may be influenced, not only by the ionic concentration in the medium, but also by the intracellular ion concentrations (Borst-Pauwels, Derks, Theuvenet & Roomans, 1974). Similarly, ion efflux kinetics may be dependent on both intracellular and extracellular ion concentrations. Ion fluxes from one compartment to another do not, however, depend on the bulk concentration in the compartments, but on the concentration at the membrane, which may be different. This concentration difference has to be taken into account when studying ion transport kinetics (Theuvenet & Borst-Pauwels, 1976), Transport kinetics will, with respect to intracellular ions, not depend on the bulk intracellular concentration, but on the concentration in the cytoplasm at the membrane. As this relevant value is not equal to the bulk intracellular concentration, determination of concentrations at the subcellular level will be of importance to quantitative studies of ion transport.

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REFERENCES

APPLETON, T. C. (1972). 'Dry' ultra-thin frozen sections for electron microscopy and X-ray microanalysis: the cryostat approach. *Micron* **3**, 101–105.

ARMSTRONG, W. McD. & ROTHSTEIN, A. (1967). Discrimination between alkali metal cations by yeast. II. Cation interactions in transport. *J. gen. Physiol.* **50**, 967–988.

AZOULAY, L. & BORST-PAUWELS, G. W. F. H. (1974). Net Rb^+ transport by Rb^+ loaded cells and the electrokinetical formulism. *Acta bot. neerl.* **23**, 505–512.

BAUER, H. & SIGARLAKIE, E. (1973). Cytochemistry on ultrathin frozen sections of yeast cells. *J. Microscopy* **99**, 205–218.

BAUER, H. & SIGARLAKIE, E. (1975). Localization of alkaline phosphatase in *Saccharomyces cerevisiae* by means of ultrathin frozen sections. *J. Ultrastruct. Res.* **50**, 208–215.

BORST-PAUWELS, G. W. F. H. (1974). n-Site 2-carrier transport. Comparison with enzyme kinetics. *J. theor. Biol.* **48**, 183–195.

BORST-PAUWELS, G. W. F. H., DERKS, W. J. G., THEUVENET, A. P. R. & ROOMANS, G. (1974). The kinetics of Rb^+ uptake by *Saccharomyces cerevisiae*. *Proc. 4th Int. Yeast Symp.*, Vienna, vol. 2, pp. 89–90.

BORST-PAUWELS, G. W. F. H., WOLTERS, G. H. J. & HENRICKS, J. J. G. (1971). The interaction of 2,4-dinitrophenol with anaerobic Rb^+ transport across the yeast cell membrane. *Biochim. biophys. Acta* **225**, 269–276.

CENTURY, T. J., FENICHEL, I. R. & HOROWITZ, S. B. (1970). The concentrations of water, sodium and potassium in the nucleus and cytoplasm of amphibian oocytes. *J. Cell Sci.* **7**, 5–13.

CONWAY, E. J. & BRADEY, T. (1947). Mechanism of high acid production by yeast and its bearing on hydrochloric acid formation in the stomach. *Nature, Lond.* **159**, 137–138.

CONWAY, E. J. & O'MALLEY, E. (1946). The nature of the cation exchanges during yeast fermentation, with formation of 0·02 N H-ion. *Biochem. J.* **40**, 59–67.

GHOSH, B. K. (1971). Grooves in the plasmalemma of *Saccharomyces cerevisiae* seen in glancing sections of double aldehyde-fixed cells. *J. Cell Biol.* **48**, 192–197.

HALL, T. A., CLARKE ANDERSON, H. & APPLETON, T. (1973). The use of thin specimens for X-ray microanalysis in biology. *J. Microscopy* **99**, 177–182.

HALL, T. A. & GUPTA, B. L. (1974). Measurement of mass loss in biological specimens under an electron microbeam. In *Microprobe Analysis as Applied to Cells and Tissues* (ed. T. Hall, P. Echlin & R. Kaufmann), pp. 147–158. London and New York: Academic Press.

HIGINBOTHAM, N. (1973). The mineral absorption process in plants. *Bot. Rev.* **39**, 15–69.

KUHLMANN, W. D. & VIRON, A. (1972). Cross-linked albumin as supporting matrix in ultrathin cryomicrotomy. *J. Ultrastruct. Res.* **41**, 385–394.

LOEWENSTEIN, W. R. & KANNO, Y. (1963). The electrical conductance and potential across the membrane of some cell nuclei. *J. Cell Biol.* **16**, 421–425.

MACKLON, A. E. S. & HIGINBOTHAM, N. (1970). Active and passive transport of potassium in cells of excised pea epicotyls. *Pl. Physiol., Lancaster* **45**, 133–138.

MACROBBIE, E. A. C. (1971). Fluxes and compartmentation in plant cells. *A. Rev. Pl. Physiol.* **22**, 75–96.

MATILE, P. & WIEMKEN, A. (1967). The vacuole as the lysosome of the yeast cell. *Arch. Mikrobiol.* **58**, 148–155.

PALLAGHY, C. K. (1973). Electron probe microanalysis of potassium and chloride in freeze substituted leaf sections of *Zea mays*. *Aust. J. biol. Sci.* **26**, 1015–1034.

PALMER, L. G. & CIVAN, M. M. (1975). Intracellular distribution of free potassium in *Chironomus* salivary glands. *Science, N.Y.* **188**, 1321–1322.

ROTHSTEIN, A. (1974). Relationship of cation influxes and effluxes in yeast. *J. gen. Physiol.* **64**, 608–621.

ROTHSTEIN, A. & BRUCE, M. (1958). Potassium efflux and influx in yeast at different potassium concentrations. *J. cell. comp. Physiol.* **51**, 145–159.

RUSS, J. C. (1974). X-ray microanalysis in the biological sciences. *J. submicrosc. Cytol.* **6**, 55–79.

SEVÉUS, L. & KINDEL, L. (1974). Dry cryosectioning of human and animal tissue at very low temperature (−140 °C). *Proc. 8th Int. Congr. Electron Microsc.*, Canberra, vol. 2, pp. 52–53.

SPURR, A. R. (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26**, 31–43.

SPURR, A. R. (1972). Freeze-substitution additives for sodium and calcium retention in cells studied by X-ray analytical electron microscopy. *Bot. Gaz.* **133**, 263–270.

THEUVENET, A. P. R. & BORST-PAUWELS, G. W. F. H. (1976). The influence of surface charge on the kinetics of ion-translocation across biological membranes. *J. theor. Biol.* (in press).

WERNER, G., MORGENSTERN, E. & NEUMANN, K. (1973). Improved contrast in dry ultrathin frozen sections. In *Electron Microscopy and Cytochemistry* (ed. E. Wisse, W. Th. Daems, I. Molenaar & P. van Duijn), pp. 393–396. Amsterdam: North-Holland Publishing.

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