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Ultrastructure of *Debaryomyces hansenii*

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Summary. The ultrastructure of the extreme halotolerant yeast *Debaryomyces hansenii* (Zopf) van Rij was similar whether the organism was grown under normal physiological conditions or at high concentrations of NaCl (12, 20 per cent w/v). A multitude of channels often seen in association with plasma membrane and/or vacuoles occur in the cytoplasm. The vacuoles contain lipid storage material. A decrease in mitochondrial size was observed with increasing salt concentration. The ultrastructure has been discussed with respect to halotolerance.

Marine occurring yeasts differ in halotolerance. The metabolic apparatus of *Debaryomyces hansenii* functions to a certain extent even at NaCl concentrations close to the solubility limit of this salt (24 per cent w/v). About 12 per cent NaCl was found metabolism limiting to *Saccharomyces cerevisiae* the other yeast preferentially used in our comparative studies (Norkrans, 1966, 1968). The strong halotolerance of *D. hansenii* seems to be due partly to an extrusion of Na and an effective uptake of K. Since the total salt level of the cells is not sufficient to counteract the osmotic potential of the medium, the cell has to meet the osmotic strain by some osmoregulating functions (Norkrans and Kylin, 1969).

It seems probable that cell functions contributing to the build-up of halotolerance of *D. hansenii* could call on a structural basis somewhat deviating from that in cells of less halotolerant yeasts. Differences might be expected *e. g.* in mitochondrial apparatus, membraneous organization, vacuole formation and cytoplasmic density. As no ultrastructural studies on *Debaryomyces* species seem to be on hand in the literature—except for some studies on ascosporal appearance performed for taxonomical purposes (*e. g.* Kodama *et al.*, 1964a, b; Besson, 1966)—such an investigation became imperative.

For comparative purposes the less halotolerant *Saccharomyces cerevisiae* was included in the studies.

Material and Methods

The organisms used were *Debaryomyces hansenii* (Zopf) van Rij (strain 26) and *Saccharomyces cerevisiae* Hansen (strain 12), previously described (Norkrans, 1966). They were grown on a reciprocal shaker at 25°C at different NaCl concentrations

in a glucose-asparagine medium (Norkrans, 1966), 500 ml per 2.5 l Fernbach flasks. Cells from logarithmic growth phase were harvested by centrifugation.

Material was fixed for *electron microscopy* in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3—7.5, for 2—4 hours at + 4°C, washed extensively and stored over night in the same buffer. Post-fixation was then carried out by one of the following treatments:

1. 2% $KMnO_4$ in Palade's (1952) veronal-acetate buffer, pH 7.2, for 2 hours at + 4°C;

2. 2% OsO_4 in veronal-acetate buffer, pH 7.2, for 2 hours at + 4°C.

The fixed cells were washed thoroughly, enclosed in 2% agar (Oxoid no 1), dehydrated in a graded ethyl alcohol series and embedded in Epon (Luft, 1961). During dehydration the material was treated over night at + 4°C in 0.5% uranyl acetate in 70% ethanol. Cells grown in the presence of 12% or 20% NaCl were fixed and washed in solutions containing 12 and 20% NaCl respectively.

Since several investigations have shown unsatisfactory preservation of the fine structure of *S. cerevisiae* with osmium tetroxide, this agent was not used for post-fixation of intact cells of *S. cerevisiae*. Studies on cells fixed in osmium tetroxide after enzymic cell wall digestion (according to Robinow and Marak, 1966) are, however, in progress for *S. cerevisiae* as well as for *D. hansenii*.

Thin sections were cut with glass knives on an LKB ultrotome and mounted on uncoated 300 mesh copper grids. Sections of material post-fixed in osmium tetroxide were stained with lead citrate (Venable and Coggeshall, 1965). The sections were examined in a Philips EM 300.

For light microscopy thick sections (0.5—1 μ) were cut from Epon embedded *Debaryomyces* cells, treated as described above in glutaraldehyde—$KMnO_4$—uranyl acetate and, for comparison, also from cells fixed in 3% glutaraldehyde only. Sections were stained for lipids or phospholipids with Sudan IV (saturated solution in 70% ethanol, 25 min), Sudan black B (saturated solution in 70% ethanol, 10 min), Nile blue, 1% and 0.02%, or aniline blue—orange G (Jensen, 1962) and for protein with mercuric bromphenol blue (Mazia *et al.*, 1953) for 2 hours (Pearse, 1968). Counterstaining with Sudan IV was made with light green (0.1% in 70% ethanol, 30 sec). The preparations were mounted in glycerol and examined in the microscope at 1600× magnification.

Observations

Micrographs of cells of *Saccharomyces cerevisiae* grown in normal nutrient solution show the general characteristics found for various strains of this yeast (Vitols *et al.*, 1961; Marquardt, 1962; Clark-Walker and Linnane, 1967).

The ultrastructure of cells grown in the presence of 12% NaCl does not seem to deviate from that of cells grown under normal conditions.

The following description of the ultrastructure of *Debaryomyces hansenii* is based on the appearance of cells grown in normal nutrient

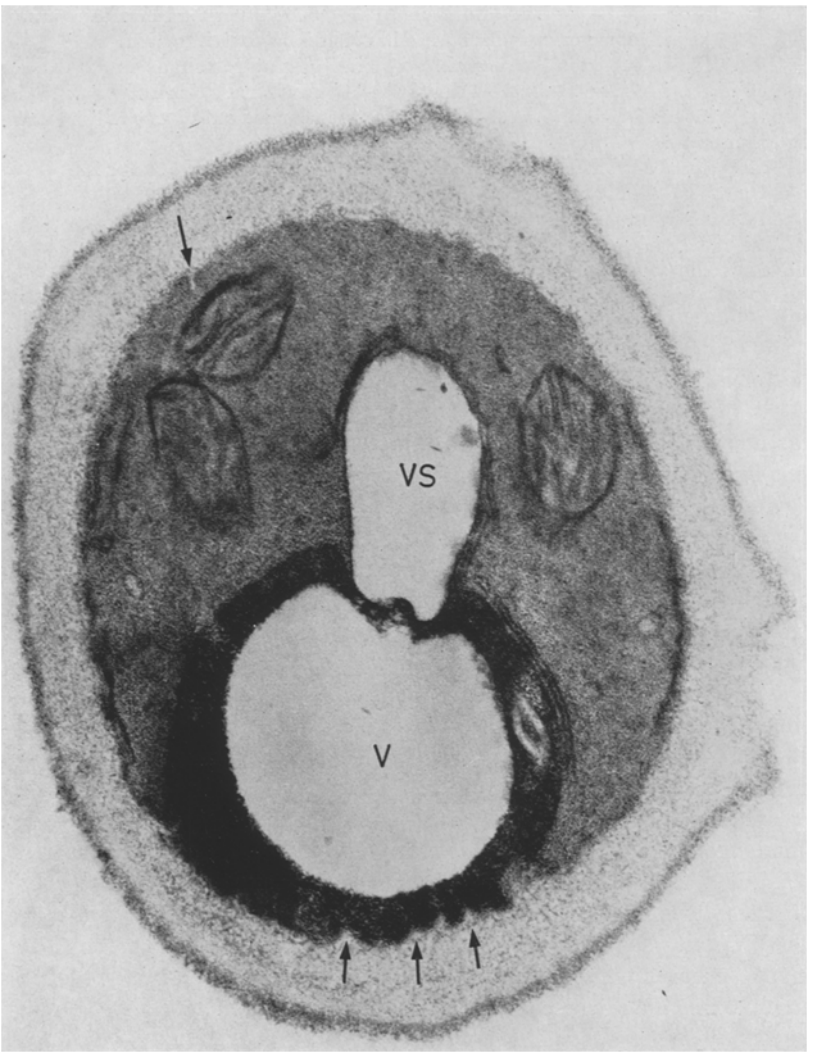

Fig. 1. Cell of *Debaryomyces hansenii* grown in the presence of 12% NaCl. The "vacuole-like structure" (*VS*) is seen in association with the storage vacuole (*V*). The plasma membrane shows numerous folds (arrows). × 51,000

solution and fixed in glutaraldehyde—potassium permanganate. Since little difference in ultrastructure has been observed between these cells and those cultivated at 12 or 20% NaCl concentration (see below), reference is also made to micrographs of cells grown in the presence of 12% NaCl (Figs. 1 and 5). So far, we have not been able to obtain good

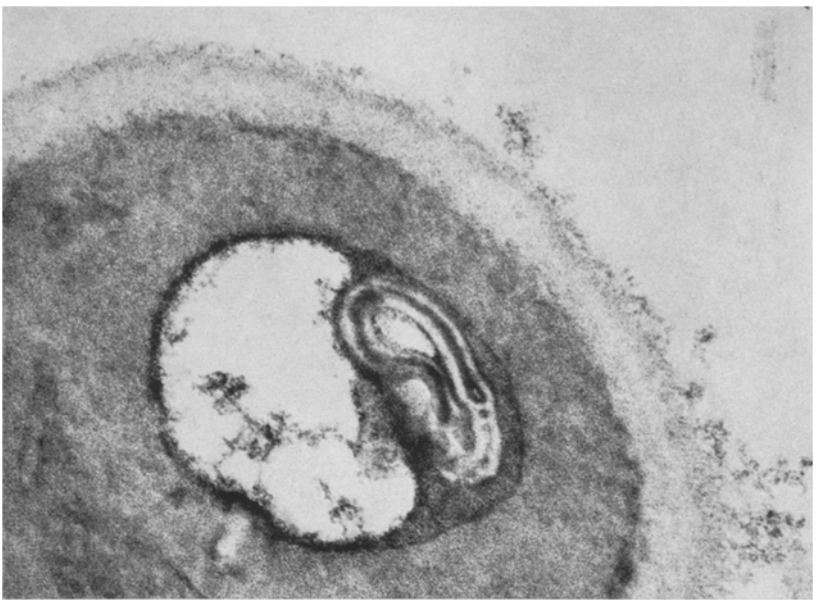

Fig. 2. Storage vacuole in *D. hansenii* with whorl-like inclusion. × 45,500

resolution with material post-fixed in osmium tetroxide. Also in material post-fixed in $KMnO_4$ the quality of fixation may vary.

The size of the cells *in vivo* is (2—4)—(2.5—4) μ (Norkrans, 1966). Consequently, the sections have round or oval profiles. The cells of *S. cerevisiae* are elongated and much larger; (5—8)—(6—9) μ *in vivo* (Norkrans, 1966).

The cell wall is thick, ca. 0.1 μ. The plasma membrane shows a great number of more or less narrow invaginations (Figs. 1 and 3). Some of these (Fig. 1) are probably folds similar to those observed in *S. cerevisiae* (Vitols *et al.*, 1961; Moor and Mühlethaler, 1963), while others form channels deep into the cytoplasm (Fig. 3).

The *mitochondria* have most varying size and shape. In cells grown under normal conditions their width (measured on the prints as the maximum diameter of 36 mitochondrial profiles) averages 0.32 μ. The inner membranes are organized as cristae.

The *vacuoles* contain storage material, often concentrated to the margins, leaving an electron transparent area (Figs. 1—5). Inclusions of varying appearance are found in the vacuoles, especially in their electron dense marginal parts (Figs. 1, 2, and 4). The inclusions may appear as profiles of cytoplasm within a unit membrane or as whorl-like (Fig. 2),

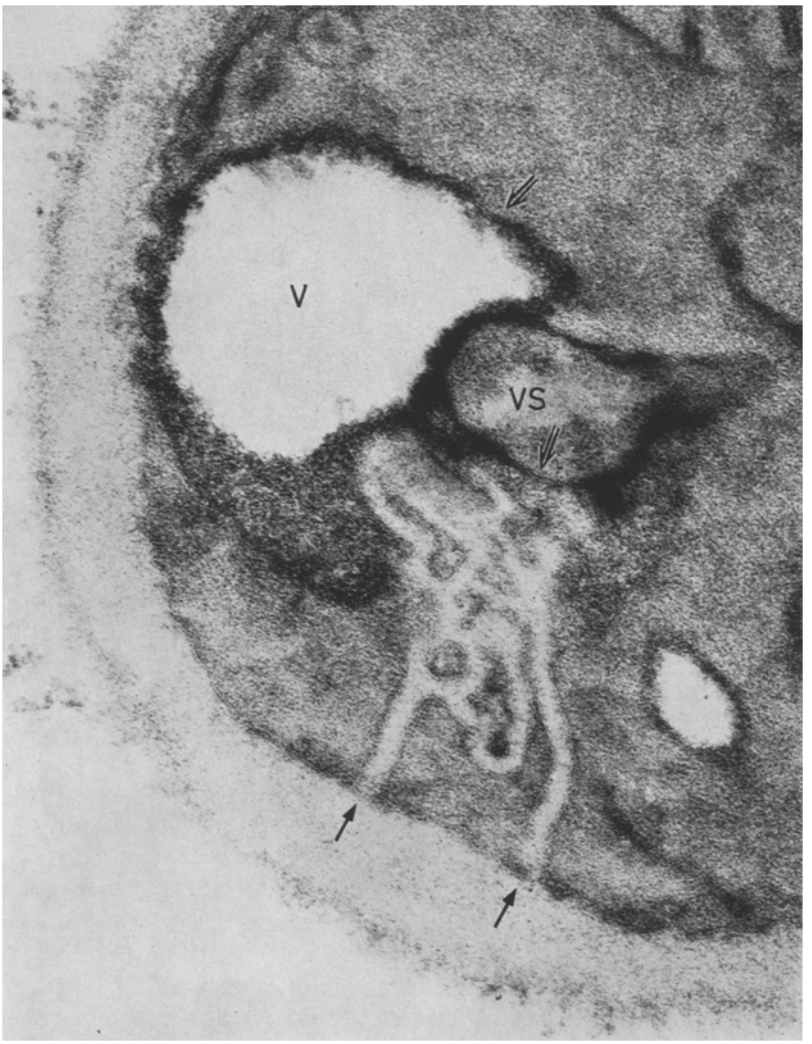

Fig. 3. Storage vacuole (*V*) and "vacuole-like structure" (*VS*) with tripartite membranes (double arrows) in *D. hansenii*. The plasma membrane shows a number of folds and two deep invaginations (arrows). × 99,000

or membraneous configurations. Similar structures may also be seen in the cytoplasm close to the vacuole (Fig. 5). Connections between the outer part of the cell and the vacuole have frequently been observed (Fig. 3).

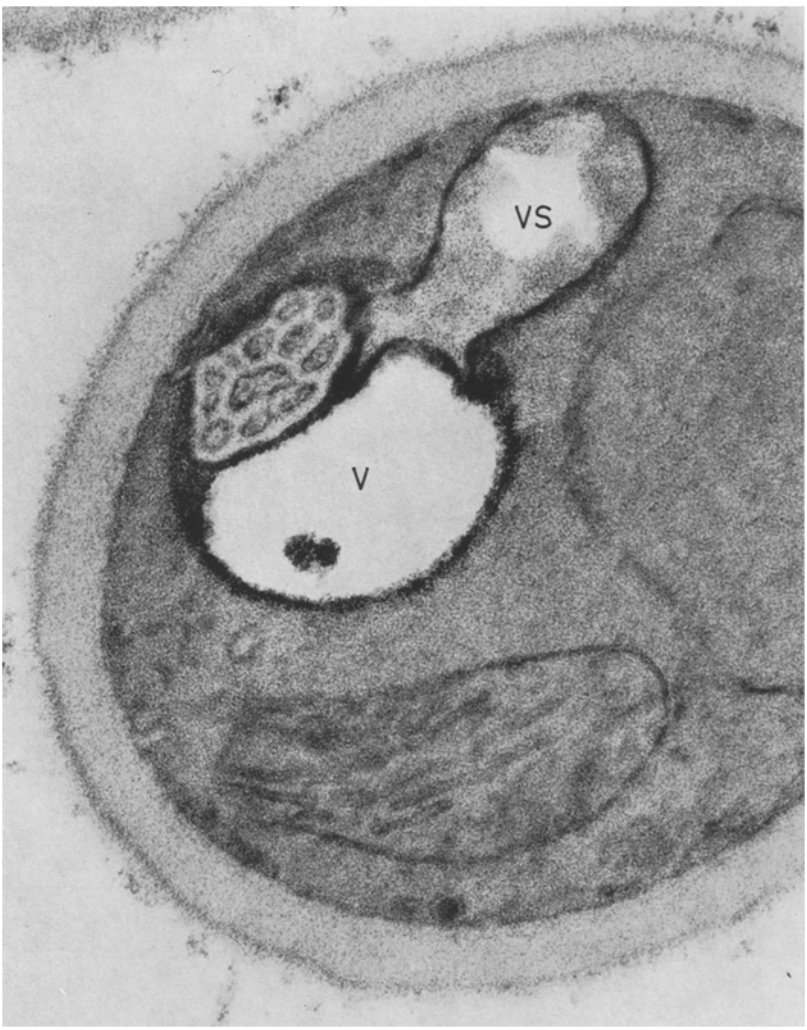

Fig. 4. Storage vacuole (*V*) with inclusions and "vacuole-like structure" (*VS*) with granular stroma in cell of *D. hansenii*. × 58,000

In cells post-fixed in OsO_4 bodies, most probably identical with the storage vacuoles, appear as round, electron dense structures. This suggests a lipid and/or protein stroma (cf. Bahr, 1954).

"*Vacuole-like structures*" similar to those observed in *Torulopsis utilis* (Linnane *et al.*, 1962) are found in close association with the storage vacuoles (Figs. 1, 3—5). They do not seem to form part of the vacuole.

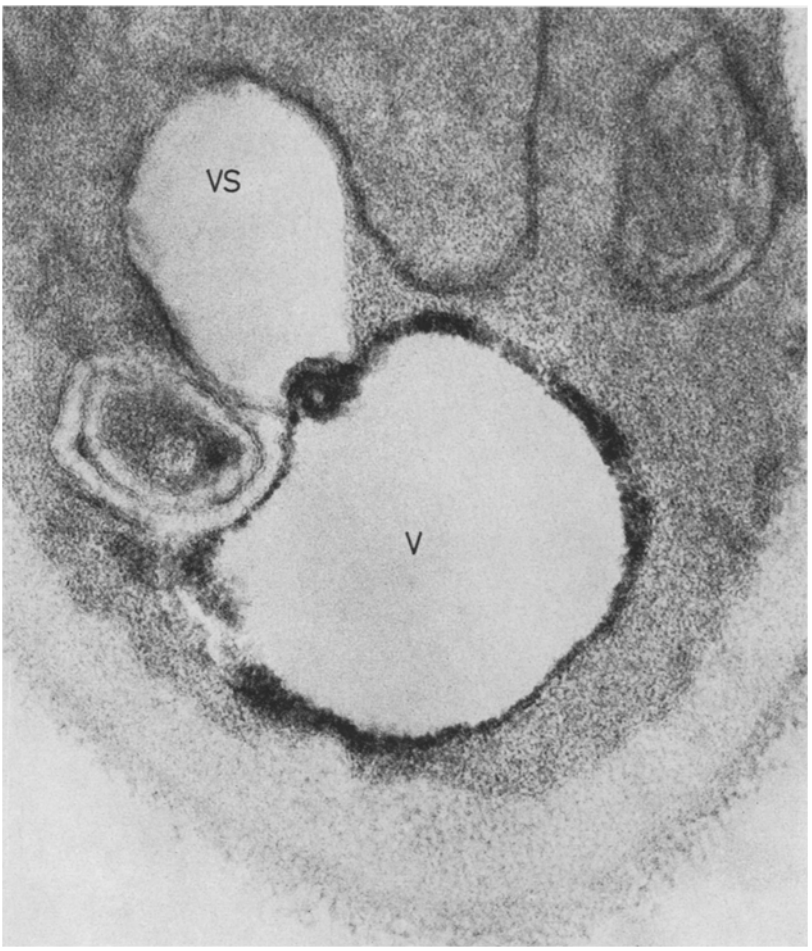

Fig. 5. Vacuole (*V*), "vacuole-like structure" (*VS*) and cytoplasmic inclusion near the vacuole in cell of *D. hansenii* grown at 12% NaCl concentration. × 99,000

In some sections they show a granular stroma, which indicates a proteinaceous content (Frey-Wyssling and Mühlethaler, 1965). The granular stroma is evident also in $KMnO_4$ treated cells, not pre-fixed in glutaraldehyde (Norkrans and Gezelius, in preparation). In such cells the storage material in the vacuoles is completely dissolved, which indicates the vacuole-like structures as separate organelles. Smith and Marchant (1968) have described similar—but smaller—organelles from *S. cerevisiae* as spherosomes.

Table. *Results of staining reactions for neutral fat, phospholipid and protein. The stains were applied to 0.5—1 μ sections of Epon embedded cells of* Debaryomyces hansenii, *grown in normal nutrient solution. GA = glutaraldehyde Hg-BPB mercuric bromphenol blue*

Fixation	Stain	Colours observed in		
		Cell walls	Cytoplasm	"Inclusions"
GA	Sudan IV	—	—	—
GA	Sudan IV + light green	light green	—	—
GA-$KMnO_4$	Sudan IV	yellow	yellow	red
GA-$KMnO_4$	Sudan IV + light green	yellow	yellow-orange	red
GA	Sudan black	—	—	(black)[a]
GA-$KMnO_4$	Sudan black	yellow	yellow	black
GA	Nile blue 1%	light blue	light blue	blue
GA-$KMnO_4$	Nile blue 1%	yellow	yellow-orange	red or blue[b]
GA	Nile blue 0.02%	light blue	light blue	blue
GA-$KMnO_4$	Nile blue 0.02%	yellow	yellow	blue
GA	Aniline blue-orange G	light blue	light blue	yellow
GA-$KMnO_4$	Aniline blue-orange G	pale yellow-green	pale yellow-green	yellow
GA	Hg-BPB	light blue	light blue	blue[c]

[a] The stained area has a much weaker colour than after $KMnO_4$ fixation.

[b] After prolonged treatment (Pearse, 1968), cell walls and cytoplasm stained blue, while the inclusions still were either blue or red.

[c] Positive reaction for all protein-rich organelles *e.g.* the nucleus. Staining of material post-fixed in $KMnO_4$ with Hg-BPB did not give reproducible results.

The results of the histochemical experiments are shown in the Table. Staining of $KMnO_4$ treated material with Sudan IV and Sudan black was positive, indicating the presence of neutral fat. Staining of glutaraldehyde (GA) fixed cells was negative with Sudan IV, due to removal of non-fixed fat during the embedding procedure; Sudan black gave a weak positive reaction, suggesting the presence of phospholipid. The reactions with 1% and 0.02% Nile blue and with aniline blue-orange G (Jensen, 1962) support the results obtained with the Sudan stains.

The staining reactions were localized to distinct areas, one or a few per cell, which with respect to size and localization seemed to agree with that of the storage vacuoles as seen in the electron microscope.

Positive reaction for protein was observed for material fixed in glutaraldehyde only. Because of the unspecificity of the method, it can, however, only be suggested that the storage vacuoles were among the stained areas.

Cells grown in the presence of 12% (Figs. 1 and 5) or 20% NaCl show the same over-all ultrastructural organization as the control cells. The mitochondria do, however, appear smaller with an average diameter of 0.25 μ (38 mitochondria measured as above) in 12% NaCl and 0.20 μ (34 mitochondria) in 20% NaCl as compared with 0.32 μ in the control. Although statistical treatment of the measurements has not been made, we consider the observed difference significant.

Discussion

The most prominent ultrastructural features of *Debaryomyces hansenii*, grown in normal nutrient solution or in the presence of high external concentrations of NaCl, are the storage vacuoles, the "vacuole-like" structures, and the channels regularly observed in the cells. Such channels are lacking in the less halotolerant *Saccharomyces cerevisiae*. *S. cerevisiae* also lacks the conspicuous storage material found in *D. hansenii*.

The storage vacuoles in *D. hansenii* contain lipid, phospholipid, and possibly also protein as concluded from the histochemical evidence (Table) in combination with the appearance of the material after $KMnO_4$ and OsO_4 fixation. Obviously the cells possess a potential for rapid assembly of membranes. Such a function has also been suggested for the storage material in spores of *Botrytis cinerea* (Buckley *et al.*, 1966).

The whorl-like and membraneous structures often observed in the storage vacuole may represent an organization of the phospholipid material, whether the structures are artifacts due to the double fixation in glutaraldehyde-potassium permanganate or not (cf. Curgy, 1968; Bowes, 1969).

The channel system observed in *D. hansenii* after glutaraldehyde-$KMnO_4$ fixation appears in some cases to originate from the plasma membrane (Fig. 3), and possibly represent plasmalemmosomes. However, membranes, in a living cell are manifestations of a dynamic system. Grove *et al.* (1968) studying membranes in *Pythium ultimum* have demonstrated an interconversion of the endoplasmic reticulum and the plasma membrane in the Golgi zone. The observed channel system in *D. hansenii* may therefore be in association not only with the plasma membrane but also with other cytoplasmic membranes.

Various functions may be ascribed to the membrane and channel system in *D. hansenii*. It may serve to transport material from the storage vacuole to sites of synthesis in the cell or to carry solutes from the outside into the interior of the cells, such functions being an obvious advantage to an organism growing under dehydrating conditions. Mobilizing of storage material for transport to sites outside the vacuole or

for osmoregulating purposes (see below) seems to imply the presence of hydrolytic enzymes. Such enzymes have been isolated from the vacuole of *S. cerevisiae* (Matile and Wiemken, 1967). The storage vacuole and/or the vacuole-like structures, may serve as enzyme pools in *Debaryomyces* and thus as the lysosome (spherosome) of this yeast.

Furthermore, the membrane-channel system seems to meet specific demands in the halotolerant *D. hansenii*. In this yeast, regulation of the intracellular K: Na ratio, more effective than the corresponding process in the less halotolerant *S. cerevisiae*, has been demonstrated (Norkrans and Kylin, 1969). As this regulation can be linked to membrane bound Na-K activated ATP'ases one might feel tempted to explain the elaborate membrane system in *D. hansenii* as a prerequisite to this cell function. The presence of phospholipid containing storage vacuoles, mentioned above, seems to fit the system very well as an indispensable potential for rapid repair and further development of the system.

The mechanism involving preferential K-uptake and Na-extrusion does not wholly explain the ability of *D. hansenii* and *S. cerevisiae* to survive and grow at high external concentrations of NaCl. For both organisms the intracellular salt concentrations are much lower than in the medium, when NaCl is present in the medium at a concentration of 8% (= 1.35 M) or higher (Norkrans and Kylin, 1969). The cells must possess some other osmoregulating capacities. Osmoregulating functions may be ascribed to mobilized storage material, *e. g.* glycerol formed from neutral fat present in the vacuoles in *D. hansenii*. This hypothesis is supported by histochemical evidence. Little neutral fat was found in cells grown at high concentrations of NaCl.

The micrographs reveal no ultrastructural differences between the control cells and those growing in the presence of high salt concentrations—with one exception: the size of the mitochondria in *D. hansenii*, where a pronounced decrease in mitochondrial diameter was observed with increasing salt concentrations. Unless this is compensated by the length of the mitochondria or an increase in the number of mitochondria per cell, which has not been observed, this decrease would correspond to a lower mitochondria capacity. The observed decrease in size correlates with a pronounced decrease in respiration found at increasing salt concentrations (Norkrans, 1968). The mitochondrial membranes are distinct even in cells from 20% NaCl and any degeneration of the cristae, such as observed in *Endomycopsis fibuligera* in 0.5 M NaCl (Hiraoka and Takada, 1968) does not occur in *D. hansenii*.

"Extreme halophilism is a genetic characteristic of the organism and not a physiological adaptation" (Brock, 1969). The lack of very marked structural differences between cells grown at as extremely different

conditions as those offered to *D. hansenii* inclined us to the same conclusions about the halotolerant yeasts.

The results of this study show, how unusual physiological properties such as the capacity of survival and growth at NaCl concentrations near the solubility limit of the salt may be correlated with ultrastructural features not present in a less halotolerant organism.

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References

Bahr, G. F.: Osmium tetroxide and ruthenium tetroxide and their reactions with biologically important substances. Exp. Cell Res. **7**, 457—479 (1954).

Besson, M.: Les membranes de levures au microscope électronique. Bull. Trin. Soc. mycol. Fr. **82**, 489—503 (1966).

Bowes, B. G.: Electron microscopic observations on myelin-like bodies and related elements in *Glechoma hederacea* L. Z. Pflanzenphysiol. **60**, 414—417 (1969).

Brock, T. D.: Microbial growth under extreme conditions. Symp. Soc. gen. Microbiol. XIX, 15—41 (1969).

Buckley, P. M., Sjaholm, V. E., Sommer, N. F.: Electron microscopy of *Botrytis cinerea* conidia. J. Bact. **91**, 2037—2044 (1966).

Clark-Walker, G. D., Linnane, A. W.: The biogenesis of mitochondria in *Saccharomyces cerevisiae*. J. Cell Biol. **34**, 1—14 (1967).

Curgy, J.-J.: Influence de mode de fixation sur la possibilité d'observer des structures myéliniques dans les hépatocytes d'embryons de poulet. J. Microscopie **7**, 63—80 (1968).

Frey-Wyssling, A., Mühlethaler, K.: Ultrastructural plant cytology. Amsterdam: Elsevier Publ. Comp. 1965.

Grove, S. N., Bracker, C. E., Morré, D. J.: Cytomembrane differentiation in the endoplasmic reticulum—Golgi apparatus—vesicle complex. Science **161**, 171—173 (1968).

Hiraoka, J., Takada, H.: Changes in mitochondrial structure of dimorphic cells of *Endomycopsis fibuligera* in saline medium. Protoplasma (Wien) **66**, 311—325 (1968).

Jensen, W. A.: Botanical histochemistry. San Francisco: W. H. Freeman & Co. 1962.

Kodama, K., Kyono, T., Naganishi, H., Takahara, Y.: Taxonomic studies on the genus *Debaryomyces* (III). *Debaryomyces fransiscae* (Capriotti) nov. comb. J. Ferment. Technol. **42**, 1—6 (1964a).

— — — — Taxonomic studies on the genus *Debaryomyces* (IV). *Debaryomyces nilssoni* (Capriotti) nov. comb. J. Ferment. Technol. **42**, 655—660 (1964b).

Linnane, A.W., Vitols, E., Nowland, P. G.: Studies on the origin of yeast mitochondria. J. Cell Biol. **13**, 345—349 (1962).

Luft, J. H.: Improvements in epoxy resin embedding methods. J. biophys. biochem. Cytol. **9**, 409—414 (1961).

Marquardt, H.: Der Feinbau von Hefezellen im Elektronenmikroskop. II. Mitt. *Saccharomyces cerevisiae*-Stämme. Z. Naturforsch. **17**b, 689—695 (1962).

Matile, Ph., Wiemken, A.: The vacuole as the lysosome of the yeast cell. Arch. Mikrobiol. **56**, 148—154 (1967).

Mazia, D., Brewer, P. A., Alfert, M.: The cytochemical staining and measurement of protein with mercuric bromphenol blue. Biol. Bull. **104**, 57—67 (1953).

Moor, H., Mühlethaler, K.: Fine structure of frozen-etched yeast cells. J. Cell Biol. **17**, 609—628 (1963).

Norkrans, B.: Studies on marine occurring yeasts: Growth related to pH, NaCl concentration and temperature. Arch. Mikrobiol. **54**, 374—392 (1966).

— Studies on marine occurring yeasts: Respiration, fermentation and salt tolerance. Arch. Mikrobiol. **62**, 358—372 (1968).

— Kylin, A.: Regulation of K:Na ratio and of the osmotic potential in relation to salt tolerance in yeasts. J. Bact. **100**, 836—845 (1969).

Palade, G. E.: A study of fixation for electron microscopy. J. expt. Med. **95**, 285—297 (1952).

Pearse, A. G. E.: Histochemistry theoretical and applied, 3rd ed., Vol. 1. London: J. & A. Churchill. Ltd. 1968.

Robinow, C. F., Marak, J.: A fiber apparatus in the nucleus of the yeast cell. J. Cell Biol. **29**, 129—151 (1966).

Smith, D. G., Marchant, R.: Lipid inclusions in the vacuoles of *Saccharomyces cerevisiae*. Arch. Mikrobiol. **60**, 340—347 (1968).

Venable, J. H., Coggeshall, A.: A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. **25**, 407—408 (1965).

Vitols, E., North, R. J., Linnane, A. W.: Studies on the oxidation metabolism of *Saccharomyces cerevisiae*. I. Observations on the fine structure of the yeast cell. J. biophys. biochem. Cytol. **9**, 689—699 (1961).

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