Biochimica et Biophysica Acta 899 (1987) 265–275
Elsevier

BBA 73560

The plasma membrane of yeast protoplasts exposed to hypotonicity becomes porous but does not disintegrate in the presence of protons or polyvalent cations

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(Received 19 December 1986)

Key words: Calcium; Plasma membrane; Protoplast; (*S. cerevisiae*)

Protoplasts of *Saccharomyces cerevisiae* swelled, lysed and disintegrated when exposed to hypotonic solutions at neutral pH. At pH 4.5 or lower the hypotonically treated protoplasts did not disintegrate and they retained their intracellular proteins, nucleic acids and nucleotides. However, they became leaky for K^+ and Ca^{2+}, indicating that pores had been created in the surface membrane, relaxing the osmotic stress. Upon readjustment of pH to neutral, the hypotonically treated protoplasts released the intracellular content and disintegrated. Also, at low pH, protoplasts did not swell in isotonic ammonium acetate and were refractory to the permeabilizing effect of nystatin and to lysis with low concentrations of detergents. Protoplasts were similarly protected against lysis and disintegration by hypotonic treatment or by detergents, even at neutral pH, if the incubation media contained polyvalent cations, especially Zn^{2+}, La^{3+}, spermine, and Ca^{2+} chelated with EDTA. The protoplasts exposed to hypotonic stress at low pH did not respire and could not regenerate into viable cells. Effects of H^+ and polyvalent cations on intramembrane forces acting between molecules of membrane phospholipids are considered along with possible changes in interactions between membrane proteins.

Introduction

For over a decade, the widely accepted 'fluid mosaic' model of biomembranes [1] has guided biological research and has received abundant experimental support. This model considers the membrane as a viscous fluid consisting of lipids in bilayer arrangement and of proteins which undergo lateral diffusion within the plane of the membrane. The lateral mobility of some integral proteins can be restricted because of their interactions with immobile matrices at the cytoplasmic or external faces of the plasma membrane.

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With tribute to the aesthetic appeal of the original formulation, recent advances may demand modification and extension of the simple model. Control of the lateral mobility of membrane proteins seems to be more complex than originally depicted [2,3] and the existence of specialized domains with distinct topology, structure and functions may be a prominent feature of many membranes [4].

In addition, it appears pertinent to redirect emphasis to systematic studies of Evans and his group on mechanical properties of erythrocyte membranes [5,6] which have led to a 'solid-liquid composite' model of the membrane [7]. The membrane's solid-like character has been ascribed to the presence of peripheral membrane proteins, in particular spectrin, which form a structural matrix

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on the cytoplasmic surface [7]. The contribution of external carbohydrate residues of glycoproteins, forming a 'lattice' over the cell surface [8], has also been considered. The lipid bilayer would act like a two-dimensional 'liquid sealer' for these matrices [7]. Spectrin, originally thought to be a protein specific to erytrocytes, giving them some unique mechanical resistance [9], has now been recognized as a general constituent of membranes, at least in vertebrates (reviewed in Ref. 10). Similar proteins may be envisaged to function in other membranes [10]. A concept of 'membrane skeleton' has emerged [11], and the forces that hold molecules of 'skeletal' proteins together add to those recognized to exist in lipid membranes.

As an approach to the problems of membrane organization, the stability of yeast protoplast membranes was studied in the present work. The yeast protoplast membrane may be simpler in organization than is the plasma membrane of higher eucaryotes and can be easily examined after the yeast cells have been stripped of their protective cell wall. The results show that protons and polyvalent cations could considerably protect the protoplast membrane against disintegration by hypotonic stress or by detergents, and allow some considerations on the nature of intramembrane forces.

Materials and Methods

Nystatin, Tween X-100 and SDS were from Serva. Radioactive adenine and L-leucine were purchased from UVVR, Prague, and $CaCl_2$ from the Institute of Nuclear Research, Swierk. Other chemicals were obtained from Lachema.

Saccharomyces cerevisiae DT XII was cultured aerobically at 30 °C for 24 h in a semi-synthetic medium containing salts, 0.5% peptone, 0.75% yeast autolysate and 2% glucose as carbon source. Protoplasts were prepared by treating the cells with snail-gut juice [12]. They were either used for experiments immediately or stored at 0 °C as a suspension in 1.2 M sorbitol for up to 5 days. The storage did not modify the properties studied in this work.

To measure the properties of protoplasts, 0.02 vol. of a dense suspension of protoplasts in 1.2 M sorbitol (approx. $2 \cdot 10^9$ protoplasts per ml) were added to slightly buffered solutions of sorbitol of varying tonicities, which had been adjusted to different pH and temperatures, and incubated for 10 min. This period was found to be long enough for osmotic equilibration of the protoplasts under all the conditions examined except for a temperature of 45 °C at which the equilibration could not be achieved and continuous slow lysis was taken place. The extent of swelling and lysis was then evaluated by four procedures: (1) by visual inspection under a phase-contrast microscope; (2) by electron microscopy of freeze-etched specimen; (3) by determining turbidity of the suspension as absorbance at 800 nm which was proportional to the number of protoplast that had not disintegrated; (4) by determining absorbance at 260 nm of the supernatant after spinning down the protoplasts as a measure of ultraviolet-absorbing material released from the lysed protoplasts. For continual measurement of swelling and lysis, the rate of absorbance decrease at 650 nm was followed in a recording spectrophotometer.

Release of other cell constituents was measured in the following way. Cells were grown in the semi-synthetic medium containing [2,8-^{3}H]adenine (0.56 μg/ml, 740 kBq/ml) and L-[U-^{14}C]leucine (2.2 μg/ml, 185 kBq/ml) for 24 h, and protoplasts were prepared in the usual way. Radioactivity of ^{3}H and ^{14}C was counted simultaneously in a scintillation counter. Washed protoplasts exhibited approx. $1.3 \cdot 10^7$ dpm of ^{3}H and $1.2 \cdot 10^6$ dpm of ^{14}C per 10^8 protoplasts. A dense suspension of the protoplasts was added into incubation mixtures to contain $1 \cdot 10^8$ protoplasts/ml. After incubation, the mixtures were centrifuged at 2500 $\times g$ for 10 min. From the supernatants, 0.2 ml were taken for determination of protein and RNA and 0.8 ml for determination of DNA by the procedure of Hartwell [13]. The difference between total ^{3}H radioactivity of the supernatant and radioactivities of DNA and RNA was assigned to free nucleotides (about 75% of releasable ^{3}H radioactivity). Approx. 9% of ^{3}H and 16% of ^{14}C radioactivity remained associated with protoplast remnants after extensive lysis of labelled protoplasts in water. In calculations, appropriate corrections were made for this non-releasable radioactivity. Protein released from protoplasts was also chemically estimated by the procedure of

Lowry et al. [51] after precipitation of 1 ml of supernatant with 10% trichloroacetic acid. In addition, the release of a specific protein, α-glucosidase (EC 3.2.1.20) was followed by determining the activity of the enzyme in the supernatants [14]; for these measurements, protoplasts were prepared from cells grown on 2% maltose instead of glucose.

To measure the release of Ca^{2+}, cells (15 mg dry weight) were loaded with radioactive Ca by incubating them in 25 mM Tris-HCl (pH 7.5) and 50 mM glucose with 0.115 mM $^{45}CaCl_2$ (approx. 100 MBq/mg Ca) at 30 °C for 40 min. Protoplasts were prepared, incubated at varying pH and tonicities, and the radioactive Ca released into the supernatants was measured by scintillation counting.

K^+ released from protoplasts into the supernatants was measured by flame photometry.

For electron microscopy, the freeze-etching technique of specimen preparation was used. A drop of a dense suspension of protoplasts was put on a copper disc and the specimen was rapidly frozen in freshly melted Freon 22 (cooling rate 100 °C/s). Platinum-carbon replicas were prepared by a standard procedure in a Balzers unit BA 360 M. The etching time was 1 min. The replicas were cleaned with 70% (w/v) sulphuric acid and washed several times in distilled water before they were mounted on formvar-coated grids and examined in a Tesla BS 500 electron microscope.

Results

Lysis and disintegration of protoplasts at different tonicities, pH and temperatures

Microscopic observation established that the lysis of protoplasts was preceded by swelling. Consequently, the two processes will not be distinguished and considered separately. Any considerable change in the turbidity of a protoplast suspension always reflected lysis in addition to swelling [15].

The extent of the lysis of yeast protoplasts in solutions of sorbitol of varying tonicities was found to be strongly dependent on pH and temperature. Fig. 1A depicts a typical dependence of the amount of ultraviolet-absorbing material released from protoplasts on the tonicity of sorbitol solutions at 20 °C and different pH. The same dependence, only symmetrically inverted, was found between the absorbance of the protoplast suspensions at 800 nm, which indicated the amount of non-lysed protoplasts, and the tonicities. Similar plots were obtained for protoplasts suspended in solutions of the same tonicities and pH, but at different temperatures. From a set of such plots, obtained from three independent experiments, the extent of lysis of protoplasts in 0.65 M sorbitol was derived, measured both by absorbance at 800 nm and by the amount of released ultraviolet-absorbing material. Fig. 1B shows the extent of the lysis, measured by the latter method, as a function of pH at different temperatures, and Fig. 1C as a function of temperature at different pH levels.

As can be seen in Fig. 1, the ability of the protoplast membrane to escape lysis and disintegration in hypotonic solutions was increasing with decreasing pH. At pH 3.0 the protoplasts did not disintegrate and released the ultraviolet-absorbing material even when suspended in water slightly buffered with 10 mM buffer. Fig. 1 also shows that the ability to withstand lysis was also increasing with decreasing temperature. It should

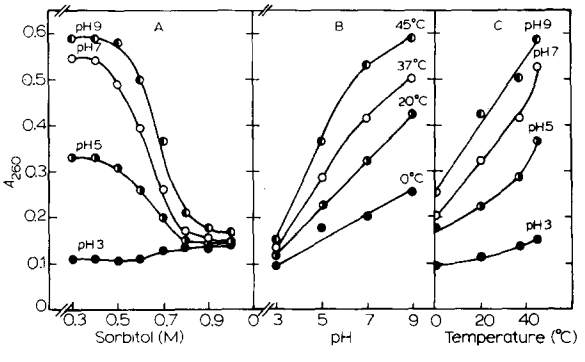


Fig. 1. Stability of protoplasts as a function of concentration of suspending medium, of pH and of temperature. Protoplasts were suspended at different pH and temperatures in the incubation medium containing 10 mM citrate-Tris buffer and sorbitol of varying concentrations, incubated for 10 min and centrifuged. Absorbance at 260 nm of the supernatant, shown on the coordinate, gives the amount of ultraviolet-absorbing material and correlates with the number of disintegrated protoplasts. A, incubation at 20 °; B and C, from plots similar as in A, but at varying temperatures, the data for protoplasts suspended in 0.65 M sorbitol are shown at different pH and temperatures.

be noted that at 45 °C the protoplasts were not stable and were undergoing slow lysis even in the hypertonic 1.5 M sorbitol.

Microscopic inspection corroborated the conclusion that the protoplasts did not lyse in hypotonic solutions at low pH. The graded response of protoplast suspensions was due to the different resistance of particular protoplasts to lysis, each protoplast apparently exhibiting an all-or-none behaviour. When compared with protoplasts suspended in an isotonic solution at neutral pH, the protoplasts in hypotonic solutions at low pH appeared smaller – somehow shrunken – and tended to agglutinate.

When the protoplasts that had been exposed to a hypotonic solution at low pH were centrifuged and resuspended in the hypotonic solution at neutral pH they lysed, but under the microscope they appeared as deformed ghosts with a highly granulated interior.

The protoplasts were also considerably protected from disintegration in hypotonic solutions by Ca^{2+} at neutral pH. Mg^{2+} was much less efficient. Interestingly, the protective effect of Ca^{2+} was strongly potentiated by EDTA – EDTA alone showing no effect – and became maximal when EDTA was present in the same concentrations as was Ca^{2+}.

In a previous report [15], protoplasts of *Saccharomyces carlsbergensis* were described to be less resistant to osmotic lysis at pH 5.3 than at pH 7.4 and some chelating agents, such as citrate and EDTA, lowered the resistance of protoplasts to lysis. With our strain we did not observe such effects and our data obtained with three other strains of *S. cerevisiae* were identical with those presented here.

Other treatments inducing lysis and disintegration of protoplasts at different pH

Yeast protoplasts swelled and lysed in isotonic ammonium acetate due to the accumulation of salt by virtue of influx of NH_3 and acetic acid. The rate of swelling and lysis was pH-dependent, increasing with increasing pH (Fig. 2). At a low pH, the protoplasts did not swell and disintegrate at all. Upon raising the low pH swelling and lysis became apparent, although the final absorbance at 650 nm of the protoplast suspensions remained higher than when the swelling and lysis had not been preceded by exposure of the protoplasts to low pH (Fig. 3). Microscopic inspection bore out that the protoplasts did not lyse at pH 4.5, but did so at pH 7.5 and 9.0. However, when first exposed to pH 4.5 and then transferred to pH 7.5 or 9.0, the protoplasts did not entirely disintegrate but remained as tiny spheres of irregular contour and shape.

Influx of K^+ into yeast protoplasts and their consequent swelling and lysis in potassium acetate could be induced by nystatin, which is known to form K^+ permeable pores in the yeast cytoplasmic membrane [16]. As shown in Fig. 4, nystatin-induced swelling and lysis did not take place at low pH. Similarly, a low pH protected the protoplasts

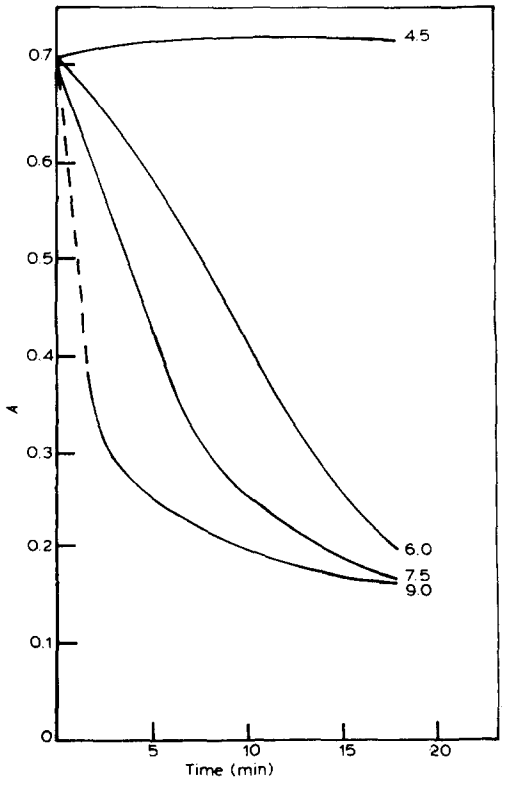


Fig. 2. Swelling and lysis of protoplasts in ammonium acetate at different pH. Protoplasts suspended in 1.0 M sorbitol (100 µl) were added to 2.0 ml of 0.5 M ammonium acetate, adjusted to pH shown on the curves, and the changes of absorbance of the suspension at 650 nm were recorded at room temperature.

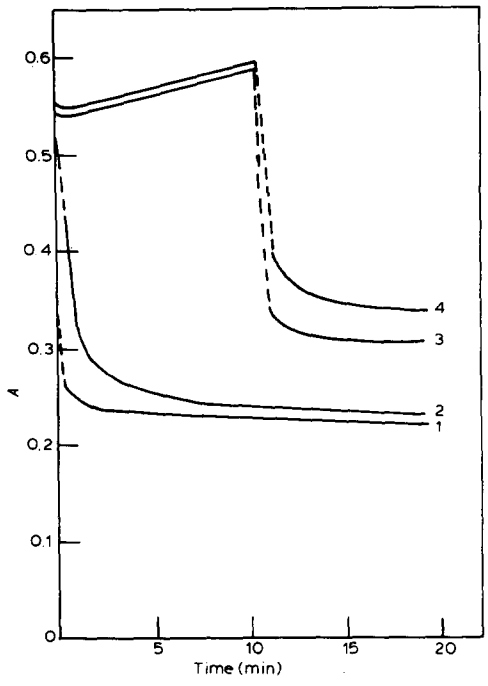
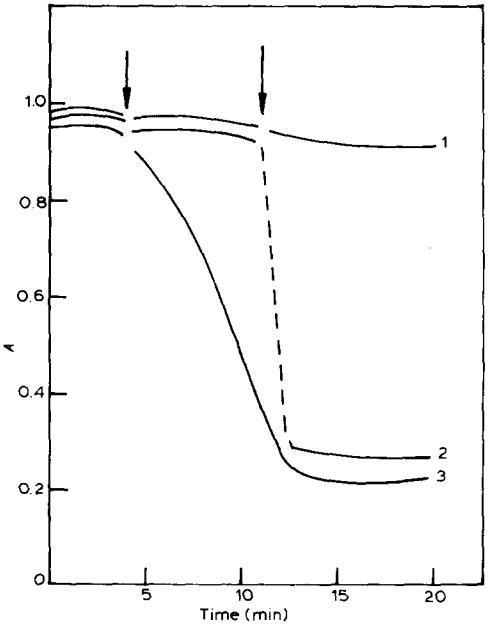


Fig. 3. Swelling and lysis of protoplasts at different pH. Protoplasts suspended in 1.0 M sorbitol (100 μl) were added 2.0 ml of 0.5 M ammonium acetate at pH 9.0 (cuvette 1), pH 7.5 (cuvette 2), and pH 4.5 (cuvette 3 and 4), and absorbance of the suspensions at 650 nm was recorded at room temperature. After 10 min, NH_4OH was introduced such that the pH changed from 4.5 to 9.0 in cuvette 3, and from 4.5 to 7.5 in cuvette 4.



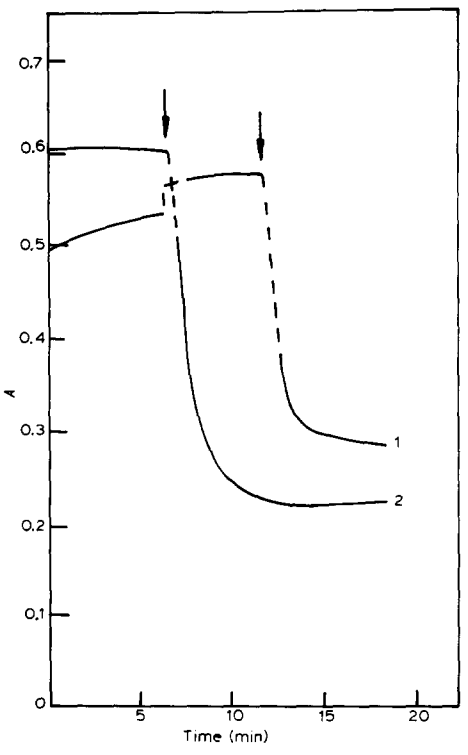


Fig. 4. The effect of nystatin on swelling and lysis of protoplasts at different pH. Protoplasts suspended in 1.0 M sorbitol (100 μl) were added into 2.0 ml of 0.5 M potassium acetate, adjusted to pH 4.5 (cuvette 1), or to pH 7.5 (cuvette 2), and absorbance of the suspension at 650 nm was recorded at room temperature. At the first arrow nystatin (250 μM) was added to both cuvettes. At the second arrow, NH_4OH was added into cuvette 1 to raise pH from 4.5 to 7.5.

against metabolic swelling and lysis [17,18], which could be observed at neutral pH.

As can be seen in Fig. 5, 0.1% Triton-X-100 caused rapid lysis of protoplasts (also detected by microscopic detection) when the protoplasts were incubated in isotonic sorbitol at neutral pH. It did

Fig. 5. The effect of Triton X-100 on swelling and lysis of protoplasts. Protoplasts suspended in 1.0 M sorbitol (100 μl) were added into 2.0 ml of 1.0 M sorbitol, buffered at pH 4.5 with 10 mM sodium succinate (cuvette 1 and 2), or at pH 7.5 with 10 mM Tris-HCl (cuvette 3), and absorbance at 650 nm was recorded at room temperature. At the first arrow Triton X-100 (0.1%) was added into all three cuvettes. At the second arrow, NaOH was added into cuvette 2 to raise pH from 4.5 to 7.5.

TABLE I

RELEASE OF K^+, PROTEINS, FREE NUCLEOTIDES AND NUCLEIC ACIDS FROM PROTOPLASTS EXPOSED TO DIFFERENT pH AND DIFFERENT TONICITIES

A small volume of a dense suspension of radioactively labelled protoplasts in 1.0 M sorbitol was mixed in the incubation mixtures and incubated at room temperature for 5 min. The mixtures were buffered at pH 4.3 with 20 mM succinate, and at pH 7.5 with 20 mM Tris-HCl. The mixtures were then centrifuged and substances released from the protoplasts were determined in the supernatants. The pellets were resuspended in the original volumes of 0.075 M sorbitol/20 mM Tris-HCl (pH 7.5), centrifuged, and the supernatants were analyzed for additional release. The latter values are shown in parentheses. The experiment was repeated with similar results.

Incubation mixture	Substances released (%)				
	K^+	proteins	free nucleotides	DNA	RNA
pH 7.5: 1.0 M sorbitol	0.5	6.5 (73.7)	4.9	9.2 (78.0)	4.2 (106.6)
0.075 M sorbitol	78.5	85.1 (1.4)	98.0	86.4 (5.6)	56.3 (52.0)
0.075 M sorbitol/ 10 mM $CaCl_2$	78.0	54.1 (12.1)	45.1	98.0 (2.3)	62.7 (47.1)
0.075 M sorbitol/ 10 mM EDTA	101.0	79.9 (7.3)	92.0	91.6 (2.4)	55.7 (22.4)
0.075 M sorbitol/ 10 mM $CaCl_2$/ 10 mM EDTA	97.5	28.6 (36.6)	10.8	29.3 (58.0)	6.0 (97.2)
pH 4.3: 1.0 M sorbitol	18.0	1.5 (77.6)	4.4	7.0 (74.0)	0.4 (112.3)
0.075 M sorbitol	96.5	3.0 (76.3)	12.3	5.0 (95.2)	0.8 (97.2)
0.075 M sorbitol/ 10 mM $CaCl_2$	85.7	15.5 (28.3)	12.1	15.6 (92.0)	2.1 (114.7)
0.075 M sorbitol/ 10 mM EDTA	101.2	3.8 (77.2)	6.5	4.0 (92.0)	0.8 (86.0)
0.075 M sorbitol/ 10 mM $CaCl_2$/ 10 mM EDTA	104.0	21.0 (44.7)	10.8	20.0 (69.6)	4.5 (98.8)

not affect the protoplasts at pH 4.3, but once the low pH was raised to neutral, rapid lysis set in.

Release of intracellular constituents from protoplasts

Various substances released from protoplasts incubated under hypotonic conditions were measured in order to assess in how far the integrity of the protoplast membranes remained intact. The results are summarized in Table I.

At pH 7.5, protoplasts incubated in 1.0 M sorbitol did not release into the medium any substantial amounts of K^+, proteins, free nucleotides, DNA or RNA. In hypotonic 0.075 M sorbitol, in which they lysed, these substances were found mostly in the medium. The fraction of proteins released, measured by radioactivity, closely corresponded to that found by chemical determination of proteins or by estimation of α-glucosidase activity liberated from maltose-grown cells. Although Ca^{2+} alone exhibited a slight protective effect in some cases, the combination of Ca^{2+} and EDTA very efficiently prevented the release of macromolecules from the hypotonically treated protoplasts. In accord with the results from optical measurements, the radioactive procedure also indicated that, in the presence of Ca^{2+} and EDTA, free nucleotides were essentially retained within the hypotonically treated protoplasts. However, all the intracellular K^+ was released.

At pH 4.3, proteins, free nucleotides, DNA and RNA remained entrapped inside protoplasts in a hypotonic mixture just as well as they did in an isotonic sorbitol. However, at the same time, all the cellular K^+ leaked out of the hypotonically treated protoplasts. Similarly, intracellular Ca^{2+} was released by hypotonic treatment at both neutral and acid pH (Table II).

When the protoplasts, protected from hypo-

TABLE II

RELEASE OF RADIOACTIVE Ca FROM PROTOPLASTS

Protoplasts ($8 \cdot 10^8$) containing 193 pmol $^{45}CaCl_2$ were incubated in the incubation mixtures for 5 min at room temperature, centrifuged and ^{45}Ca released was measured in the supernatants by scintillation counting. The mixtures were buffered at pH 7.5 with 20 mM Tris-HCl, and at pH 4.0 with 20 mM sodium phthalate.

Incubation mixture		^{45}Ca released (%)
pH 7.5:	1.0 M sorbitol	3.2
	0.1 M sorbitol	99.9
	0.1 M sorbitol/10 mM $CaCl_2$	83.3
	0.1 M sorbitol/10 mM EDTA	107.4
	0.1 M sorbitol/10 mM $CaCl_2$/ 10 mM EDTA	107.9
pH 4.0:	1.0 M sorbitol	5.1
	0.1 M sorbitol	100.1

tonic disintegration by either Ca^{2+} + EDTA or low pH, were resuspended in 0.075 M sorbitol at pH 7.5 they disintegrated and released the remaining material. This indicates that the protection was reversible and could not be accounted for by protein denaturation or by some other irreversible processes.

The effects of different cations upon protoplasts

The dramatic effect of Ca^{2+}, in combination with EDTA, upon the stability of protoplasts in hypotonic solutions was compared with the effects of other cations. Both measurement of absorbance of protoplast suspensions and microscopic inspection indicated that, in 0.075 M sorbitol buffered with 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$ alone or in the presence of 10 mM EDTA hardly prevented the disintegration of protoplasts; neither did $SrCl_2$ or $BaCl_2$. $ZnCl_2$ (10 mM) exhibited a very powerful protective effect, also in the absence of EDTA; the protoplasts did not disintegrate, although their shape became irregular, and they did not release ultraviolet-absorbing substances and proteins. Essentially similar stabilizing effects were found with $CuCl_2$ and $CdCl_2$, and slightly less with $CoCl_2$, $MnCl_2$ and $NiCl_2$. Stabilization was also observed with $LaCl_3$, even at 1 mM concentration, at which the other salts were ineffective, and also with spermine (5 mM), polylysine (200 μg/ml) and protamine (50 μg/ml).

TABLE III

DISINTEGRATION AND LYSIS OF PROTOPLASTS TREATED WITH $ZnCl_2$

A small volume of a dense suspension of protoplasts in 1.0 M sorbitol was added into tubes containing 1.0 M sorbitol buffered with 20 mM Tris-HCl (pH 7.5), and, where indicated, 10 mM $ZnCl_2$. Detergents were mixed in and absorbance of the suspensions measured at 650 nm. A drastic drop of absorbance corresponded to complete lysis of protoplasts detected microscopically.

Additions into sorbitol	Absorbance
None	0.78
0.05% Triton X-100	0.06
0.15% SDS	0.07
$ZnCl_2$	0.86
$ZnCl_2$/0.8% Triton X-100	0.85
$ZnCl_2$/0.15% SDS	0.94
$ZnCl_2$/0.25% SDS	0.91
$ZnCl_2$/0.8% SDS	0.10 [a]

[a] Absorbance after 10 min with SDS. Without $ZnCl_2$, lysis with the detergents was complete within 1 min, in the presence of $ZnCl_2$ it was delayed with SDS and no lysis was observed with Triton X-100 within 1 h.

The effect of $ZnCl_2$ was examined in more detail. As indicated by the results of Table III, protoplasts treated with 10 mM $ZnCl_2$ in isotonic sorbitol at neutral pH did not lyse in the presence of Triton X-100, and they were more refractory to lysis with SDS. The responses were similar in hypotonic (0.075 M) sorbitol.

Respiration and viability of hypotonically treated protoplasts

The respiration of protoplasts exposed to low pH (< 4.5) was considerably diminished even in an isotonic solution – when compared to protoplasts respiring at neutral pH – and was completely abolished in a hypotonic solution. Ca^{2+} or EDTA did not afford much protection to the respiratory capacity, either.

Protoplasts exposed to hypotonicity hardly regenerated to viable cells, and neither low pH nor Ca^{2+} + EDTA afforded protection against this loss in regeneration ability.

Electron microscopy of protoplasts exposed to hypotonicity

Morphology of control protoplasts, incubated in 1.0 M sorbitol at pH 7.5, is shown in Fig. 6A

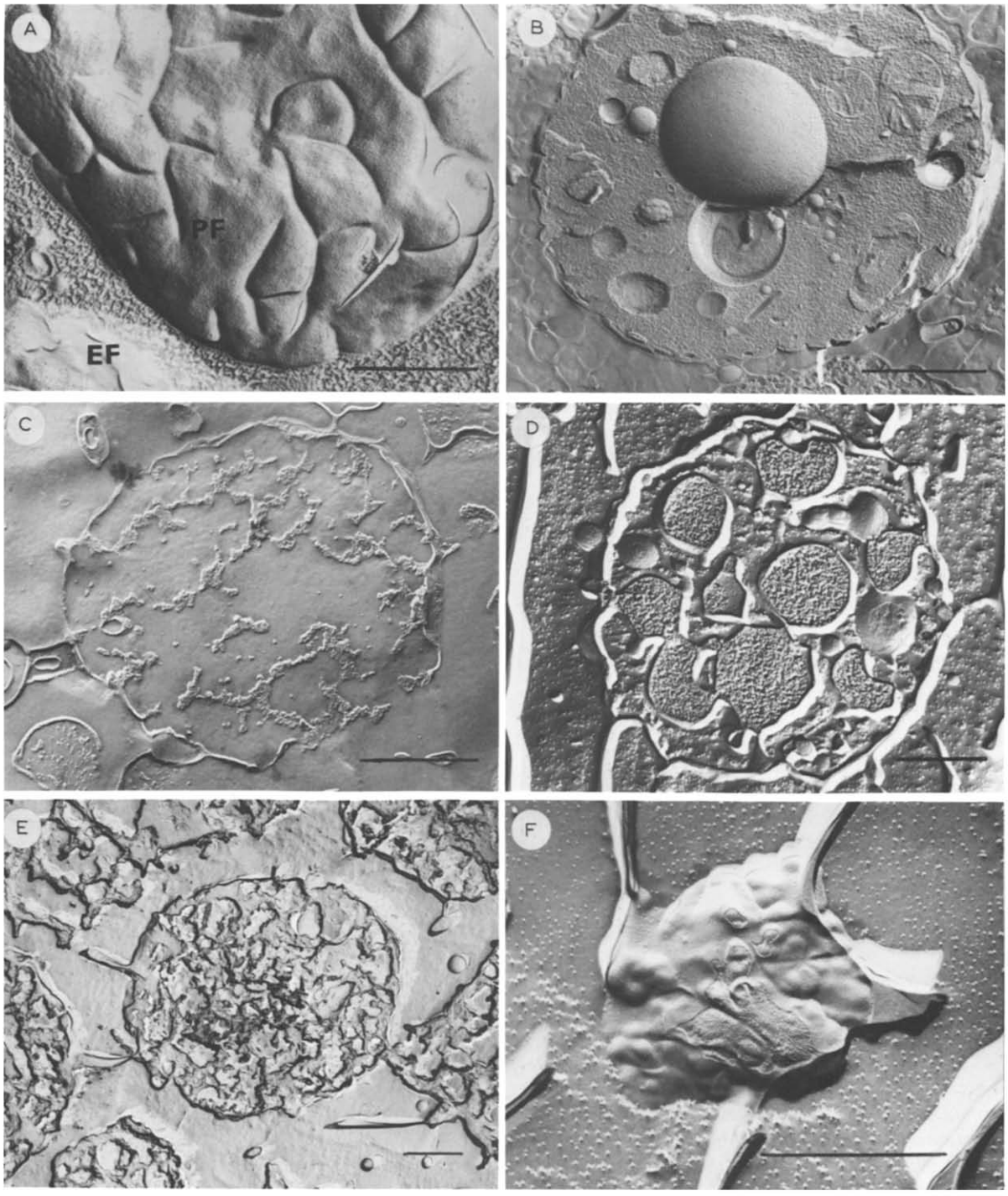


Fig. 6. Freeze-fracture electron microscopy of protoplasts exposed to different pH and different tonicities. A and B, 1.0 M sorbitol (pH 7.5); C, 0.1 M sorbitol (pH 9.0); D, 0.1 M sorbitol (pH 7.5); E, 0.1 M sorbitol (pH 4.0); F, 0.1 M sorbitol (pH 7.5), 10 mM $CaCl_2$. PF, plasmic fracture face. EF, exoplasmic fracture face. Bars represent 1 μm.

and B. The plasma membrane exhibited invaginations joined end-to-end and forming long furrows. The plasmic fracture face (PF) of the split plasma membrane was densely covered with plasma membrane particles, partly in hexagonal arrays. The plasma membrane particles were only sparsely represented on the exoplasmic fracture face (EF). The interior of the protoplasts was normal in appearance, with finely granulated cytoplasm. The same morphology was found in protoplasts kept in 1.0 M sorbitol at both acidic and alcaline pH.

Protoplasts treated hypotonically (0.1 M sorbitol) were modified in all cases. Effects of pH were apparent in their detailed morphology. At pH 9.0, those protoplasts that escaped complete disintegration were nearly empty and the plasma membrane was clearly discontinuous (Fig. 6C). At pH 7.5, protoplasts which had not completely disintegrated retained organelles and their ghosts, indicating that the holes in the plasma membrane were not large enough to allow expulsion of the organelles (Fig. 6D). At pH 4.0, the intracellular content was preserved, but in the form of a dense mesh of artificial trabeculae (Fig. 6E). A protective effect of Ca^{2+} in a hypotonic treatment in 0.1 M sorbitol at pH 7.5 is evident from Fig. 6F, showing that the plasma membrane of the protoplasts retained cohesion and continuity.

Discussion

Yeast protoplasts exposed to hypotonicity at neutral or alkaline pH largely disintegrated. Under the osmotic pressure the plasma membrane was broken down and the intracellular content expelled into the surrounding solution. At low pH, or in the presence of polyvalent cations at neutral pH, the protoplasts submitted to osmotic stress preserved their gross morphological appearance and essentially retained their intracellular proteins, nucleic acids and nucleotides. However, they became leaky for K^+ and Ca^{2+}, their capacity to respire was impaired, their intracellular structures were considerably damaged and they were not viable. It can be envisaged that under the latter conditions the membrane did not break down, but only pores large enough to allow ions and water equilibrate across the membrane and relax the osmotic stress were formed in the stretched plasma membrane. Among polyvalent cations, Zn^{2+} was particularly efficient in protection. The stabilizing effect of Zn^{2+} on biological membranes had already been described [19] and exploited in procedures for isolation of animal plasma membranes [20,21]. Ca^{2+} was found more eficient when complexed with EDTA. The potentiation of biological effects of Ca^{2+} by its chelation with EDTA or EGTA had also been described previously at several occasions [22–27].

The similarity in the effects of protons and polyvalent cations in lipid membranes has been observed in many studies and is largely accounted for by the ability of protons and polyvalent cations to neutralize repulsive charges of the polar groups of membrane phospholipids [28–35]. In protoplasts, such a neutralization may have two consequences. First, a decrease in repulsive charges may effectively increase the forces that hold the membranes together, allowing the membrane exposed to osmotic disbalance to stretch and became porous, but not disintegrate. The forces would include hydrogen bonds between phosphates in head groups and van der Waal's interactions between alkyl chains [34]. Second, the neutralization of charges would diminish hydration of the head groups, allowing tighter packing of phospholipids and simultaneously an increase in hydrophobic forces. The tight packing would impede the intercalation of nystatin and detergents into the lipid bilayer. This would explain why the membranes were found resistant toward these compounds as long as the packing was not relaxed by removing divalent cations or restoring neutral pH.

In the same vein, the monotonous decrease in resistance of protoplasts to hypotonic treatment with rising temperature suggests that the forces that oppose the osmotic pressure may become weaker as the thermal motion in the membrane gets more intensive and, at the same time, the water content on the plasma surface may increase, pushing the lipid head groups apart [36].

If these simple considerations, based on phospholipid behaviour, were valid protons and polyvalent cations should affect artificial lipid vesicles in a manner similar to their effect on the protoplast membranes. Unfortunately, as stated in a recent review [37], "it is amazing that there are so few experimental works on the rupture of mem-

branes of giant liposomes in osmotic swelling". Any relevant observations are lacking. On the other hand, an apparent resistance to hypotonic disintegration at low pH was observed with protoplasts of *Bacillus megaterium* [39] and *Escherichia coli* [39], and was vaguely interpreted as being due to a 'reduction of the solubility of protoplasm' [39]. A stabilizing effect of polycations on membranes of bacterial protoplasts [40–43], of erythrocytes [44] and of animal non-erythroid cells [45] has also been observed. The observations may recall the once famous idea of Heilbrunn [46] about the 'surface precipitation reaction' whereby Ca^{2+} caused stiffening of newly formed cytoplasmic boundaries.

The deformed yeast protoplasts obtained upon hypotonic treatment at low pH or at neutral pH in the presence of polyvalent cations resemble erythrocyte ghosts. The latter exhibit the shape of intact erythrocytes and, under appropriate conditions, can be completely resealed [47]. Intact erythrocyte ghosts became fragmented during removal of spectrin and they also lost the coating of filamentous material normally present on the inner surface of the membrane [48]. Proton-induced aggregation of spectrin was considered responsible for changes in mechanical properties of erythrocytes at low pH [49].

Whereas yeast protoplasts retained their shape only under specific conditions delineated in this paper and, under usual conditions, disintegrated in hypotonic solutions, Ehrlich ascites tumor cells were found to swell in hypotonic solutions at neutral pH but to retain their shape (Kováč, L. and Böhmerová, E., unpublished data; see also Ref. 50).

Combining the observations made on yeast protoplasts with those on bacterial protoplasts, erythrocyte ghosts and tumour cells, the following interpretation can be put forward. Along with electric, van der Waal's and hydrophobic forces, implicated in liquid-like ordering of phospholipids in biological membranes, additional forces acting between molecules of proteins may be important for membrane stability. The protein 'membrane skeleton' [11] may be more complex in animal cells than in fungal or bacterial cells and give the membrane some solid-like features and mechanical stability. In fungi and in bacteria it is mainly the cell wall that affords mechanical protection. Under particular conditions, stiffening can be induced in membranes of fungi and bacteria, making them similar to animal plasma membranes. Such a process may involve restructuring of membrane proteins in the electric fields produced by protons and polyvalent cations. Although induced artificially in experiments, such stiffening may not be without physiological relevance. Its further study may provide a deeper insight into the membrane architecture.

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