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OSMOTIC EFFECTS ON THE PLASMA MEMBRANE OF THYMOCYTES

H.C. BAUER, V. SPETH and G. BRUNNER
Max-Planck Institut für Immunbiologie, Freiburg (G.F.R.)
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Summary

Rat thymocytes were treated with media of different hypotonic osmolarity and the resulting damage of the plasma membrane was monitored by measuring the permeation of biochemical markers (K^+, fluorescein, lactate dehydrogenase) and by observation of morphological changes in the plasma membrane using the freeze-etch technique. Release is dependent on the molecular weight of the markers; those with higher molecular weights permeate predominantly at a lower osmolarity of the medium and those with lower molecular weights permeate at a higher osmolarity. The marker release is temperature dependent: at 4°C it takes place at a lower osmolarity of the medium than at 37°C.

With decreasing osmolarity of the medium at 4°C a decrease in the number of the intramembranous particles and increasing aggregation of the intramembranous particles occurs.

Introduction

Use of osmotic effects is one of the oldest methods for obtaining information about the structure and function of the plasma membrane [1]. In contrast to erythrocytes, for which there are numerous detailed studies on the effect of osmotic shock on the cell membrane [2], there is little information on cells possessing nuclei. There are, however, several good reasons why use of this classic method in membranology suits the study of our current problems.

(i) A number of methods for preparing plasma membranes have already been established using hypotonic shock either for cell swelling followed by mechanical cell disruption [3], or for cell breakage [4]. Use of the latter method, however, gives no data about the differential breakage of other cell organelles. The ideal osmotic shock would therefore be a breakage sparing all other cell components which may contaminate the plasma membrane fraction.

(ii) Close investigation of the process of cell disruption should provide information on the concept [5] of the cell membrane having an heterogeneous

structure. By heterogeneity of the plasma membrane we mean that its various constituent molecular components are not randomly distributed in the plane of the membrane. Apart from osmolysis there is at this time no method available to study the structure of the plasma membrane during different stages of cell disruption. In addition, the temperature dependence of cell disruption can be followed during osmolysis. It would seem interesting to focus attention on this point because there are many recent reports and ideas about phase separation of lipid mixtures [6,7]. Temperature-dependent changes in the architecture of natural membranes have been reported [8].

In this paper the damage or breakage of cellular membranes during hypotonic shock and its temperature dependence have been studied using different markers for its integrity (fluorescein diacetate, ethidium bromide, lactate dehydrogenase, acid phosphatase and K^+). In addition, freeze-etch studies show that osmotic treatment of thymocytes induces a change in the structure of the plasma membrane, i.e., an aggregation of intramembrane particles and a decrease in their number.

Materials and Methods

Chemicals

Ethidium bromide and fluorescein diacetate were obtained from Serva (Heidelberg, G.F.R.); NADH and pyruvate from Boehringer (Mannheim, G.F.R.); diaminoethanetetraacetic (EDTA) was obtained from Merck (Darmstadt, G.F.R.); phosphate-buffered saline was from Serva (Heidelberg, G.F.R.) and 10^5 units/l of penicillin were added.

Preparation and general procedure

Thymocytes were prepared from thymi of 6—8-weeks old Lewis rats. The cell suspension was prepared in phosphate-buffered saline (310 mosM; pH 7.2) using a loosely-fitting Potter homogenizer. Stroma and dead cells were separated by filtering the suspension through nylon wool. The cells were washed twice in phosphate-buffered saline at 190 $\times g_{av}$ for 7 min at 4°C. The cell suspension, mainly consisting of small thymocytes, was adjusted to approximately $1 \cdot 10^8$ cells/ml. The studies on the effect of hypotonic shock were performed using a borate buffer (20 mM = 38 mosM), at either pH 7.2 or pH 9.2, or phosphate-buffered saline of this osmolarity mixed with different amounts of phosphate-buffered saline of 310 mosM, pH 7.2. A series of samples was prepared of 40, 90, 140, 170, 200, 270 and 315 mosM. Most of these experiments were done using borate buffer, pH 9.2, in order to compare our results with previously described methods. The osmolarity of the solutions was measured with a Knauer-Osmometer Type M (Knauer, Berlin, Germany).

Cell suspensions (0.5 ml) in phosphate-buffered saline were added to 5 ml of the shocking buffer which shifted the final osmolarities to 65, 110, 155, 183, 210 and 255.

Shocking was studied at 4 and 37°C and was continued for 5 min with slow stirring.

K^+ release, fluorescein diacetate, Trypan Blue, and lactate dehydrogenase were used as markers for cell membrane damage.

Marker determination

(1) Plasma membrane damage. After spinning down the cells at 270 $\times g_{av}$ for 5 min, the K^+ released into the supernatant was measured using a Perkin-Elmer 290 B atomic absorption spectrophotometer. In experiments using fluorescein diacetate as a marker for membrane damage [9] the cells were first incubated with fluorescein diacetate dissolved in phosphate-buffered saline (10 mM final concentration) for 45 min at 37°C. The cells were then pelleted at 190 $\times g_{av}$ for 7 min, and resuspended in the different hypotonic mixtures. After shocking, the cells were collected by centrifugation and the fluorescence of fluoresceine in the supernatant was measured by a Zeiss spectrophotometer PMQ 2 adapted for fluorescence measurements (excitation 460 nm, emission 514 nm).

(2) Nuclear membrane damage. Ethidium bromide (10^{-4} M final concentration) was added directly to the mixtures. After shocking, the damage to the nuclear membrane (i.e., the release of DNA) was determined by measuring the increase in the fluorescence of ethidium bromide at 590 nm. The excitation was done at 340 nm.

Enzyme assays

Lactate dehydrogenase (EC 1.1.1.27) was determined according to the method of Bergmeyer [10], in the supernatant after shocking and removal of cells. The acid phosphatase activity (EC 3.1.3.2) was determined according to the method of Fishman [11] using a Boehringer test combination kit.

Electron microscopy

After shocking, cells were fixed with glutaraldehyde (final concentration 2%, v/v) for 20 min. The influence of glutaraldehyde on the release of K^+ and lactate dehydrogenase was investigated and the results are presented later in the text. The cells were washed with phosphate-buffered saline and transferred stepwise into glycerol solutions, the last step having a final concentration of 25% (v/v). Freeze-etching was performed as described in ref. 13. Particle counts were performed on calibrated positives, evaluating only flat areas of the plasma membrane P-face.

Results

Cell membrane damage

Four different marker methods were chosen for monitoring the leakage, damage or breakage of the cell membrane: Trypan Blue uptake; the release of fluorescein after incubation with fluorescein diacetate; the release of cytoplasmic lactate dehydrogenase and K^+ release (Fig. 1). For a comparison of the information given by the curves in Figs. 1—3 (enzyme activities, appearance of fluorescence, K^+ release) we have defined 50% release of the respective markers as half-marker-activity (MD_{50}, membrane damage 50%) which indicates damage of the plasma membrane or the membranes of cell organelles.

The most sensitive marker for the plasma membrane is the release of K^+. It shows an MD_{50} for plasma membrane damage of about 205 mosM. The release of fluorescein (M_r 332.3), which is produced by the action of cytoplasmic nonspecific esterases on fluorescein diacetate, shows an MD_{50} in a more hypotonic

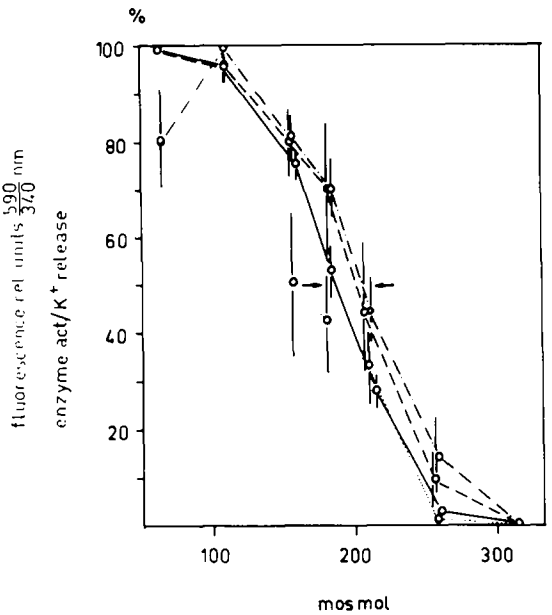


Fig. 1. Relation between plasma-membrane markers and osmolarity at 4°C. Arrows indicate the MD_{50}. ———, Lactate dehydrogenase (0% = 0.67 nM · min^{-1} · mg^{-1} protein, 100% = 2.17 nM · min^{-1} · mg^{-1} protein); – – – –, fluorescein (0% = 95 relative transmission units/ml, 100% = 1458 relative transmission units/ml); — · — · —, K^+ (0% = 2.2 µl K^+/ml, 100% = 8 µg K^+/ml); · · · · · ·, Trypan Blue. n = 4—7.

region of about 200 mosM. The largest molecule of the markers used, lactate dehydrogenase (M_r 140000) has an MD_{50} of about 185 mosM.

Trypan Blue was found not suitable as a marker because of its insensitivity to small osmolytic changes. In addition, it is difficult to discriminate between nuclei and damaged whole cells in the case of thymocytes. This is seen in the abnormal curve for Trypan Blue uptake in Fig. 1.

Temperature dependence of plasma membrane and cell organelle damage

Using osmotic cell disruption for plasma membrane preparation it would be advantageous to find an osmotic range which mainly breaks the plasma membrane without damaging other cell organelles.

As expected, with decreasing hypotonicity at 4°C, the plasma membrane was damaged first (MD_{50} for K^+, 205 mosM; fluorescein diacetate, 200 mosM; lactate dehydrogenase, 185 mosM) followed by damage to other intracellular organelles (see Fig. 2).

Acid phosphatase, commonly used as a marker enzyme for lysosomes and having a smaller molecular weight than lactate dehydrogenase (25000), shows a lower MD_{50} (140 mosM) than lactate dehydrogenase. The nucleus requires the greatest hypotonicity for significant damage, having an MD_{50} with ethidium bromide of 120 mosM.

When the experiments were carried out at 37°C the damage to the different cell organelles was clearly quite different when compared with the situation at 4°C, as depicted in Fig. 3. Damage to the plasma membrane at 37°C requires a greater osmotic shock than at 4°C. The MD_{50} at 37°C for K^+ release was found

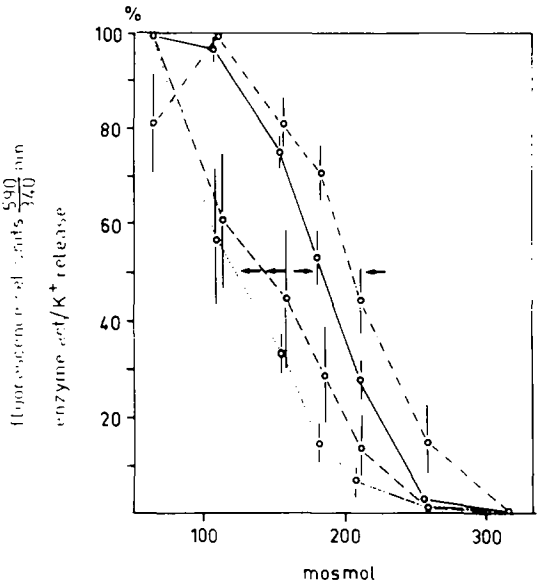


Fig. 2. Relation between membrane markers of cell organelles and osmolarity at 4°C. Arrows indicate the MD_{50}; ———, Lactate dehydrogenase; — — — —, acid phosphatase (0% = 4.2 nM · min^{-1} · mg^{-1} protein, 100% = 36.6 nM · min^{-1} · mg^{-1} protein); — · — · —, K^+; · · · · · ·, ethidium bromide (0% = 3.7 relative transmission units/ml, 100% = 31.6 relative transmission units/ml). n = 4—7.

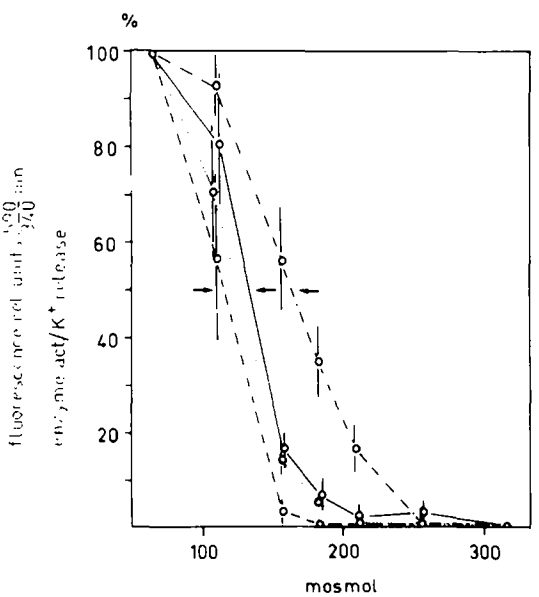


Fig. 3. Relation between membrane markers and osmolarity at 37°C. Arrows indicate the MD_{50}. ———, Lactate dehydrogenase (0% = 0.3 nM · min^{-1} · mg^{-1} protein, 100% = 1.73 nM · min^{-1} · mg^{-1} protein); — — — —, acid phosphatase (0% = 4.3 nM · min^{-1} · mg^{-1} protein, 100% = 26.4 nM · min^{-1} · mg^{-1} protein); — · — · —, K^+ (0% = 3.1 µg K^+/ml, 100% = 8.1 µg K^+/ml); · · · · · ·, ethidium bromide (0% = 4 relative transmission units/ml, 100% = 32 relative transmission units/ml). n = 4—7.

to be 165 mosM and for lactate dehydrogenase to be 130 mosM. In contrast to the situation at 4°C, lysosomal damage, nuclear damage and lactate dehydrogenase release, all took place over a narrow hypotonic range.

The cells are more resistant to osmolysis at 37°C than at 4°C. It is also interesting to note the differing osmolarity ranges for the MD_{50} release of K^+ and lactate dehydrogenase when determined at 37°C or 4°C. Whereas the range for K^+ is 40 mosM (165—205 mosM), it is 55 mosM for lactate dehydrogenase (130—185 mosM).

Freeze-etching

Freeze-etching of thymocytes in the osmotically active media used, showed differences in three aspects: (i) the number of the intramembranous particles of the plasma membrane; (ii) the distribution of the particles and (iii) the reversibility of the particle distribution.

(i) The particle density in the thymocyte plasma membrane was decreased by lowering the osmotic activity of the media. This seems to be temperature-independent. As shows in Table I, in isotonic buffer of 310 mosM the P-face of the plasma membrane has about 1000 particles per μm^2 at 4°C as well as at 37°C. At 155 and 110 mosM the number of particles is decreased to about 800 at 4°C and 37°C. Further lowering of osmolarity (65 mosM) results in a further decrease of particle density to about 350 per μm^2 at 4°C.

(ii) The distribution of intramembranous particles in the plasma membrane showed obvious differences depending on the osmolarity and temperature used. In isotonic buffer at 310 mosM a random distribution of intramembranous particles is found at both the temperatures used (Fig. 4a). This is in accordance with previous studies [12] which showed that between 37 and 4°C there is no change in the number and distribution of intramembranous particles in the plasma membrane. At 155 mosM, this aspect is unchanged irrespective of temperature. At 110 mosM, particles remain evenly distributed when the temperature is 37°C (Fig. 4c). However, if cells are shocked and incubated at this osmolarity at 4°C, they show a strong aggregation of intramembranous particles (Fig. 4b). At 65 mosM an even stronger aggregation is found at low temperature (4°C; Fig. 4e).

At 65 mosM and high temperature (37°C) the situation is unclear, because almost no tangential fracture faces are exposed, and the discrimination between

TABLE I

RELATION BETWEEN OSMOLARITY OF THE MEDIUM AND NUMBER OF THE INTRA-MEMBRANOUS PARTICLES

Means and standard deviations of 7—19 experiments are given.

Temperature	Number of particles			
	310 mosM	155 mosM	110 mosM	65 mosM
4°C	994 ± 37	808 ± 79	825 ± 59	355 ± 35
37°C	1097 ± 80		757 ± 55	

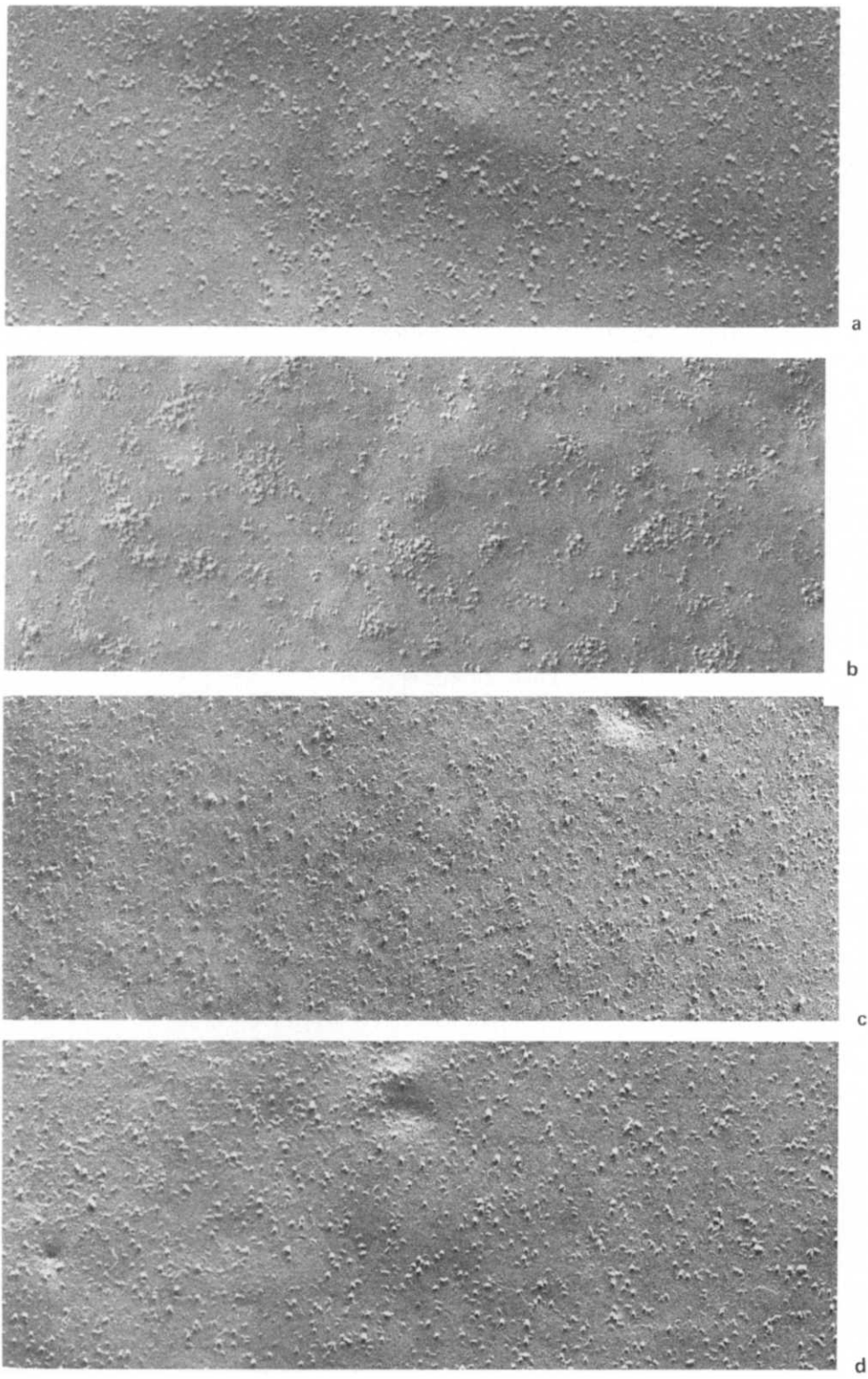
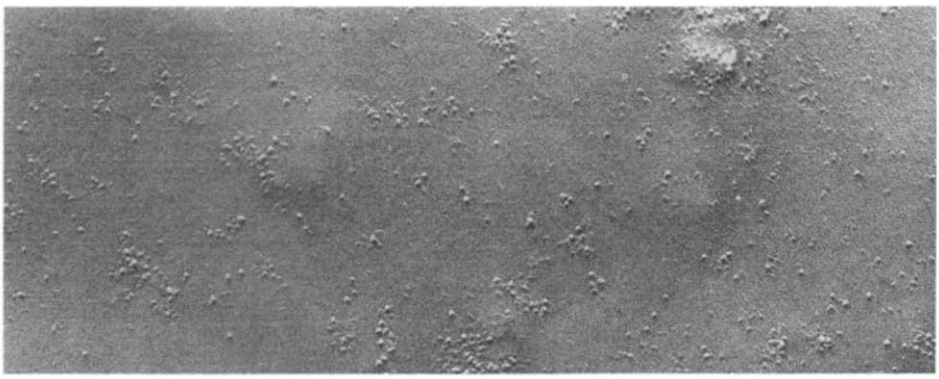


Fig. 4, a, b, c and d. For legend see opposite page.



e

Fig. 4. Freeze-etch aspects of the plasma membrane (P-face) of rat thymocytes at different osmolarity and temperature. a, 310 mosM, 4°C: membrane particles are randomly distributed, small aggregates up to five particles can occasionally be seen. Magnification × 80 000. b, 110 mosM, 4°C: membrane particles are heavily aggregated, the particle number has slightly decreased. Magnification × 80 000. c, 110 mosM, 37°C: membrane particles are randomly distributed. Magnification × 80000. d, same as b, but reincubated in 37°C. Membrane particles are again randomly distributed. Magnification × 80000. e, 65 mosM, 4°C: at this osmolarity drastic and irreversible aggregation of particles and diminuition of particle number takes place. Magnification × 80000.

broken plasma membranes and other types of intracellular membranes of the disrupted cells is difficult.

(iii) In cells treated with 110 mosM media at 4°C, and then incubated at 37°C at this osmolarity, particles are randomly redistributed again (Fig. 4d).

Each of the different aspects described can be fixed and conserved with glutaraldehyde in such a way that the subsequent impregnation with 25% (v/v) glycerol has no influence on either the number or the distribution of intramembranous particles.

All the reported biochemical, as well morphological, data are independent of the use of borate buffer or diluted phosphate-buffered saline.

Discussion

There is little data available about the effect of osmotically-active media on the plasma membrane, on cell disruption or on cell organelles in mammalian cells which are nucleated. Hypotonic swelling and/or hypotonic shock have been used for cell disruption and subsequent isolation of plasma-membrane fractions (ref. 4 and Bauer, H.C., Ferber, E., Golecki, J. and Brunner, G. (1977), unpublished). Different biochemical markers were used in the present study to follow the course of cell membrane damage under conditions of increasing hypotonicity. In order to compare the effect of hypotonicity on different markers, the "half-marker-activity" (i.e., release of metabolites, enzymes or increase in fluorescence), MD_{50}, for membrane damage was used.

It has been shown from the release of plasma-membrane markers that their MD_{50} value is dependent on their molecular weight under increasing hypotonicity: the MD_{50} at 4°C is 205 mosM for K^+ release (M_r 39), 200 mosM for fluorescein (M_r 332.3) and 185 mosM for lactate dehydrogenase (M_r 190 000). However, the MD_{50} at 4°C for acid phosphatase (M_r 15000), is approximately

140 mosM and the MD_{50} at 4°C for ethidium bromide is 120 mosM. These findings suggest that at 4°C the breakage of intracellular components occurs at a later stage in osmolytic cell disruption.

The strong temperature dependence of the osmolysis is quite remarkable, particularly the markers for the plasma membrane showing lower MD_{50} values at 37°C than at 4°C. These effects cannot be simply explained by different permeability properties of the membrane for the marker release. It appears that the plasma membrane has a greater stability at 37°C because the cell has a higher plasticity at this temperature. The cell is therefore capable of withstanding a volume increase, while at 4°C the plasma membrane is more rigid and breaks more easily due to its inflexibility. This type of osmotic temperature behaviour is well known in erythrocytes [14]. Aloni et al. [8], however, have shown that liposomes, produced from erythrocyte lipid extracts, do not show this temperature behaviour. The fragility of liposomes increased as the temperature increased. This finding demonstrated that the lipid phase is not responsible for this type of temperature behaviour and it was suggested [8] that the cytoskeleton may be involved in producing these effects.

The osmotically induced changes in the cell which have been monitored by biochemical methods are also accompanied by morphological alterations. These are shown by freeze-etch studies and evaluated from changes in the number of intramembranous particles per surface area and their degree of aggregation. A decrease in the number of intramembranous particles was found with increasing hypotonicity. This effect could be interpreted as either (i) emergence of the intramembranous particles from the hydrophobic core; or (ii) by an increase in the cell surface area due to osmotic swelling. Which of these two interpretations is correct is not clear. On consideration of the second suggestion, it can be calculated from the observed particle number that the osmotic effect would result in a cell volume increase of about 30%, since the same number of particles is merely spread over a larger area.

The aggregation of intramembranous particles in the plasma membrane occurs in an osmolytic range that corresponds to the MD_{50} of the marker used to follow nuclear membrane damage (ethidium bromide, 120 mosM). The plasma-membrane markers (K^+, fluorescein diacetate, lactate dehydrogenase) indicate that cell damage takes place between 220 and 140 mosM. However, the aggregation of the intramembranous particles does not occur until a much lower osmolarity (110 mosM) is reached. This discrepancy reflects the difference in monitoring the damage biochemically as opposed to morphologically. The biochemical damage or leakage of membranes implies that there is an occurence of pores having a diameter of 1 nm for K^+ and about 30 nm diameter for lactate dehydrogenase. The latter pore size occurs at a hypotonicity in which morphological alterations are also clearly visible (155—110 mosM).

It should be noted that the different buffer systems used (borate and phosphate-buffered saline) for the hypotonic skock do not affect the morphological changes seen. This means that the reversible particle aggregation is not dependent on the chemical constituents of the buffers, but rather on the osmotic effect. Secondly, the fact that aggregation at 110 mosM is reversible suggests that aggregation is not caused by release of cellular metabolites.

At an osmolarity of 65 mosM a real fragmentation of the plasma membrane

can be observed. This is accompanied by severe irreversible aggregation of membrane particles and a drastic decrease in particle number per μm^2. It could be explained by the fact that during osmotic lysis of cells many peripheral proteins are solubilized. As a consequence, rearrangement and displacement of membrane components, i.e., aggregation of intramembranous particles and their disappearance out of the fracture plane, takes place.

A loss of particles by extraction of the particle material itself seems unlikely, since it is well established that intramembranous particles are integral membrane proteins.

At the present time there is no conclusive information on the structure of intramembrane particles or their involvement in cellular function. It has been suggested that the intramembrane particles may be, in part, 'anchorage' proteins situated at the attachment points of the cytoskeleton and plasma membrane. It would seem more likely that aggregation of the plasma-membrane intramembranous particles would be caused by disarragement of the fibrillar system under the plasma membrane during swelling of the cell, rather than by a phase separation of membrane lipids. This idea is supported by the temperature dependence of the reversibility of aggregation which does not occur in fragmented plasma-membrane fractions. Presumably, a functional cytoskeleton is necessary. This means that an osmotic shock at 4°C, which does not rupture the cell membrane, may cause disturbance of the cytoskeleton in a way that membrane particles become aggregated. The original status of the cytoskeleton and the particles are restored at phyiosloglcal temperature. Real rupture of cell membrane implies irreversible disruption of the cytoskeleton accompanied by irreversible aggregation of the membrane particles.

The morphological observations also do not favour the idea that aggregation is caused by phase separation. Aggregation induced by lipid phase separation should have produced smoother lipid planes and more distinct areas.

In summary, the results show that the effects of an osmotic shock can be seen with the biochemical methods in a less hypotonic range than with electronoptical methods. Therefore, the membrane seems to become more permeable at first before the membrane particles start to aggregate and/or to disappear.

Acknowledgements

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