Microbial and Plant Protoplasts

edited by

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PREFACE

The collection of papers in this volume forms the Proceedings of the Fourth International Symposium on Yeast and Other Protoplasts held in the University of Nottingham, England in September 1975. The papers present a broad and comparative view of the four major areas of study that use protoplast systems, and it is gratifying to see that the momentum of interest in these "naked cells" and their diverse abilities proceeds with great vigour. The major pre-occupations with protoplast systems have concerned cell-wall biosynthesis during regeneration, and fusion leading to somatic hybridisation. It is significant that knowledge of wall biosynthesis is advanced in all of the groups of organisms used in protoplast studies, namely bacteria, yeasts, fungi and higher plants. However, this fundamental property of protoplasts, to regenerate and revert to the normal form of the organism, is intrinsic in many areas of protoplast work. Fusion of protoplasts with its resulting genetic possibilities is already established in the higher plant field and the more recent developments with fungi may set the stage for similar work with bacterial and yeast protoplasts. In certain fields the full potential of the protoplast systems has yet to be realised and in others their application has not yet been considered. Some of the latter are represented in this volume for the purpose of presenting a comparable survey of the four fields of study. The papers provide valuable up-to-date reviews of the protoplast field, and it is hoped they will give encouragement and

highlight new areas of investigation for those workers entering this area of exciting investigation.

October, 1975

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CONTENTS

ISOLATION AND PROPERTIES OF BACTERIAL PROTOPLASTS

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The isolation of wall-less protoplasts in the early 1950's (1,2) set off a flurry of activity that resulted in major advances in knowledge of the basic cell physiology of prokaryotes. The primary observation was that the bacteria, *Bacillus* cells in the first studies, lost their characteristic rod shape when the wall was removed by lysozyme action. They also became osmotically sensitive and stable only in concentrated solutions of relatively impermeant solutes. These observations were interpreted in terms of a rigid shape-retaining cell wall and a highly compliant, fragile cytoplasmic or protoplast membrane. Subsequent studies have led to significant modification of this interpretation, especially in regard to wall rigidity. However, the function of the cell wall in maintaining cell shape and in osmotic protection was clearly established and a reasonably complete picture was developed of the means by which living bacterial cells are able to adapt to major changes in osmotic pressure of natural environments. Moreover, osmotically lysed protoplasts yielded ghosts that were similar to erythrocyte ghosts. Thus, in a sense,

bacterial protoplasts became the erythrocytes of the prokaryotic cell physiologists, and the protoplast membrane became the equivalent of the erythrocyte membrane.

I RELEASE OF PROTOPLASTS FROM THE CONFINES OF THE CELL WALL

There are many means for partially or completely removing walls from bacterial cells—they have been reviewed previously (3,4). However, it seems worthwhile here to consider what happens to a bacterial protoplast when its protective wall covering is removed.

The intracellular osmolality of a growing bacterial cell is generally higher than that of its growth medium. There is, therefore, a constant tendency for water to flow into the cell to equalize chemical activities on both sides of the water-permeable protoplast membrane. Movement of water into the cell results in swelling and a stretching of the elastic cell wall, which becomes tense and loses compliance. The movement stops when the water activity within the cell is equal to that outside, and the protoplast water is under hydrostatic pressure that may be as great as 30 atm. Movement of water into and out of cells can occur very rapidly, and equilibrium may be reached in less than 50 msec after transfer to a new medium (5).

If the wall of a cell with full turgor is removed, the protoplast swells rapidly and bursts explosively. To avoid this catastrophe, one generally suspends cells for protoplast preparation in media that are hypertonic. The cells then shrink because of water loss and the elasticity of the wall. Often the protoplast contracts more than the whole cell, and plasmolysis vacuoles are formed between the membrane and the

wall. The vacuoles may or may not be visible microscopically, depending on number per cell and position, but their volumes can be measured accurately with solutes such as raffinose that penetrate into vacuoles but do not cross the protoplast membrane. In gram-positive bacteria, plasmolysis results in eversion of mesosomal membranes into vacuoles (6), and when the wall is removed, the everted mesosomes may remain attached to protoplasts as long, beaded tails.

Once the protective wall is removed, the protoplast quickly comes to an osmotic equilibrium with the medium. This adjustment is commonly accompanied by cytological alterations, such as dispersion of the nuclear body and detachment from its mooring on the membrane. Protoplast release may also be accompanied by phospholipid degradation due to autolysin with phospholipase C activity (7,8). The extent of these losses depends in part on the ambient magnesium concentration. Mesosomal tags are lost from *Bacillus* protoplasts when the magnesium concentration is less than about 20 mM (9,10), and lipoteichoic acids are lost from *Lactobacillus* and other protoplasts if the magnesium level is less than about 5 mM (8).

In contrast, high concentrations of magnesium ion act to fix the membrane and render protoplasts resistant to osmotic stresses. If *Bacillus licheniformis* cells are suspended in solutions containing more than 40 mM magnesium ion prior to wall removal, the resultant protoplasts retain the rod shape and are osmotically resistant (10). Many other agents, including polyvalent ions, detergents, hexachlorophene, heat and protein cross-linking agents, cause similar fixation and shape retention. Moreover, natural "fixation" can also occur.

Bacillus stearothermophilus cells grown at high temperatures routinely yield elongate, osmotically resistant protoplasts (11,12). Rod-shaped protoplasts have been prepared also from *Bifidobacterium bifidum* cells inhibited in wall synthesis (13). Moreover, when cultures of mesophilic *Bacillus* organisms are grown at pH of about 5 prior to lysozyme treatment, the protoplasts retain the rod shape of the intact cells and are osmotically resistant (14,15). Recently, it has been shown (16) that the outer layer of the *Spirillum serpens* wall forms a shape-retaining array independent of the murein sacculus of the cell. Clearly, under at least some circumstances, the protoplast membrane can be a shape-retaining structure and can have mechanical properties that one ordinarily associates with the cell wall.

A highly pertinent example here is that of the mycoplasmas. These prokaryotes have no cell walls and are surrounded only by a membrane that looks in electronmicrographs very little different from protoplast membranes. And yet, this membrane seems to be a shape-retaining structure, and there are mycoplasmas that have characteristic ovoid or rod shapes, and even one, *Mycoplasma gallisepticum*, that has a characteristic, asymmetric, bilobate structure (17). Moreover, mycoplasmas such as *Acholeplasma laidlawii* are remarkably resistant to osmotic shock. In order to lyse them osmotically, one must first soak them in glycerol solution and then transfer them rapidly to deionized water. Obviously, we have here a membrane that is differentiated to serve mechanical functions ordinarily served by cell walls.

The change in the protoplast membrane during cell growth at low pH from a limp to a shape-retaining structure has been associated with a change in lipid composition (14,15). However, changes in growth conditions also result in changes in membrane proteins and inorganic ion content (12,18) and these latter changes may in fact be the cause of altered mechanical properties.

II BACTERIAL PROTOPLASTS AS OSMOMETERS

Bacterial protoplasts are often considered to be perfect osmometers, and therefore, it should be possible to predict their osmotic responses by use of the van't Hoff-Boyle equation, $V-b = a/\pi$, where V is the volume of the protoplast, b is the osmotically inactive volume, a is a constant and π is the external osmolality. However, the results presented in Fig. 1 indicate very non-ideal behaviour in concentrated and dilute solutions. Here cell volumes were determined microscopically; similar results were obtained when relative volumes were determined from light scattering measurements. Over the range of inverse osmolalities from about 0.25 to 0.60 (osmolalities from about 3.9 to 1.7), the protoplasts behave approximately as perfect osmometers. A straight line constructed by use of the points in this region, indicated by the dashed line, intersects the ordinate line at a value for "b" of 1.6 μm^3, which corresponds closely to the expected volume of dry matter in the protoplasts. However, it is not possible practically to dehydrate the protoplasts completely, and there appears to be water that cannot be withdrawn osmotically, possibly because of impermeability of the contracted membrane to water or to increased osmotic coefficients of internal solutes in very concentrated solution.

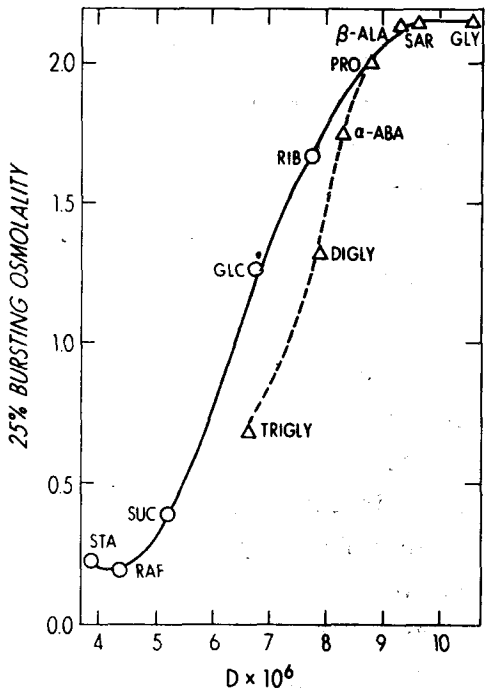

Fig. 1. Average volumes of *Bacillus megaterium* protoplasts in relation to suspending medium osmolality. The dashed line indicates the relationship predicted by the van't Hoff-Boyle equation with constants chosen to fit the results obtained with protoplasts in sucrose solutions of between 1.7 and 3.9 osmolal. The slope of the line is 15.5 μm^3-osmol/kg and the intercept is 1.6 μm^3. Details are given in reference 19.

In dilute media, the protoplasts again deviate from ideal behaviour. It appears that the membrane is somewhat elastic and can build up a fair degree of tension. In these experiments, protoplast lysis was insignificant and solute leakage from cells was minimal. At the extreme shown in Fig. 1, the apparent difference in osmolality across the protoplast mem-

brane, indicated by the distance between the dashed line and the solid curve, is some 6.7 atm. The membrane tension is some 556 dynes/cm, according to the general Laplace equation, $T = P(R/2)$, where T is the tension, P is the pressure difference and R is the protoplast radius and the bulk elastic modulus of the membrane appears to be some 10^7 dynes/cm^2. These values are probably somewhat overestimated because there was minor leakage during the experimental period. However, we have found (20) that during wall removal, protoplasts retain nearly all of the cell potassium, which is then slowly released over periods of hours along with other small solutes such as phosphate ions.

The potassium that is counterion for cell polymers and Mg^{2+} are retained for days at 4°C in the absence of lysis. Protoplasts are stable for very long periods if their metabolism is restricted.

III PHYSICAL PROPERTIES OF THE PROTOPLAST MEMBRANE

A. *Permeability and Porosity*

The first experiments with bacterial protoplasts showed that they were stable only in osmotically buffered media, that is, media that contain high concentrations of impermeant solutes. Perhaps it would be better to say relatively impermeant solutes because most of the compounds that are used as stabilizers can penetrate the protoplast membrane but do so slowly. In many instances, stabilizing solutes are relatively impermeant only because of the experimental conditions. For example, *B. megaterium* is strictly aerobic and can concentrate glucose aerobically, but protoplasts are stable in concentrated glucose solutions under anaerobic conditions or at 4°C. However, if the protoplasts are aerated at higher temperatures, they quickly take up glucose, swell and burst. Abrams

(21) found that protoplast swelling is due in part also to uptake or resorption of potassium ions during glycolysis.

Early attempts to interpret the osmotic stabilizing capacities of various solutes in physicochemical terms proved to be futile, and Weibull (2) concluded that "the stabilization phenomenon can hardly be explained in osmotic terms". Our subsequent studies (22,23) showed that the protoplast membrane acts as a porous differential dialysis membrane and that its effective porosity increases when it is stretched. For any particular solute, there is a threshold concentration for osmotic stabilization. Thus, if one has a suspension of protoplasts stabilized in a solution containing a high concentration of some particular solute and one transfers the cells to progressively more dilute solutions, there is initially little or no osmotic lysis associated with dilution. But then, once bursting does ensue, it is complete in the population over a relatively narrow range of solute concentrations. When we carried out this sort of procedure with a range of stabilizing solutes, we found that the osmolality at which bursting occurred varied greatly depending on the particular test solute. We were able to make some sense out of this variation by use of a series of chemically related compounds with differing molecular sizes. The data presented in Fig. 2 show osmolalities at which 25% bursting of protoplast populations initially stabilized in 2.38 osmolal sucrose solution occurred following slow, dropwise addition of large quantities of diluents containing the indicated solutes. The plots here are of osmolalities causing 25% bursting against diffusion coefficients of the solutes in water. These coefficients are reflections