ABSTRACT

EFFECTS OF pH ON THE OSMOTIC PROPERTIES OF *BACILLUS MEGATERIUM* PROTOPLASTS

By

Ronald J. Brown

Protoplasts released from *Bacillus megaterium* exhibited decreased stability when the ambient pH was raised above neutrality, although sensitivity to osmotic pressure changes was retained. This destabilization was found to occur regardless of the external osmotic pressure and almost no protoplasts survived at any external osmotic pressure when the ambient pH was 9.3. Acid treatment of protoplasts suspensions made them resistant to osmotic shock beginning between pH 5 and 6 and they became insensitive to osmotic pressure changes as well. Thus, the number and final average volume of survivors determined for protoplast suspensions at pH 4 was constant regardless of the external osmotic pressure or the molecular size of the stabilizing solute. However, slightly fewer acid treated protoplasts survived in NaCl than in sucrose solutions, possibly indicating an interaction between ionic strength effects and acid effects. This was supported by leakage of 260 nm absorbing material at high but not low salt concentrations and decreased optical density at high salt concentrations. Butanol lysed untreated but not acid treated protoplasts. Acid stabilization was lost upon raising the ambient pH of acid treated protoplasts suspensions but destabilization of the protoplasts resulted

even in hypertonic media. Extensive denaturation of cell components by acid treatment is suggested by those results. The buffering capacity of intact and burst protoplasts was equivalent below pH5, although the equalibration time required was 2h longer for intact protoplasts. That difference in equilibration times suggested retention of at least a partial membrane barrier. An intact membrane was also suggested by a low level of leakage of 260 nm absorbing material from pH4 treated protoplasts in a 0.3 osmolal NaCl solution. The membrane permeability barrier to hydronium ions appeared to be complete above pH5 but leaky below pH5, which corresponds to the approximate pH where acid stabilization became obvious. Therefore, acid stabilization may involve both membrane and cytoplasmic effects.

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By

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To My Wonderful Wife Lily . . .

With Love and Appreciation

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I am grateful to Thomas R. Corner, Ph.D., assistant professor of microbiology at Michigan State Universidy, for excellent council, instruction, encouragement and technical advise.

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INTRODUCTION

Bacterial protoplasts can be prepared from several different organisms by enzymatic removal of cell wall material. The resulting structure is the osmotically fragile protoplasmic unit of a bacterium (22, 40, 41), namely a membranous bag of cytoplasmic materials which assumes a spherical shape due to the viscoelastic properties of the membrane. It will imbibe water, swell, and burst unless suspended in isotonic medium containing solutes unable to cross the membrane barrier (i.e. lipophobic compounds), in which case an equilibrium is reached between the internal and external water activity following the influx or efflux of water and resultant swelling or shrinking according to the osmotic pressure gradient. Thus, the bacterial protoplast behaves as a perfect osmometer (9, 19) within a moderate range of external osmotic pressure. This property has been used (2, 18) to assess protoplast permeability to different compounds, and it has been found that the ability of lipophobic sugars to stabilize nonrespiring protoplasts is directly related to their molecular size.

Further studies (2) on the osmotic behavior of *Bacillus megaterium* protoplasts have suggested the existance or occurrence of small aqueous channels through which lipophobic stabilizing molecules could diffuse in accordance with their molecular size and which are extended by swelling of the protoplast in response to an osmotic pressure gradient. The bursting of protoplasts in hypotonic solutions has been

postulated to be a result of brittle fracture. Apparently, when the aqueous channels are extended sufficiently by swelling of the protoplast to permit entry of stabilizing solute molecules, a rapid, cascading influx of solutes and water ensues. This causes rapid stretching of the protoplast membrane leading to brittle fracture. Such bursting can be avoided by slow osmotic dilution (i.e. dropwise addition of diluent, or dialysis), and tremendous extension of the membrane, up to 300% increase in surface area with no apparent bursting, has been achieved by the use of dialysis procedures.

The foregoing studies indicate some of the mechanical properties of the membrane which accurate models of membrane structure will be required to satisfy. Additional information can be gained about membrane properties and structure by physical and chemical perturbations of bacterial protoplasts and subsequent testing of osmotic behavior. For example, it was found (2) that urea and glycerol relax protoplast membranes while formaldehyde toughens them. The known effects of the former compounds as protein denaturants and the latter as a cross-linking agent suggests the importance of protein in determining the mechanical properties of bacterial membranes. Since the osmotic behavior of bacterial protoplasts is determined by the interaction of the three components of the osmotic system: the internal phase, the external phase, and the intervening membrane barrier, perturbation studies need to include investigation

of which phase is affected.

Among the previous studies done are perturbations of growth temperature (6), incubation temperature after protoplast formation (6, 26) surface tension (31), stabilizing solute size and conformation (2, 33), hydronium ion concentration (5, 13, 21, 24) and the addition of various chemicals to the suspending medium (2, 17, 23, 27, 36). Some of these studies have not fully characterized the changes in osmotic properties resulting nor determined the phase or phases affected. Perturbations of pH are an example.

Although acid pH was known to diminish the lytic effects of physical, chemical, or enzymatic treatment on bacteria, Edebo (5) was the first to investigate acid stabilization of bacterial protoplasts. He found by microscopic observations and optical density determinations that *B. megaterium* protoplasts did not burst when subjected to osmotic shock at an ambient pH of 5 or less. Stabilization was decreased (i.e. more bursting occurred) above pH 5 and nearly complete bursting occurred at a pH of 6 or greater. He reported the acid stabilization to be reversible by raising the ambient pH during the process of osmotic dilution and speculated (3, 4, 5) that acid treatment might cause gelling of the cytoplasm, thus hindering solubilization of the cytoplasm and preventing the completion of lysis. Edebo, as well as other investigators (13), have reported acid stabilization of *Escherichia coli* and

Engerobacter aerogenes spheroplasts.

Op den Kamp et al. (24) also observed acid stabilization of *B. megaterium* protoplasts. They found that cells grown in a yeast extract - peptone medium without glucose yielded a membrane fraction of different phospholipid composition than when glucose was added. With added glucose a decrease was observed in the total amount of phosphatidyl glycerol present and an additional phospholipid, glucosaminyl phosphatidyl glycerol, was found in a substantial amount. Glucose also caused a lowering of the culture pH to about 5 during overnight growth and artificial lowering of the culture pH to 5 one hour before harvesting (no glucose added) reportedly produced alterations in the phospholipid composition similar to the addition of glucose. Protoplasts prepared either from cells grown in the medium containing glucose or from cultures in which the pH was artificially lowered with HCl did not lyse when osmotically diluted in a pH 6.2 buffer but leaked 260 nm absorbing material.

Failure to maintain pH5 during protoplast formation and the stabilization test might be the reason for the observed leakage since acid stabilization of protoplasts is reversible (5, 21). Furthermore, it is not known whether any relationship exists between the reported changes in phospholipid composition and osmotic behavior of the protoplasts, and it is not known for certain whether this stabilization resulting from altered pH during growth is comparable to that reported by Edebo which was a result of altering the

ambient pH or non-respiring protoplasts.

Similarly, Haest et al. (11) observed a decrease in phosphatidylglycerol and an increase in lysyl phosphatidylglycerol in the membrane fraction of *Staphylococcus aureus* upon decreasing the pH during growth from 6.5 to 5. These cells showed a decrease in cation permeability and an increase in nonelectrolyte permeability. Results obtained with liposomes composed of purified phosphatidyglycerol or lysyl phosphatidylglycerol confirmed the effects of phospholipid composition on permeability. Permeability was tested at neutral pH because lowering the ambient pH at the time of testing was found to decrease cation permeability, possibly due to increased protonation of the amino groups in the membrane proteins and lipids. They postulated that nonelectrolyte permeability is controlled by packing of lipid molecules, whereas cation permeability is dependent on the charges on the polar headgroups. However, the osmotic properties of protoplasts from cells of *S. aureus* grown at pH 5 were not tested.

Acid stabilization of *Streptococcus fecalis* protoplasts was reported by Marquis et al. (21). Titrations of intact cells, butanol treated cells and isolated cell components revealed that hydronium ions could displace this bound magnesium, but the membrane acted as a barrier to ion movement at pH values greater than 3. Membranes completely depleted of Mg++ ion were shown to remain structurally intact, and again acid treated protoplasts

at pH 5 or less were resistant to lysis by osmotic shock. Bringing the ambient pH back to neutrality reversed acid stabilization of those protoplasts in hypotonic solutions.

In contrast to the results obtained with bacterial protoplasts, acid treatment has been shown (14) to decrease the osmotic stability of protoplasts from the yeast, *Saccharomyces carlsbergensis*. Similar results have been reported (28) for mycoplasma. Alkaline treatment has been observed (21, 27, 28) to decrease the osmotic stability of all the bacterial protoplasts, spheroplasts, L-forms, and mycoplasma tested.

Other investigators have demonstrated that the bacterial protoplast membrane is a significant barrier to hydronium ion, at least above pH 3, by titrating intact and burst *E. coli* (43) and *Micrococcus lysodeikticus* (8) cells. Yet the ambient pH has a profound effect on the growth rate of bacteria. Growth of *S. fecalis* is known (21) to be reversibly stopped at pH values between 4.9 and 3, but irreversibly at pH 2. Sakharova (29) showed an optimal ambient pH range of 5.5 to 7.5 for growth of *B. megaterium*, 50% reductions in growth rate at pH 5.0 or 8.2, and an abrupt increase in the effect of pH on growth rate at pH 5.3.

However, it is not known whether the effects of pH on growth rate involve the membrane, the cytoplasm, or both, and investigations (7, 8, 15, 43) on the relation of the external to the internal pH of bacteria have given conflicting results. Similarly, it is not known which cell components

are affected during acid stabilization and alkaline destabilization of bacterial protoplasts. Furthermore it is not known whether the effects of pH on the growth rate of bacteria are related to the effects of pH on the osmotic stability of protoplasts, or whether either of these is related to the effects of pH on the phospholipid composition and permeability of bacterial membranes. Furthermore, additional information is lacking concerning the effects of ambient pH on the osmotic properties of bacterial protoplasts. All previous studies have employed a single, extremely hypotonic osmotic concentration to test osmotic stability, and nothing is known about behavior at intermediate concentrations. Some investigators have not adequately controlled or monitored the pH during testing, and optical density measurements plus qualitative microscopic observations have been the only reported methods of determining osmotic stability. The present investigation was undertaken to characterize more fully the effects of pH on the osmotic properties of bacterial protoplasts and to determine which cell components are affected.

METHODS AND MATERIALS

Organism and Growth Conditions. The asporogenous KM strain of *Bacillus megaterium* was maintained in stock culture on Trypticase Soy Agar (BBL) plates at 25 C. The organism was grown in 400 ml aerated batch cultures of 2% (w/v) Oxoid peptone broth (Flow Laboratories, Rockville, Md.) at 30 C in two liter flasks on a gyratory shaker.

The cells were harvested at the late exponential phase of growth (from 15 to 18 h) when the optical density reached about 2.3 or about 1.3 mg of cells (dry weight)/ml. Harvesting was done by centrifugation of 30 ml samples at 10,000 x g for 10 min in a Sorvall RC-2B refrigerated centrifuge at 4 C. The cell pellets were used immediately for protoplast preparation.

Optical densities of cultures were measured at a wavelength of 700 nm (A_{700}) and optical densities of protoplast suspensions were measured at a wavelength of 650 nm (A_{650}) using a Spectronic 20 spectrophotometer (Bausch and Lomb) equipped with 1.12-cm light path cuvettes. Distilled water was used as a blank.

Preparation of Protoplasts. Protoplasts were prepared by the action of lysozyme (N-acetylmuramide glucanohydrolase, Sigma Chemical Co., St. Louis, Mo.) on whole cells suspended in a 2.0 osmolal (1.25 M) sucrose solution. Crystalline lysozyme dissolved at a concentration of 20 mg/ml in 0.1 M potassium phosphate buffer (pH 7) was added to the cell pellet. Only 0.2 ml of the lysozyme solution was added,

immediately followed by 5 ml of 2.0 osmolal sucrose solution, resulting in a final concentration of 770 μg lysozyme/ml, about 7.6 mg of cells (dry weight)/ml, and a negligible change in sucrose concentration.

The resultant suspensions were incubated at room temperature (about 25 C) until protoplast formation was about 98% complete as observed by phase contrast microscopy, usually about 30 minutes.

All suspensions prepared at one time were combined and 5 ml samples taken for subsequent testing.

Adjustment and Measurement of pH. The pH of the above protoplast suspensions was usually 6.4. Values between 2 and 6 were obtained by the addition of H_2SO_4 or HCl to suspensions in sucrose solutions and values between 7 and 10 by the addition of NaOH. The concentrations of acid or base solutions were prepared such that 0.05 ml added to 5 ml of the protoplast suspensions would give the desired pH (e.g. 0.05 ml of 0.6 N H_2SO_4 into 5 ml protoplast suspension usually lowered the pH to about 4.0). The sucrose concentration was thus negligably affected. Controls were prepared by adding 0.05 ml of distilled water to protoplast suspensions in place of acid or base solutions. Na_2SO_4, Li_2SO_4, and LiCl in the same concentration as H_2SO_4 were also added to different 5.0 ml samples of protoplasts.

Reversibility of acid effects was tested by raising the pH of acid-treated protoplast suspensions to near neutrality with from 0.1 to 0.2 ml amounts of appropriately

concentrated solutions of NaOH or Ha_2HPO_4. Controls were prepared by adding distilled water, containing added hydronium ion, in place of base solutions to acid-treated protoplast suspensions.

Protoplast suspensions were maintained at room temperature for 15 min after the addition of ions, during which time the pH was monitored with a Corning research model 12 pH meter equipped with a single thin-stemmed glass electrode. The temperature was then lowered to 4 C in an ice bath and maintained during subsequent testing of osmotic properties.

The contribution of added ions to final osmolality was always less than 0.05 osmol/kg.

Titration of Protoplasts. Preparation of protoplasts for titration was done using volumetric flasks and pipettes. Intact protoplasts were prepared by suspending whole cells, with 0.2 ml lysozyme solutions added, in 2.5 ml distilled water, half the usual volume. They were incubated at room temperature until bursting was complete as observed by phase contrast microscopy, usually 30 min. The volume was increased by adding 2.5 ml of twice concentrated sucrose solution, followed by vigorous pipetting or sonication to shear DNA and break up aggregates. This treatment resulted in suspensions of burst protoplasts identical to the intact protoplast suspensions in concentration of sucrose, lysozyme phosphate buffer, cell dry weight, and total volume. The intact protoplast suspensions were pooled, as were the suspensions of burst protoplasts, and

a 5 ml sample was taken of each for titration. The remaining volume of burst protoplasts was divided into two equal portions. Deoxyribonuclease (0.005 mg/ml) was added to one sample, which was incubated at 30 C for 30 min. Both samples were then sedimented at 30,000 x g for 30 min to separate the membrane fraction from the soluble fraction and cell wall fragments. The supernatants were decanted and the membrane pellets washed once in water. Each pellet was then suspended in 2 osmolal sucrose-lysozyme-phosphate buffer solution at the original concentration and samples were taken for titration. In addition, the supernatant and wash from the tube to which no dexyribonuclease was added were combined and a sample taken for titration.

The volumes of samples from the membrane fraction or the soluble fraction were adjusted so that the total amount of cell component present was equal to the amount of that component normally present in a 5 ml sample of original suspension. Several 5-ml samples of a solution of 2 osmolal sucrose-plus lysozyme were also titrated and the amount of hydronium ion bound was subtracted from the amount of bound by protoplasts or cell fraction suspensions in the same solution. The titrations were carried out with 0.1 N H_2SO_4. A Corning research model 12 pH meter was used and readings were taken after 2 min equilibration.

<u>Osmotic Dilutions of Protoplast Suspensions</u>. The ambient osmotic presure of protoplast suspensions prepared in 2 osmolal sucrose solution was rapidly lowered by transferring

0.1 ml into 3.9 ml of NaCl solution at 5 C. The solutions ranged in concentration from 2 osmolal to 0.3 osmolal. This provided sufficient dilution in numbers that counts and volume measures of surviving protoplasts could be efficiently made. The contribution of sucrose to osmotic pressure in those NaCl solutions was less than 0.03 osmol/kg. Occasionally, sucrose solutions whose concentration ranged from 2.0 to 1.3 osmolal were used for osmotic stabilization to compare with stabilization in NaCl solutions. However, since the refractive index of sucrose solutions increases rapidly with concentration, NaCl usually had to be used as an osmotic stabilizer in place of sucrose so that absorbance of light at a wavelength of 650 nm (A_{650}) could be used to estimate stabilization. Furthermore, the use of NaCl provided a system in which protoplasts and suspending medium were separable by filtration and leakage of cytoplasmic materials could be monitored. Protoplasts treated with acid or other ions were maintained at the appropriate pH or ion concentration during the dilution process by adding ions to the diluents prior to adding the 0.1 ml sample of protoplast suspension. Following dilution, the protoplasts were incubated at 5 C for 30 min before use. The 5 C temperature was maintained during all manipulations, which were made as rapidly as possible.

Osmolality is a concentration term defined as molality times the osmotic coefficient of the compound concerned.

Osmolalities used in this study were calculated from data in a recent _Handbook of Chemistry and Physics_.

Counting and Sizing of Protoplasts. Counts of surviving protoplasts suspended in various osmolalities of NaCl or sucrose solutions were carried out by means of a Petroff-Hauser counting chamber and a Leitz-Leica binocular phase contrast microscope equipped with a 100X oil immersion objective and 15X oculars. For each suspension counted, the chamber was filled twice and two separate counts made for each filling, according to the method of Corner and Marquis (2).

A calibrated ocular micrometer was used to measure protoplast diameters. One hundred protoplasts were measured for each determination, and average diameters, average radi, and average volumes were calculated.

Filtering of Protoplast Suspensions and A_{260} Measurement. To monitor RNA leakage, protoplasts were separated from NaCl solutions by filtration through 0.22 μm millipore filters. The filtrates were tested immediately for absorbance at 260 nm by use of a Beckman DBG scanning spectrophotometer.

Butanol treatment. Susceptibility of protoplasts to lysis by membrane disruption was tested by butanol treatment of suspensions in NaCl solutions. Prior to butanol treatment, protoplast suspensions were raised to room temperature to enhance the dissolution of butanol in water. The final concentration of butanol was 0.3 M. Following the addition of butanol, the suspensions were mixed several times by

inversion. The suspensions were incubated at room temperature and the time course of lysis followed by means of A_{650} determination.

RESULTS

<u>Protoplast Stability at Different pH Values</u>. In accordance with past observations, the optical density of untreated protoplast suspensions (ca. pH6 .5) decreased as the ambient osmotic pressure in the suspensions was reduced (Figure 1B). This osmotic sensitivity was retained at higher pH values. However, general destabilization or destruction of protoplasts was apparent, resulting in lower optical density readings with increasing pH at every osmolality tested (Figure 1C). Microscopic observations confirmed that the number of protoplasts/ml decreased with increased pH, even at 2.0 osmolality, and very few intact protoplasts were seen at pH 9.3.

Acid treated protoplasts appeared to have lost sensitivity to osmotic changes, beginning at about pH 6 (Figure 2), and microscopic observations confirmed that intact protoplasts were present even in 0.3 osmolal NaCl solutions, at least below about pH 5.5. However, the changes in optical density observed as a result of lowering pH were complex (Figure 1A,B). Between pH 6.4 and about pH 4.5 the optical density values decreased in 2.0 osmolal NaCl but increased in 0.3 osmolal NaCl. Below pH 4.5, increases in optical density were seen in all NaCl solutions. This suggested that "optical artifacts" not attributable to osmotic behavior were involved. Extensive aggregation was seen below pH 4, but that should cause decreasing rather than the observed increasing optical density measurements, due to both a reduction in the total number of particles and an increase in their effective volume.

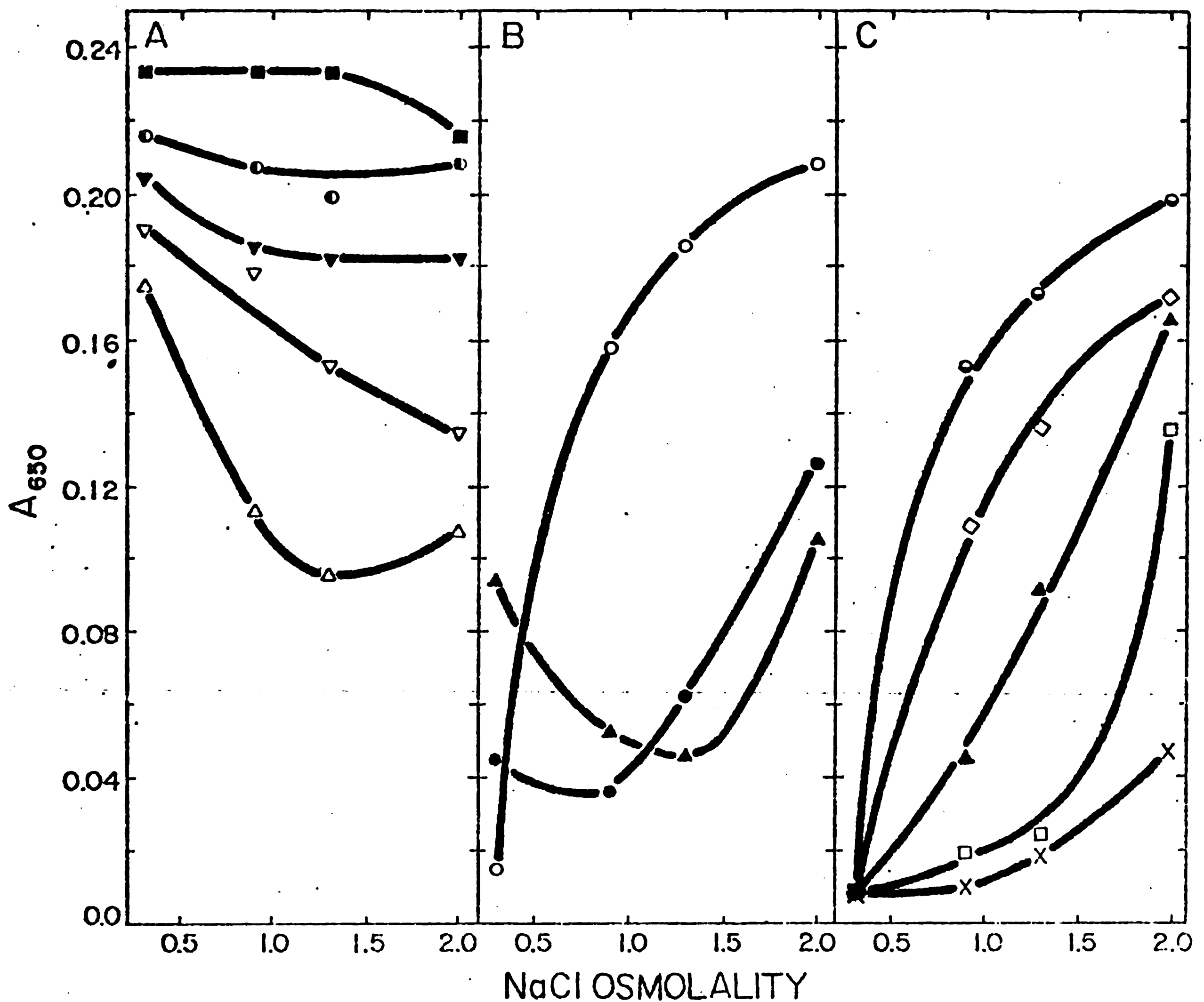


Figure 1. Osmotic behavior of protoplasts at different pH values estimated by optical density determinations. Protoplasts were released in 2.0 osmolal sucrose prior to adjusting the ambient pH with H_2SO_4 or NaOH and transfering samples to NaCl solutions of various concentrations at the pH levels of (A): 2.4 (■), 2.8(◐), 3.2(▼), 3.6(▽), 4.1(△); (B): 4.7(▲), 5.2(●), untreated (○); (C): 6.7(◉), 7.0(◇), 7.4(▲), 8.3(□), and 9.3(X).

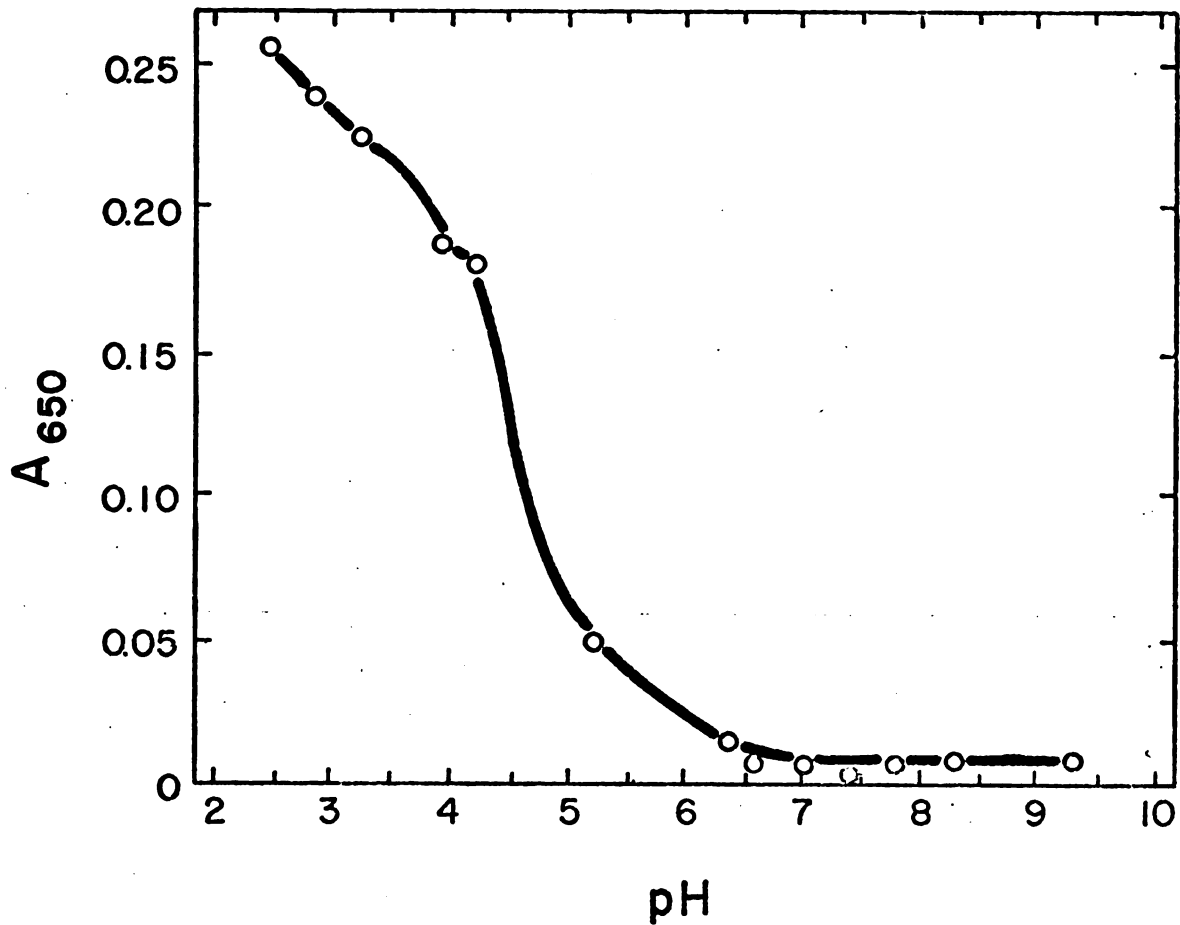


Figure 2. Effect of pH on the resistance of protoplasts to osmotic shock. Protoplasts were released in 2.0 osmolal sucrose prior to adjusting the ambient pH with NaOH or H_2SO_4 and transferring samples to 0.3 osmolal NaCl solution at the adjusted pH.

Quantitation of the extent of aggregation of protoplasts in NaCl solutions at pH 4.1 to 4.3 gave variable results but indicated no relation between NaCl concentration and the degree of clumping. This was strongly supported by the fact that almost no aggregation occurred in any solution with control protoplasts. Therefore, it appears that aggregation was mainly related to hydronium ion concentration and probably had little, if any relationship to NaCl concentration in the range used for these studies.

Direct microscopic counts (Figure 3B) verified the sensitivity of untreated protoplasts to lysis by rapid osmotic dilution. The number of survivors in NaCl solutions declined moderately between a 2.0 and 1.3 osmolal concentration but rapidly below 1.3, and less than 5% of the input number survived at 0.3 osmolality. Similar lysis was evident in sucrose solutions, but survival was greater with 40% surviving at 0.3 osmolality. In contrast, acid treated protoplasts suspended in either NaCl or sucrose solutions at an ambient pH of 4.1 to 4.3 were not subject to osmotically induced lysis. About 60% of the input protoplasts survived at all NaCl concentrations (Figure 3B) and about 75% in sucrose solutions. This observation verified the resistance of acid treated protoplasts to osmotic lysis suggested by optical density measurements and indicated a lack of sensitivity to ambient osmotic pressure changes.

The latter was confirmed by determining the volume of protoplasts in both NaCl and sucrose solutions (Figure 3C).

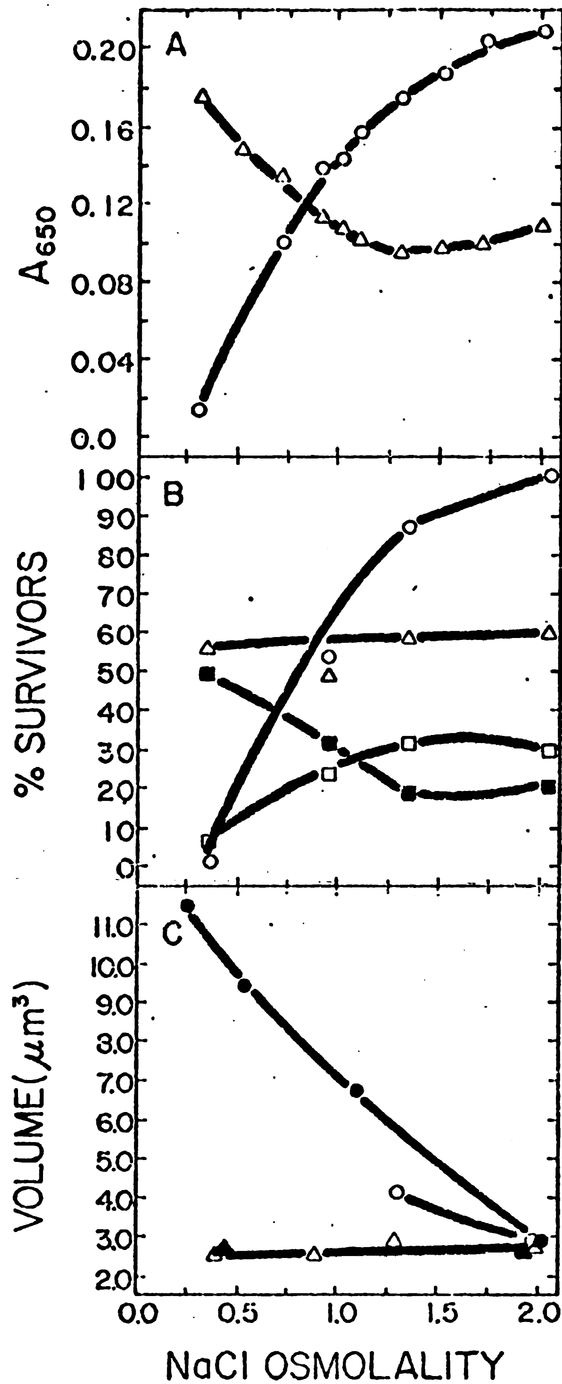


Figure 3. Acid effects on the osmotic properties of protoplasts. Optical density and the number and final average volumes of survivors in NaCl solutions are shown for untreated (O) and pH4.1 to 4.3 treated (Δ) protoplasts in A, B, and C respectively. Final average volumes of survivors in sucrose solutions are shown for untreated (●) and treated (▲) protoplasts in C. The number of less dense (ghosty) (□) and normal (■) appearing survivors in NaCl solutions is shown in B. All protoplasts were prepared in 2.0 osmolal sucrose solutions at neutral pH.

Acid treated protoplasts (i.e. those treated at pH 4.1 to 4.3) showed no significant changes in volume with reduction of ambient osmolality and measurements made in NaCl and sucrose solutions were nearly identical at every concentration. Untreated protoplasts, on the other hand, in sucrose solutions swelled from a final average volume of about 3 m^3 at 2.0 osmolality to greater than 11 m^3 at 0.3 osmolality. Swelling of untreated protoplasts also occurred in NaCl solutions, but measurements could only be made at 2.0 and 1.3 osmolality because of the great amount of bursting at lower concentrations. The increase in final average volume of survivors from about 3 m^3 to slightly greater than 4 m^3 was less than in sucrose solutions of similar osmotic concentration.

The degree of phase darkness exhibited by intact acid treated protoplasts was variable. The occurrence of protoplast 'ghosts' following lysis is well known (42), but the apparent optical density observed here was intermediate between normal protoplasts and true 'ghosts'.

No evidence of membrane disruption was apparent. Differential counts showed that this phenomenon was related to NaCl concentration (Figure 3B). The shape of the curve generated resembles that for A_{650} values when plotted against NaCl osmolality, which is reasonable since both are light scattering phenomena. Less dense appearing protoplasts were not seen in acid treated sucrose suspensions or in control suspensions in either NaCl or sucrose solution, indicating the phenomenon may be a result of the combined effects of hydronium ion concentration and NaCl concentration.

Thus, protoplasts were destabilized a pH values above neutrality but gained resistance to osmotic lysis at acid pH and were, in fact, insensitive to changes in ambient osmotic pressure at pH 4.1 to 4.3.

The optical density measurements made with protoplasts in NaCl solutions correlated well with direct counts at neutral pH and above but not at acid pH (Figure 3A,B), suggesting the existence of optical artifacts related to both NaCl and hydronium ion concentration. Further characterization of the acid effect was done with protoplasts at pH 4.1 to 4.3 to assure that they had developed adequate resistance to osmotic lysis with a minimal amount of aggregation. Protoplasts referred to hereafter as acid treated were suspended in solutions at an ambient pH within that range.

The Specificity of Hydronium Ion in Acid Stabilization. The substitution of hydrochloric acid or citrate-phosphate buffer in place of sulfuric acid to achieve pH 4.1 to 4.3 gave nearly identical optical density measurements, whereas treatment with LiCl, Li_2SO_4, or Na_2SO_4 at similar concentrations gave results (Figure 4A,B) nearly identical to untreated protoplast suspensions. This indicated the observed stabilization is specifically related to hydronium ion concentration.

Reversibility of Osmotic Dilutions. Sequential osmotic dilution from 2.0 to 0.3 osmolality and sequential concentration from 0.3 to 2.0 osmolality of a single sample of protoplasts, instead of using separate samples for each osmotic dilution

A 650

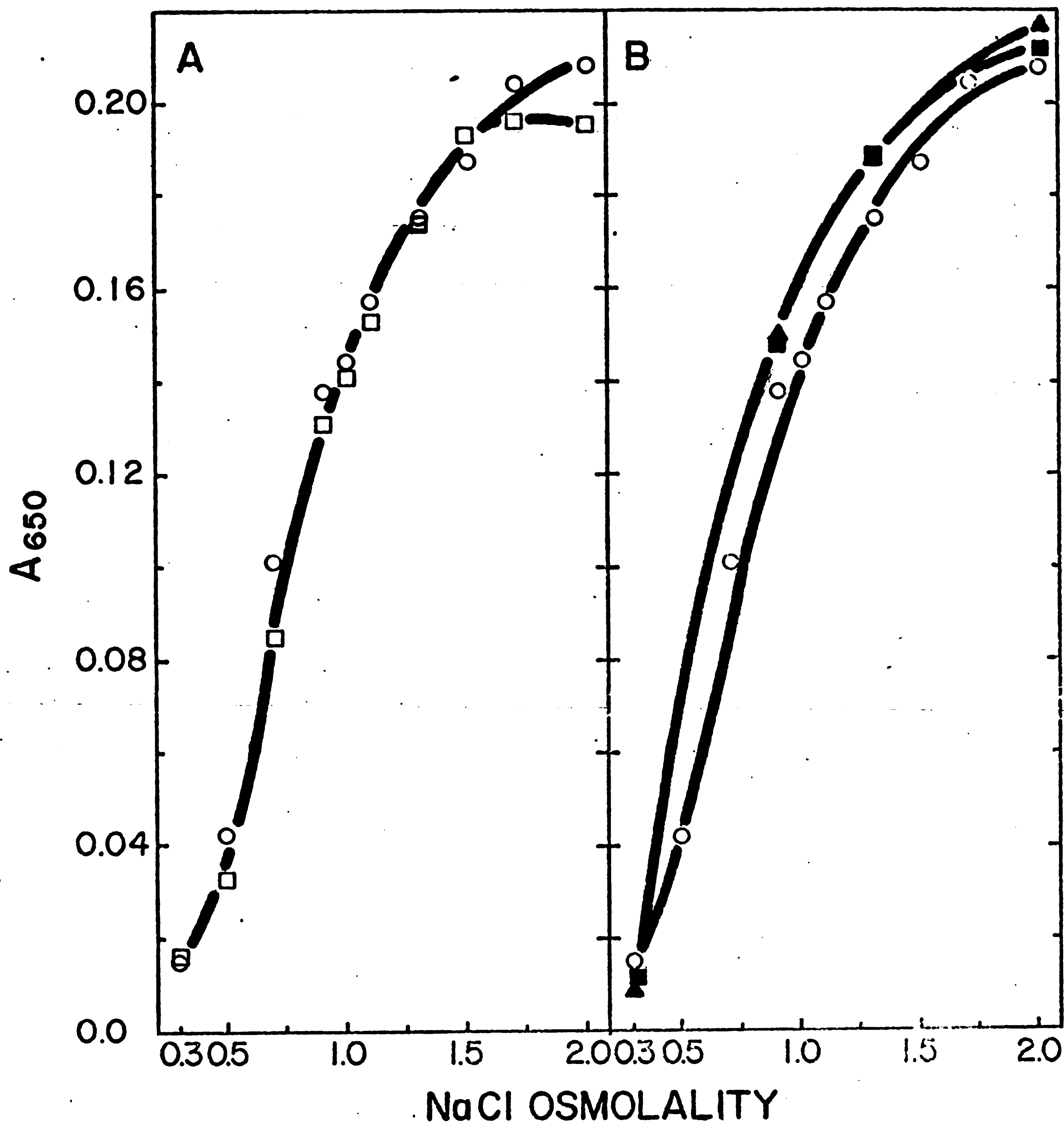


Figure 4. Specificity of the hydronium ion in acid stabilization. Protoplasts prepared in 2.0 osmolal sucrose solution at neutral pH were subjected to a concentration of (A) Na_2SO_4 (□), (B) Li_2SO_4 (■) or LiCl (▲) equivalent to that required for H_2SO_4 to give a pH of 4.1 to 4.3. Untreated protoplasts (O) are shown for comparison.

was done to test the reproducibility and reversibility of the effects of NaCl concentration on optical density values. Dilution was carried out with distilled water and concentration with a 5M NaCl solution, both of which contained an appropriate concentration of acid to maintain pH 4.1 to 4.3 when acid treated suspensions were being studied. Optical density measurements were taken at each osmolality after 10 min equilibration and corrected for the dilution in numbers.

As expected (Figure 5), values generated by sequential dilution of untreated protoplasts were nearly identical to values from separate one step dilutions, but are not reversible by concentration because of bursting. In contrast, the effects of osmotic concentration and dilution on the optical density values obtained with acid treated protoplasts were readily reversible. Although identical values were not obtained at each concentration, it is clear that the 'optical artifacts' observed for acid treated protoplasts are a reversible function of NaCl concentration.

Reversibility of Acid Effects. It has previously been reported (5, 21) that acid stabilization of bacterial protoplasts is readily reversible. Tests of the reversibility of acid effects, done by raising the pH of acid treated protoplast suspensions with 0.02N NaOH, are reported in Figure 6. Counts and optical density measurements showed that acid stabilization was lost when the pH of acid treated protoplast suspensions was raised to near neutrality. However, those protoplasts appeared to be destabilized at all

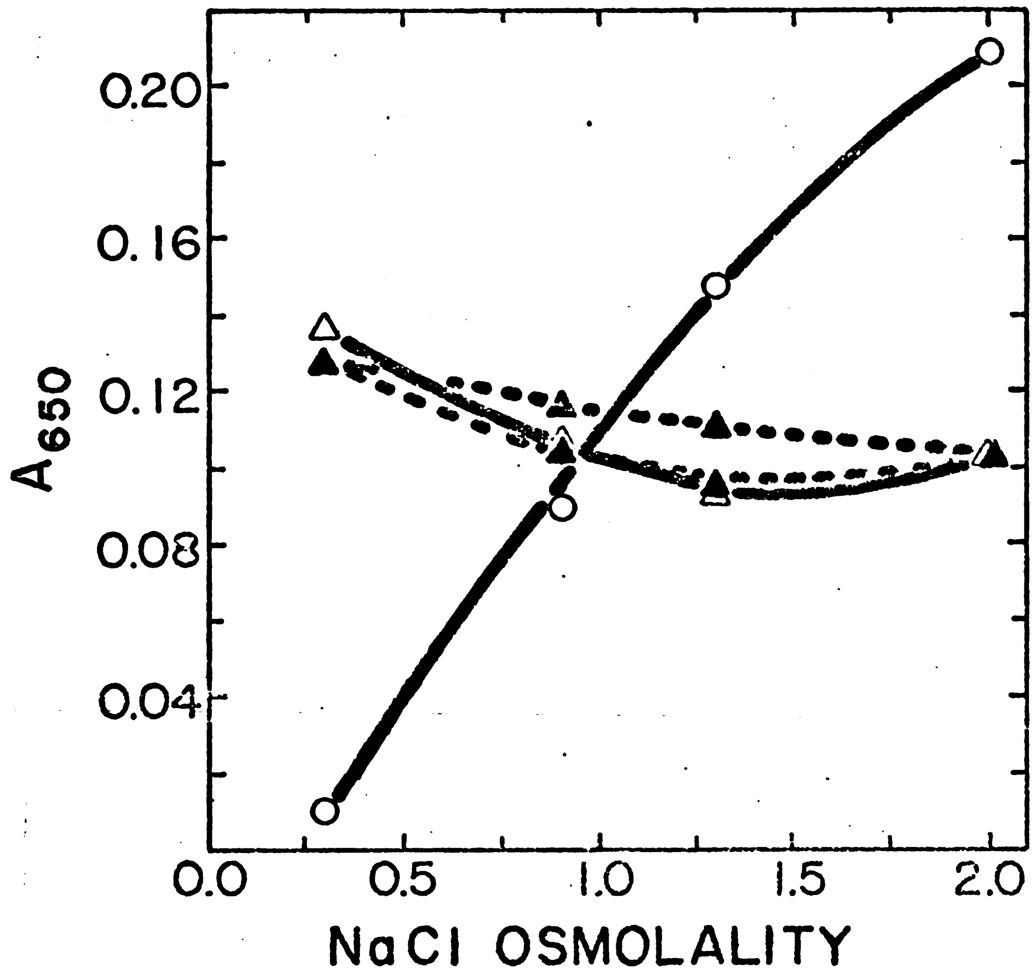


Figure 5. Effect of NaCl concentration on the optical density of acid treated protoplast suspensions. Samples of protoplasts prepared in 2.0 osmolal sucrose solution were sequentially diluted and concentrated (▲--▲) in NaCl solutions by the addition of acidified H_2O or 5M NaCl solution. Usual single-step dilutions of separate samples were done for untreated (O) and treated (Δ) protoplasts for comparison.

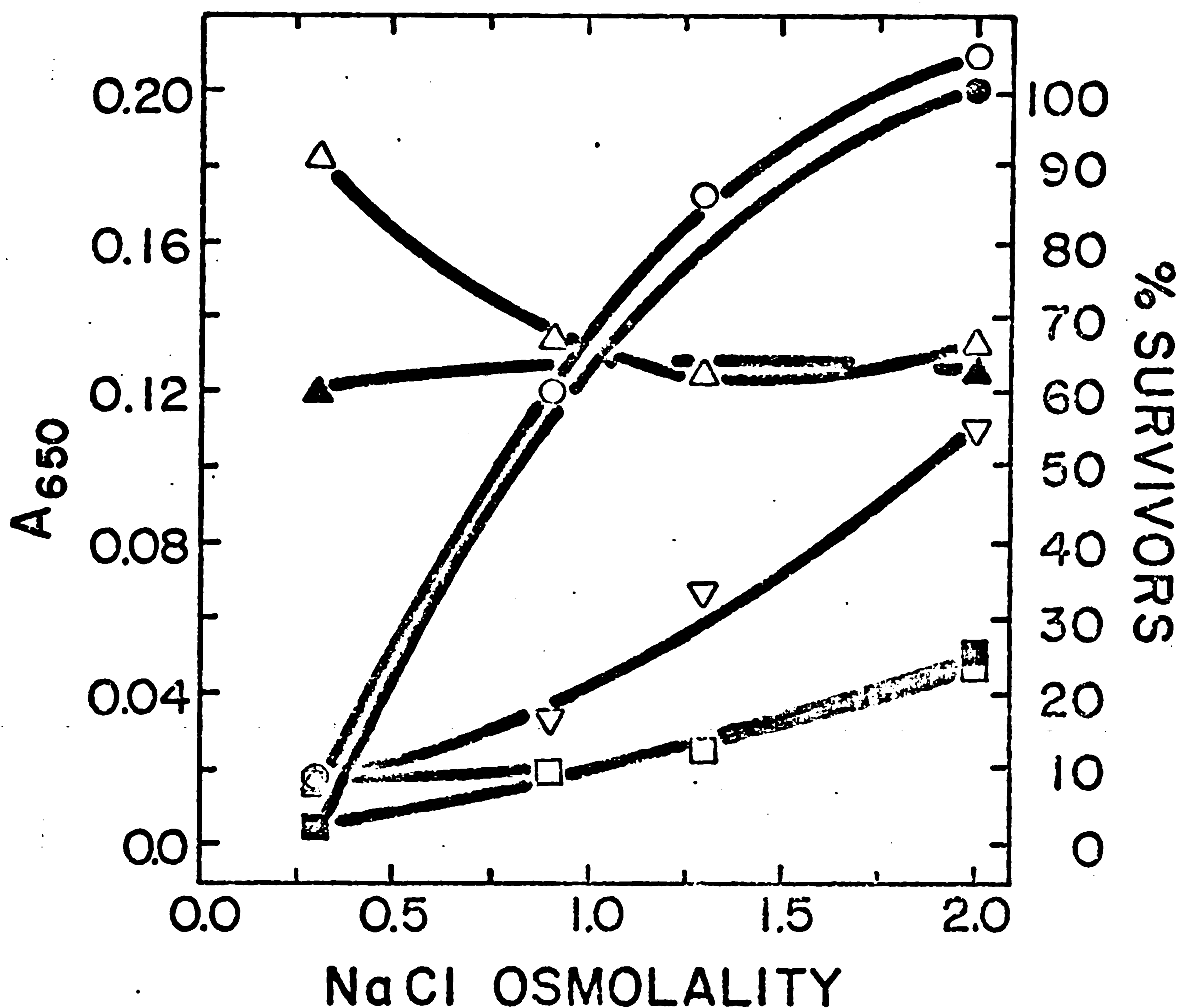


Figure 6. Reversibility of acid stabilization. Acid treated protoplast suspensions in sucrose solutions were bact titrated with 0.02N NaOH. Samples were taken at pH 4.1 to 4.3 (△,▲), pH 6.3 (▽), and pH 6.5 (□,■) and diluted in NaCl solutions. Optical density measurements (A_{650}) are represented by open symbols and microscopic counts by closed symbols. Results were similar when the pH was raised after transferring acid treated protoplasts to NaCl solutions and when 0.02N Na_2HPO_4 was used in place of NaOH. The optical density (O) and number of survivors (●) of untreated protoplasts is shown for comparison.

omsolalites. Less than 25% of input protoplasts (40% or acid treated survivors) survived when the pH was brought back to 6.5 even at 2.0 osmolality. At an intermediate pH of 6.3 destabilization was much less pronounced in 2.0 osmolal NaCl. Results were similar whether the pH was raised before or after transferring the protoplasts from sucrose to NaCl solutions. Using 0.2N Na_2HPO_4 in place of NaOH also gave similar results.

Leakage of 260 nm Absorbing Material. Additional information on the stability and integrity of acid treated as opposed to control protoplasts was sought by measuring leakage of certain cytoplasmic materials. Considering the dramatic differences in osmotic sensitivity observed, one would expect a marked difference between them in the amount cytoplasmic material leaked out by swelling or released by bursting. UV obsorbance was selected to measure this parameter. Both amino acids (280nm absorbance) and nucleic acids (260 nm absorbance) can be monitored in this way.

Filtrates from suspensions in NaCl solutions were prepared as described for this purpose. Peak absorbance in the range 230 to 300 nm occurred at 260 nm. The ratios of absorbance at 260 nm to absorbance at 280 nm (Table I) indicated that primarily nucleic acid material, presumably low molecular weight RNA, was released from acid treated protoplasts. Untreated protoplasts apparently released both protein and nucleic acid materials, which is consistent with lysis observed microscopically. In accord with microscopic

Table 1. Influence of acid treatment and NaCl concentration on the 260 to 280 nm absorbance ratios of filtrates from protoplast suspensions.

NaCl OSMOLALITY	$A_{260}:A_{280}$ RATIOS	
	FILTRATES OF UNTREATED PROTOPLAST SUSPENSIONS	FILTRATES OF pH 4 PROTOPLAST SUSPENSIONS
2.0	1.8	1.8
1.3	1.6	2.0
0.9	1.7	2.0
0.3	1.7	2.0

observations reported here and elsewhere (2, 18), untreated protoplasts showed increased release of cytoplasmic materials as they swelled and burst in response to decreased ambient osmotic pressure (Figure 7).

However, when applied to acid treated protoplasts, the results were not as clear. The small amount of 260 nm absorbing material released in 0.3 osmolal NaCl solution, compared to control protoplasts, agrees with the resistance to osmotic lysis observed microscopically and suggests that membrane integrity is maintained, since membrane damage usually is accompanied by a significant release of low molecular weight RNA (16). At higher NaCl concentrations, a greater amount of 260 nm absorbing material was apparently released (Figure 7) and the ratio of 260 to 280 nm absorbance (Table I) for the filtrate from acid treated protoplasts in a 2.0 osmolal NaCl solution was 1.8, suggesting the release of both proteins and nucleic acids. In other experiments with authentic RNA it was demonstrated that the combined effects of acid treatment and NaCl concentration on RNA alone could not account for the increased 260 nm absorbance. Furthermore, the increase in absorbance was reproducible with a single sample of protoplasts by concentrating them from 0.3 to 2.0 osmolality prior to filtration for analysis (Figure 7, dotted line). The corresponding dilution of 2.0 osmolal sample was not done because of technical difficulties in accurately measuring the resulting low levels of absorbance.

The resistance to osmotic lysis of acid treated protoplasts

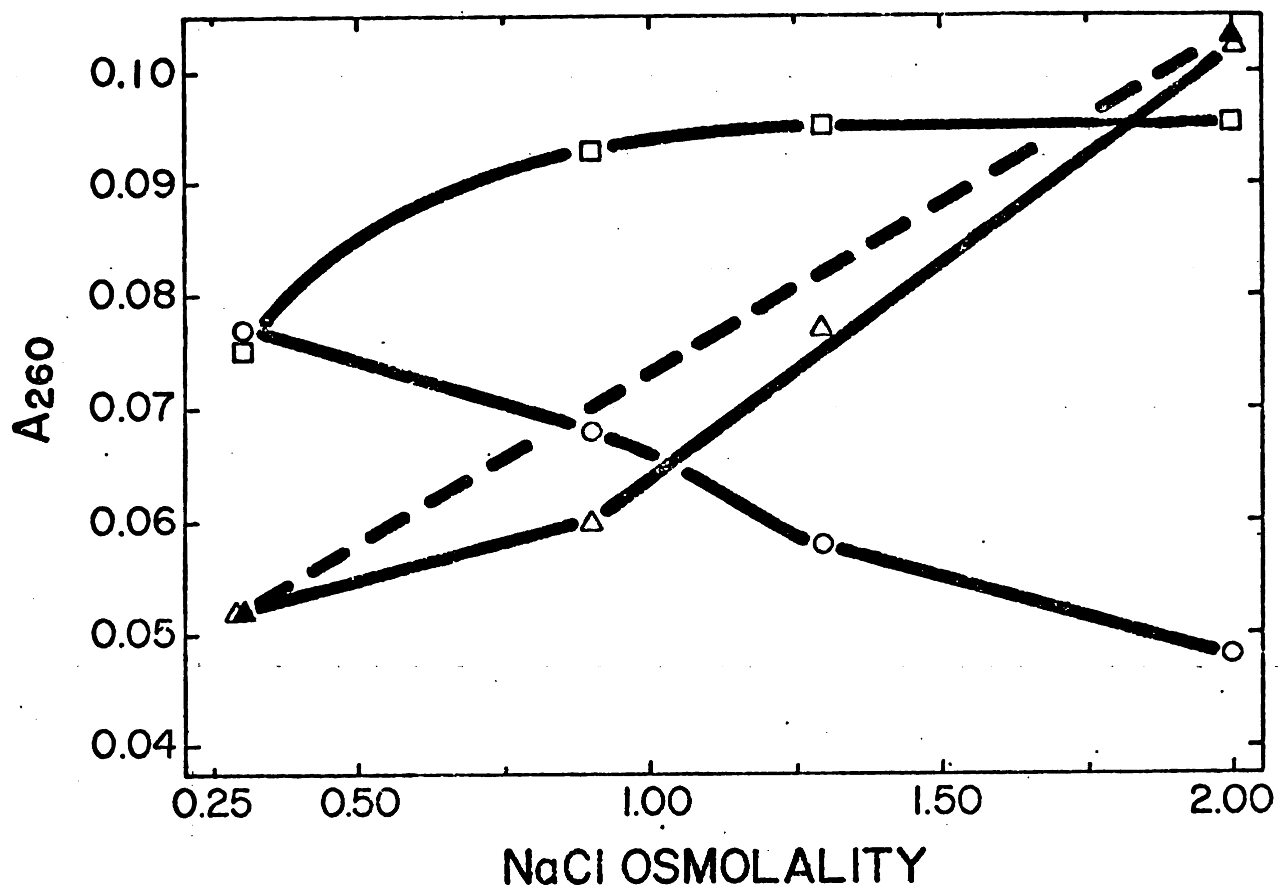


Figure 7. Influence of acid treatment and NaCl concentration on protoplast leakage. Filtrates were prepared from untreated (○) and acid treated (△) protoplast suspensions in NaCl solutions. Filtrates were also prepared from acid treated suspensions in which either the pH had been reversed to 6.5 with 0.02N NaOH before transfer from 2.0 osmolal sucrose (□) or the NaCl osmolality had been increased from 0.3 to 2.0 (▲---▲) by the addition of acidified 5M NaCl solution.

and their membrane integrity at 0.3 osmolality is strongly supported by these results. However, membrane integrity at high NaCl concentrations is questionable, especially considering the correlation of these measurements to the optical artifacts seen in A_{650} measurements and to the occurrence of less dense appearing protoplasts by phase microscopy at similar NaCl concentrations.

On the other hand, the stability of ribosomes reported by Peterman (25) and studies by Truab and Normura (37) on the reaggregation of ribosomes indicate high salt and hydronium ion concentration could disrupt ribosomes. One component that could be released and might permeate the membrane is the 5S ribonucleic acid component.

Filtrates from acid treated suspensions whose pH had been raised to 6 . 5 with 0.02N NaOH prior to osmotic dilution in NaCl solutions contained amounts of 260 nm absorbing material (Figure 7) in agreement with the destabilization previously described.

Butanol Lysis. It is known that addition of butanol to normal protoplasts in hypertonic solution at neutral pH causes membrane disruption and resultant lysis (31). Edebo (3, 4, 5) described lysis as a process requiring both membrane destruction and subsequent solubilization of the cytoplasm in the surrounding medium. Therefore, if the failure of acid treated protoplasts to lyse is dependent on membrane integrity rather than extensive denaturation and fixing of the cytoplasm, disruption of the membrane would lead to lysis.

Addition of butanol at a final concentration of 0.3 M to untreated protoplast suspensions caused rapid lysis at 25 C as evidenced by an almost complete loss of optical density at every osmolality (Figure 10). In contrast, acid treated protoplasts treated similarly showed no change in optical density even after several hours incubation at 25 C.

From these results it can not be determined whether the membrane is so toughened that it resists disruption by butanol or the cytoplasm is indeed denatured to the point of forming an insoluble gel.

Titrations. Intact and burst protoplast suspensions as well as crudely prepared soluble and membrane fractions were titrated in an attempt to determine whether acid treatment was restricted to effects on the membrane by the barrier it presents or if hydronium ion was also bound or taken up by the cytoplasm (i.e. soluble fraction). A marked difference was apparent in the amount of hydronium ion bound by intact as opposed to broken protoplasts (Figure 9); the latter bound considerably more. Very little binding was observed by the membrane fraction, the soluble fraction gave results very near those obtained with burst protoplasts, and the amount bound by the soluble fraction was much greater than the difference observed between whole and burst protoplasts.

It was found that treatment of the membrane fraction with deoxyribonuclease (DNase) made no difference in the titration, indicating DNA was probably not present in that fraction. Since the pK of the membrane fraction was approximately 2.35, the

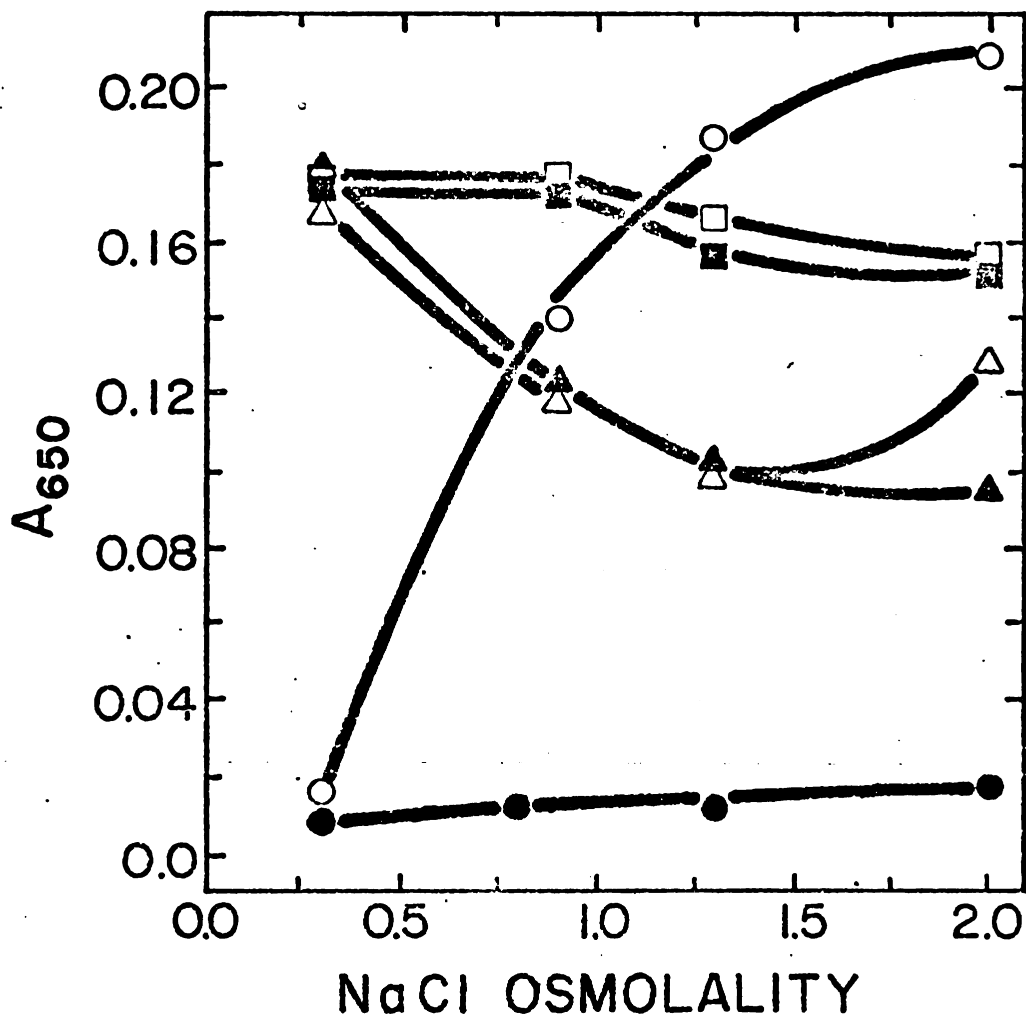
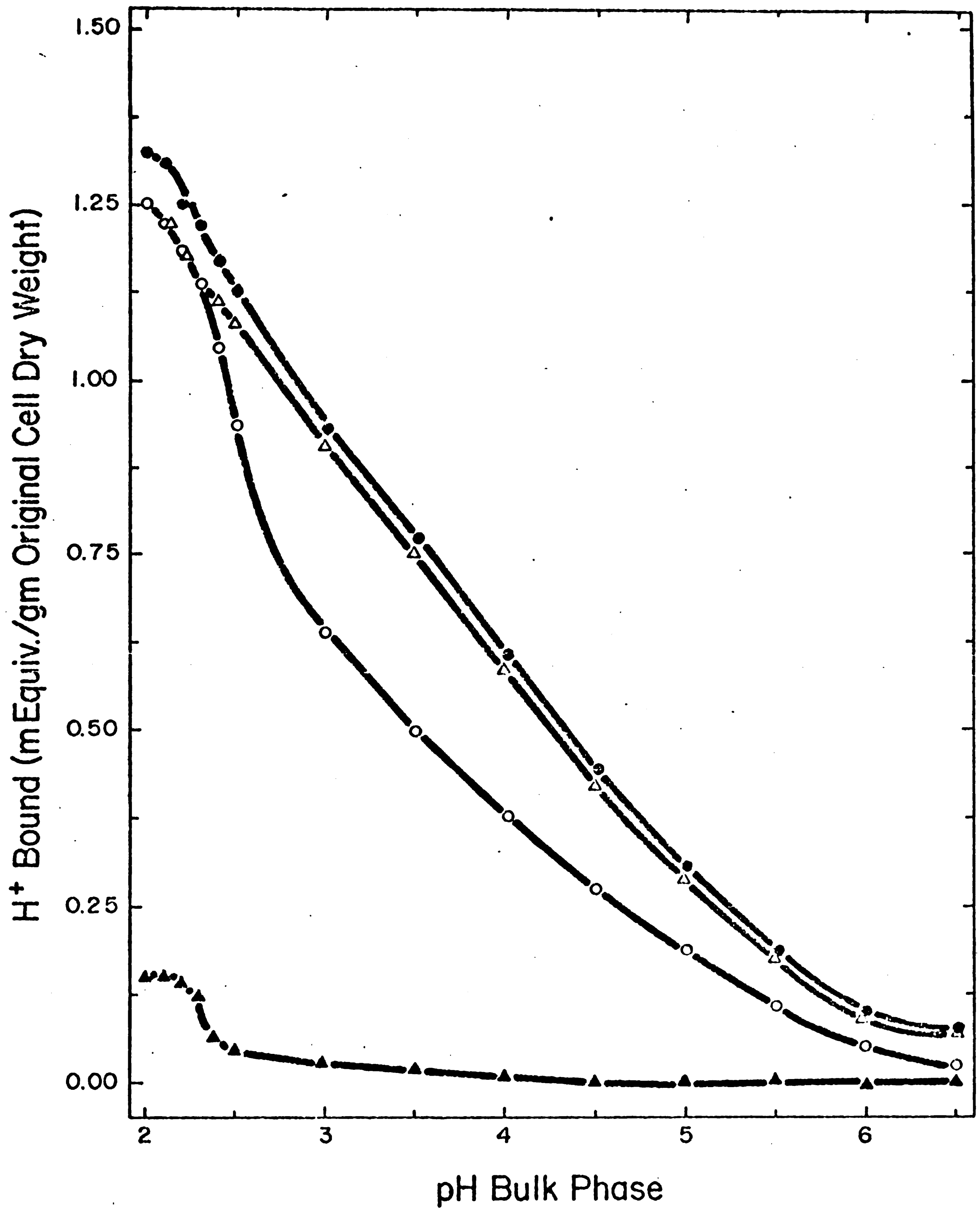


Figure 8. Resistance of acid treated protoplasts to butanol lysis. n-Butanol was added at a final concentration of 0.3M to untreated (○,●) pH4.1 to 4.3 treated (△,▲) and pH 3.6 treated (□,■) protoplasts in NaCl solutions. Open symbols represent optical density determinations before and closed symbols after butanol treatment.

Figure 9. Binding of hydronium ion by protoplasts and cell components. Intact (O) and burst (●) protoplasts and crude membrane (▲) and soluble (△) fractions, separated by centrifugation of burst suspensions at 30,000 X g for 30 min., were titrated with 0.1 N H_2SO_4. The concentrations of separated fractions were adjusted to be equivalent to their concentrations in the intact or burst protoplast suspensions. Binding by the suspending medium of 2.0 osmolal sucrose containing 770 µg lysozyme/ml and 0.004M potassium phosphate buffer (pH 7.0) was subtracted from the total amoutn bound by the cell suspension or cell fractions.



1.50
1.25
1.00
0.75
0.50
0.25
0.00
H+ Bound (mEquiv./gm Original Cell Dry Weight)
2
3
4
5
6
pH Bulk Phase

groups largely responsible for proton binding were probably the phosphate groups of membrane phospholipids. No binding was observed above pH5.

Intact and burst protoplast suspensions, but not the soluble fraction, also showed inflections in their binding curves between pH 2.5 and 2.0, which probably corresponded at least partly to binding by the membrane components present. In the case of intact protoplast suspensions, that inflection was partly due to breakdown of the membrane permeability barrier, shown by the fact that binding eventually (pH 2.3) became equivalent for intact and burst protoplast suspensions.

It was noticed when performing titrations with whole protoplasts that the bulk pH reached an apparent equilibrium in about 2 min (the time allowed during titrations), but was not maintained at pH values below 5.0. A steady increase in pH occurred after about 5 min, which was not seen with burst protoplasts, the soluble fraction, or the membrane fraction.

To clarify that apparent flow of hydronium ions, burst and whole protoplast suspensions were prepared at three different pH values and readings were obtained at fixed times after the addition of hydronium ion. The results are reported in Figure 10. It appeared that the membrane was a barrier to hydronium ion at pH 6 and 6.8, maintaining a lower pH for whole as opposed to burst protoplasts for the entire 2.5 h tested. However, at a pH near 4, a rather rapid flow of hydronium ions was apparent in whole protoplast suspensions. The initial pH was 0.8 units lower than with burst protoplasts containing the

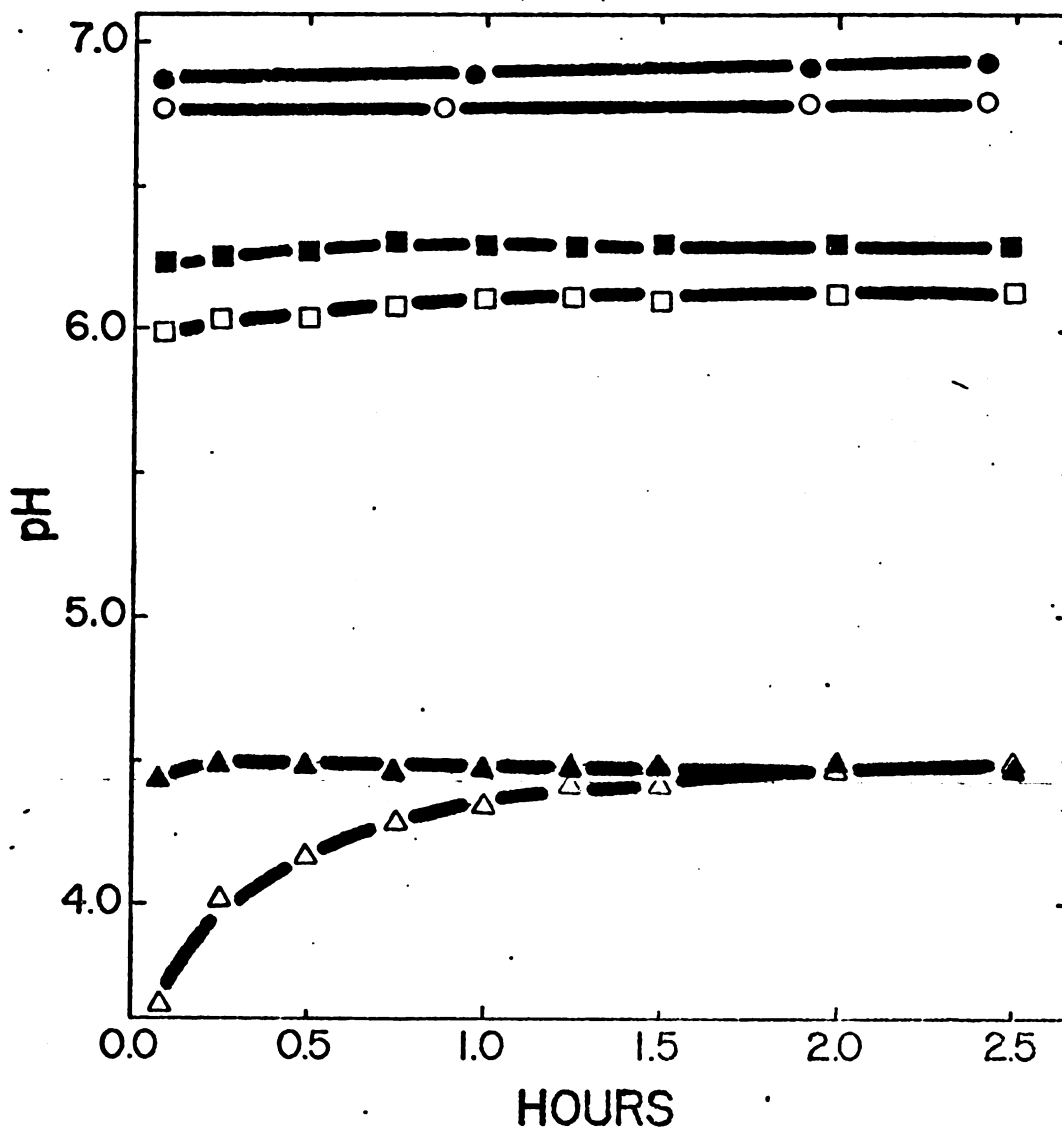


Figure 10. Time dependence of pH for acid treated protoplasts. The pH of untreated (○,●), and acid treated protoplast suspensions adjusted to pH 6 (□,■) or pH 4 (△,▲) was measured at 15 min. intervals. Intact protoplasts are represented by open symbols and burst protoplasts by closed symbols. All Protoplasts were released in 2.0 osmolal sucrose at neutral pH.

same amount of added acid, but the readings were identical in less than 2.0 h. Gilby and Few observed a similar phonomenon with *Micrococcus lysodeikticus*, but they reported the flow was much faster (5 min), apparently was not related to breakdown of the membrane barrier, and the breakdown of the membrane barrier to hydronium ion occurred below pH3. The present studies with *B. megaterium* indicated that the membrane barrier to hydronium ion gradually failed at pH values less than 5. The observed flow of ions could be due to the cytoplasm or additional binding on the interior of the membrane.

DICUSSION

The results presented in this report clearly indicate that protoplasts of *B. megaterium* are destabilized when the bulk phase pH is raised above neutrality, which is in agreement with results reported by Marquis et al. (21) for protoplasts of *Streptococcus fecalis.* Marquis et al. also found that divalent but not monovalent cations could overcome alkaline destabilization. Alkaline treatment has been found (27, 28) to destabilize all the bacterial protoplasts, spheroplasts, L-forms and mycoplasma tested. The present results show alkaline destabilization is not related to ambient osmotic pressure, suggesting a general loss of membrane integrity. One could speculate, considering the cohesional forces (1, 38) and structural models (10, 34, 35, 39) proposed for membranes, that changes in the charge distribution on membrane components by alkaline treatment causes conformational changes leading to weakened bonding and thermodynamically favored dissolution of the membrane. On the other hand, a reasonable speculation is that permeability to stabilizing solute is changed so drastically that the membrane no longer provides an effective barrier by which osmotic stabilization can be obtained. Alternatively, the physical state of the internal phase of the system, the cytoplasm, could be so altered as to greatly increase the internal osmotic pressure (e.g. increased hydration or dispersion of cytoplasmic components). Marquis et al. (21) also reported that very high concentrations of solute afforded some protection against alkaline destabilization, which lends support to the latter hypothesis. The ability of

divalent cations to overcome alkaline destabilization could fit any of these speculations. In any event, a bulk phase hydronium ion concentration of 10^{-7} M or greater is apparently necessary for protoplast stabilization in the absence of excessive amounts of divalent cation (i.e. 5×10^{-2} M).

Increasing the hydronium ion concentration in the bulk phase of a bacterial protoplast suspension to greater than 10^{-6} M (pH6) clearly changes the osmotic properties of the protoplasts. Edebo previously reported (5) that *B. megaterium* protoplasts gained resistance to osmotic shock when the ambient pH was less than 5.5 during the osmotic dilution and observed a marginal degree of resistance between pH 5.5 and 6. Marquis et al. (21) reported similar acid stabilization of *S. fecalis* protoplasts beginning at pH 5, but since the final pH after osmotic dilution was neither regulated nor monitored, it is likely that the reported stabilization actually began somewhere between pH 5 and 6. The present results confirm this acid stabilization of bacterial protoplasts and the pH range found effective is similar to that previously reported. In addition, these results indicate that such acid treated protoplasts no longer behave as osmometers, being insensitive to changes in the osmotic pressure of the external environment. The number of survivors and their final average volume remained constant at every ambient osmolality tested and the volumes were nearly identical whether NaCl or sucrose, which are of different molecular size, was used as stabilizing solute. This is in sharp contrast to the results obtained with untreated

protoplasts, the number of survivors and final average volume of which were dependent on the external osmolality as well as the molecular size of the stabilizing solute as previously reported (2, 18). The slightly greater number of acid treated survivors found in sucrose than in NaCl solutions (i.e. 75% and 60% of input number respectively) could be an indication of the interaction of ionic strength and hydronium ion concentration, although it may simply reflect random error due to the small number of acid treated sucrose suspensions counted. Certainly, it does not appear to be related to either osmotic pressure or permeability consideration, since ambient osmotic pressure changes had no effect on survival and no differences in the volumes of acid treated protoplasts were found with either different osmotic pressures or stabilizing solutes of different molecular size.

Evidence for the interaction of ionic strength and acid treatment was also obtained from the leakage studies and the optical anomalies reported. It is tempting to conclude a cause and effect relation between the phenomena of increased leakage (A_{260}) at high salt concentration and decreased optical density which was observed both microscopically (phase contrast microscope) and spectrophotometrically (A_{650}). Surely leakage of cytoplasmic materials could decrease optical density in the absence of a volume change. Increasing the NaCl concentration was found to effectively induce all three measured parameters simultaneously, and the optical density anomalies were found to be reversible by altering the NaCl

concentration. Furthermore, microscopic phase darkness of sucrose stabilized, acid treated protoplasts was constant from 2.0 to 0.3 osmolal. This could mean acid treated protoplasts are insensitive to osmotic pressure change but affected by changes in ionic strength, which is reasonable since the perturbation is caused by ions.

The source of the material leaked at a high salt concentration is not known, but disaggregation of ribosomes is possible considering the reported effects (25, 37) of pH and salt concentration on ribosomes, and the 5S RNA component could probably permeate the intact membrane. Alternatively, the 260 nm absorbing material observed could be of some other source by the degradative action of nucleases. Of course, this leakage could be a result of membrane damage or destruction, thus altering or destroying the permeability properties of the protoplast, but probably not as a result of acid treatment alone because little leakage was observed in solutions of low ionic strength and low osmolality (i.e. 0.3 osmolal NaCl). Therefore, it appears that acid treated protoplasts had a structurally intact membrane, at least in solutions of low ionic strength. However, additional data need to be obtained by studying leakage of cytoplasmic materials other than 260 nm absorbing substances, determining the nature and likely source of the leaked substance measured in this study, and comparing these parameters between NaCl or sucrose stabilized acid treated protoplasts.

Edebo (5) and Marquis et al. (21) also reported that

acid stabilization of bacterial protoplasts is readily reversible. They assumed that bursting of acid treated protoplasts in hypotonic media when the ambient pH was raised to neutrality indicated a return to the former, untreated state. However, the stability of acid treated protoplasts in hypertonic media after back titrating to neutrality was not tested. The results reported here indicated that the small percentage of protoplasts surviving acid treatment and back titration had regained sensitivity to ambient osmotic pressure changes even at a pH as low as 6.3. However, destabilization was evident regardless of the external osmolality and increased greatly from pH 6.3 to pH 6.5. It could be that acid treatment caused such extensive denaturation of cell components that renaturation was not readily achievable, which is consistent with the lack of susceptibility of acid treated protoplasts to n-butanol.

Actually, the inability of n-butanol to lyse acid treated protoplasts provides little absolute information on the state of the membrane or the cytoplasm, except that the simultaneous occurrence of an intact, butanol susceptible membrane and a readily soluble cytoplasm cannot be the case (3, 4, 5). However, the membrane could be intact and susceptible, but the cytoplasm denatured to an insoluble gel; or the membrane could be intact, but toughened so that butanol is unable to distupt it, and the cytoplasm either affected or unaffected. It is probable that conditions which would denature and gel the cytoplasm would have a similar effect on the membrane. The

effect of other lytic agents of specific membrane activity on acid treated protoplasts, as well as electron microscopy, might provide additional information on the physical state of the membrane and cytoplasm in these protoplasts.

Attempts were made in this study to ascertain which cell components were affected by the acid treatment. The approach was to study the binding of hydronium ion by whole and burst protoplasts and crude subcellular fractions. Little is known about the relation of the ambient pH to the internal pH of bacteria, but it has previously been reported (8, 21, 43) that the bacterial membrane of *E. coli*, *M. lysodeikticus*, *and S. fecalis* is a permeability barrier to hydronium ion even as low as pH 3 or 4. Those conclusions were drawn from titrating both burst and whole, cell or protoplast suspensions and observing a difference in buffering capacity. Similar results were obtained in the present studies with *B. megaterium* by titrating both burst and whole protoplasts. This supported the notion (7, 8, 43) that the membrane acts as a barrier to hydronium ion. If it were a complete barrier, then binding by separated membrane and soluble fractions would account for the difference in binding by whole and burst protoplasts, and one would predict the membrane fraction to bind an amount similar to that bound by whole protoplasts, and the soluble fraction to bind an amount similar to the difference in binding by burst and whole protoplasts. Those results were not obtained. The membrane fraction bound much less than predicted, and the soluble fraction bound much more

than predicted. This could be due to inclusion of cell wall material in the soluble fraction and in the protoplast suspensions but not in the membrane fraction, because of the extensive degradative action of lysozyme (28, 30). Cell Walls from *B. megaterium* can bind a considerable amount of hydronium ion. However, results reported by Marquis (21) on the titration of isolated cell walls and on the % cell dry weight attributable to cell wall material, when compared to the present results, indicated that the observed difference in binding between whole protoplasts and the membrane fraction can not be accounted for by binding to cell wall material. It was also noted that whole protoplasts suspensions yielded a nonlinear titration curve compared to results with suspensions of burst protoplasts or subcellular fractions, especially below pH3, which could be an indication of a gradual failure of the permeability barrier to protons.

The results of studying pH as a function of time with acid treated protoplasts confirm that the membrane is not a complete permeability barrier to hydronium ion when the bulk phase pH is brought to 5 or less, since an apparent flow of hydronium ions was observed resulting in an equivalent equilibrium for intact and broken protoplasts suspensions within 2.0 h. Gilby and Few (8) observed a change in pH with time when titrating *M. lysodeikticus* but the time required to reach equilibrium was about 5 min and the observed flow apparently did not involve failure of the membrane barrier until the pH was less than 3. It is possible that they

allowed insufficient time (15 min) to observe the permeability failure reported here. However, the effect of an intact cell wall on this phenomenon is not known.

The flow of hydronium ions observed in the present study is important because pH readings were taken during titrations after 2 min equilibration. If they had been taken after 1 or 2 h equilibration, the titrations curves for intact and burst protoplasts would probably be identical below pH 5. Furthermore, a single sample of protoplasts was used for an entire titration. Therefore, the 2 min equilibration times were cumulative , which is probably the reason for a nonlinear titration curve for whole protoplast suspensions.

Although it has not been determined where the additional binding involved in this gradual change of pH occurs, involvement of the cytoplasm is strongly suggested since intact and burst protoplasts eventually reach the same equilibrium. Therefore, since most measurements were made 1 h or more after acid treatment, the stabilization effects previously described probably involve the cytoplasm as well as the membrane. It is noteworthy that the membrane barrier to hydronium ion was observed to fail at about pH 5 and acid stabilization of protoplasts apparently began between pH 6 and 5.5. This could mean that only the membrane is involved in acid stabilization above pH 5.

It should be noted that the flow of hydronium ions was a relatively slow process, indicating the membrane barrier was not destroyed, but the permeability properties must have been

altered. In this regard, the decreased permeability for cations reported (11) for cells grown at pH5 may seem to conflict the present results, but those studies (11) were carried out by testing the valinomycin mediated exchange of Rb^+ using cells from cultures grown at pH5 rather than nonrespiring protoplasts.

This observed net flow of protons across the protoplast membrane below pH5 but not above pH5 may be significant in regard to testing for the active extrusion of protons, which has been reported (12) for *Streptococcus fecalis*. It could be that the apparent permeability barrier to protons observed above pH5 is a net effect involving an extrusion process which fails at pH5 or lower. This hypothesis warrants further investigation.

In conclusion, it is evident that acid stabilized protoplasts are devoid of measurable osmotic responses but the cell components involved and their physical state remains uncertain. Little leakage at low osmolality, insensitivity to butanol, and destabilization upon raising the ambient pH suggest acid treated protoplasts are structurally intact, including the membrane, but extensively denatured. Studies on the change of pH of protoplast suspensions with time confirm the cytoplasm is involved in acid treatment below pH5. Probably both the membrane and cytoplasm are acid fixed or denatured below pH5. The nature of acid stabilization between pH5 and 6 warrants further investigation, since involvement of the cytoplasm has not been indicated in acid treatment above pH5.

Membrane chemistry and permeability characteristics also need to be investigated at different ambient pH values and compared to the effects of pH on growing cells, since the relation between acid stabilization of non-respiring protoplasts and protoplasts from cells grown at acid pH is unknown.

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