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Effects of pH and osmolarity on the composition and distribution of membrane phospholipid of Bacillus stearothermophilus

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THE EFFECTS OF pH AND OSMOLARITY ON THE COMPOSITION AND DISTRIBUTION OF MEMBRANE PHOSPHOLIPID OF

BACILLUS STEAROTHERMOPHILUS.

By

Jonathan K. Trautman

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1987

Approved by

Chairman, Board of Examiners

Dean, Graduate School

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The effects of pH and osmolarity on the composition and distribution of membrane phospholipid of Bacillus stearothermophilus (83 pp.)

Director: Dr. George L. Card

This study was guided by the hypothesis that changes in the anionic membrane lipids of Gram positive bacteria are part of the cellular mechanism for adapting to the pH, ionic strength, and osmolarity of the growth medium. Bacillus stearothermophilus was grown under varying culture conditions and analyzed for changes in the relative lipid composition and distribution of the different phospholipids between the inner and outer layers of the membrane bilayer. In growth studies, cells showed increased sensitivity to the addition of both electrolytes and nonelectrolytes as the pH of the growth medium was increased. Increasing the pH of the culture medium resulted in an increase in total cellular lipid which was primarily due to an increase in anionic lipid concentration (especially phosphatidylglycerol). The increase in pH also resulted in an increase in the amount of lipid released from the cells during growth. The ratio of anionic to zwitterionic lipid was higher in the extracellular lipid fraction than the cellular fraction, which suggested that lipid released from the cells was derived from the outer layer of the membrane bilayer. The addition of electrolytes resulted in a decrease in total cellular lipid in medium buffered at pH 5.8 and inhibited growth at pH 7.0 and 8.0. Under all growth conditions, over 95% of the lipid at the outer membrane surface consisted of anionic lipid. The results were consistent with a model in which the potential and, therefore, the pH, ionic strength, and osmolarity at the outer membrane surface is a function of charge density and controlled by regulating the anionic lipid concentration.
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INTRODUCTION

The lipid bilayer model for membrane structure was first proposed some 60 years ago (20) and has undergone a number of revisions. Today, the generally accepted concept is that of the Fluid Mosaic Model, put forth by Singer and Nicholson (75), based upon freeze-etching and freeze fracturing studies of membranes. In this model, the membrane is composed of a lipid bilayer in which proteins are embedded. The membrane is comprised of 60-80% proteins and 20-40% phospholipids. Of the phospholipids, 20% are associated with proteins while the remaining 80% make up the bilayer (80).

Phospholipids are amphipathic molecules consisting of a hydrophobic region (the fatty acids) and a hydrophilic region (the glycerophosphate group). The molecule consists of a glycerol to which long chain fatty acids are usually esterified to the 1 and 2 positions. The first position is generally occupied by a saturated fatty acid and the second position by an unsaturated fatty acid. The ratio of the two fatty acid types determine the nature of the membrane fluidity (15). A polar headgroup is linked to the sn-1,2-diacylglycerol-3-phosphate by a phosphodiester bond. The most common phospholipids found in the bacterial membranes are the zwitterionic lipid phosphatidylethanolamine (PE) and the two anionic lipids phosphatidyglycerol (PG) and cardiolipin (CL) (Fig. 1). The relative percentage of each phospholipid will vary among bacteria but under any given condition will remain constant for a particular bacteria. For example, Gram nega-
tive bacteria, as a group, consist primarily of PE, which represents 70–90% of the total membrane lipid. In Gram positive bacteria, however, PG and CL together represent 40–90% of the total lipid. In both cases, these percentages will remain invariable for a specific growth condition. A physiological explanation for the differences in lipid composition among different organisms remains to be established. In fact, it is not clear why any particular organism contains different types of phospholipids.

Although the phospholipid biosynthetic pathways are well known, at least for Escherichia coli (16), the mechanisms that control phospholipid composition remain unclear (78). This includes the regulation of the ratio of zwitterionic to anionic lipids. It has been suggested (14,65,79) that the branch point of phospholipid synthesis at CDP-diglyceride, is involved in polar headgroup regulation. It is at this point where the division is made for zwitterionic or anionic lipid headgroup attachment.

In studies of mutants of phospholipid synthesis of E. coli, a number of suggestions were made about the regulation of phospholipid synthesis (28, 64). Phosphatidylserine synthetase (pss) attaches the phosphatidyl moiety to the hydroxyl group of L-serine, the first step of PE production (64). In a mutant that causes a 6–15 fold increase in pss, there was only a slight change in the relative lipid composition. This led to the conclusions that other factors such as intracellular concentration of precursors or accessibility of the enzyme to membrane substrates were involved in membrane composition regulation. From another mutant of E. coli, it was suggested that a certain ratio of anionic to zwitterionic lipid was im-
important for some vital membrane function (28). This mutant was deficient in phos-
phatidylserine decarboxylase which decarboxylates phosphatidylserine and forms
PE. The inhibition of PE synthesis caused an accumulation of phosphatidylserine
(PS), an anionic lipid, which resulted in the stopping of cell growth upon a 20-40%
replacement of the total membrane phospholipid with PS. It was also suggested
that the level of activity of an enzyme may be more important for regulation, than
the actual amount of the enzyme present in the cell (28).

The phospholipid composition is also influenced by conditions in which the
bacteria are grown. The membrane composition has been shown to be influenced
by temperature. The CL content increased in Bacillus stearothermophilus as the
growth temperature was increased (46). The rate of aeration of the cells also in-
fluenced the relative concentration of PG and CL. Cessation of aeration caused PG
to be converted to CL, and uptake of precursors was halted (8). The relative con-
centration of CL was shown to increase in both E. coli (11) and B.
stearothermophilus (8) when cells reached the stationary phase of growth. In the
case of B. stearothermophilus, the concentration of PE also increased whereas PG
was seen to decrease due to CL increase.

Both pH and osmolarity have been shown to influence the membrane com-
position as well as the ratio of zwitterionic to anionic lipids. In a chemostat study
involving two strains of Bacillus subtilis (51), the relative percentage of a lysine
derivative of PG was shown to decrease as the pH was raised. When B. subtilis
var. niger was grown in a Mg**+-limited chemostat, the level of PG increased while
PE disappeared when the pH was increased from 5.0 to 7.0. The CL level remained
constant while a polar glycolipid, diglucosyl diglyceride (DGDG) decreased slightly. When the pH of the chemostat was increased to 8.0, both CL and lysyl-PG disappeared while PG and DGDG increased substantially. For B. subtilis var. niger, the relative percentage of CL doubled at the expense of PG and lysyl-PG, whereas PE and DGDG remained constant when the pH was increased from 5.0 to 7.0 in a phosphate-limited chemostat. In the case of B. subtilis NCIB 3610, both PE and lysyl-PG disappeared when the pH was increased from 5.0 to 7.3. The level of PG decreased, the CL level doubled, and the DGDG level tripled, when the pH was increased. From these results it was suggested that: 1) the increase in positively charged lipids was a physiological response to the change in the proton concentration and 2) the PE level was important for ionization of the membrane surface (51).

Cells of Staphylococcus aureus and Streptococcus faecalis were observed to form lysyl-PG as the pH was lowered (31). These results suggested that the derivative might function in amino acid transport. Op den Kamp et al. (57) studied the effect of pH on the lipid composition of Bacillus megaterium MK10D. They observed that in a growth medium at pH 5.0, a glucosamine derivative of PG was formed which was not seen in cells grown at pH 7.0. The relative percentage of PG went from 40% at pH 7.0 to 8% at pH 5.0, whereas glucosaminyl-PG made up 32% of the total lipid of cells grown at pH 5.0. PE, lysyl-PG, and CL remained constant throughout the pH shift. In B. stearothermophilus B65 (45), no amino derivatives were formed. However, PE was shown to increase as the pH was lowered, regardless of the temperature at which the cells were grown.
Bacteria are classified into four groups based on their requirement for NaCl: non-halophiles, halotolerants, moderate halophiles, and extreme halophiles (44). Extreme halophiles will be discussed later. Non-halophiles and halotolerant bacteria do not require NaCl to grow. The difference between the two groups is that non-halophiles are inhibited by a high NaCl concentration (0.5 M) whereas halotolerant bacteria are capable of growth in medium containing up to 4.0 M NaCl (41). Moderate halophiles require a minimum of 0.5 M NaCl and are able to grow in media up to 3.0 M NaCl (83). There are similarities between these three groups. The first similarity is in their cell envelope structure, where the phospholipids are formed from the sn-1,2-diacylglycerol-3-phosphate (32,54,60,88) previously mentioned. The second similarity is that the osmoregulatory mechanism for adapting to increased solute concentration involves increasing the levels of internal “compatible solutes” (32,48,60). Generally, these solutes are amino acids such as proline, glutamic acid, and the amino acid derivative betaine. Exceptions to requiring a high amino acid concentration internally were observed by Oren (60) in Haloanaerobium praevalens and Halobacteroides halobius, which were found to contain high intracellular K⁺ and Na⁺ concentrations in a medium of high NaCl concentrations.

Extreme halophiles require a minimum of 3.0 M NaCl and have a different membrane structure. They do not have typical phospholipids but rather ether linked isoprenoid alcohols similar to other Archaebacteria (19,42). The compatible solute for growth for these bacteria is K⁺. The intracellular concentration of K⁺ increases as the NaCl concentration of the medium increases.
There was also a change in the phospholipid composition of halotolerant and moderately halophilic bacteria in response to increasing NaCl concentration. An increase in osmolarity caused an increase in anionic lipid. In a study of *S. aureus* 209P (a halotolerant organism), Kanemasa et al. (33) reported that the relative percentage of CL markedly increased from 10% to 50% when cells were grown in media of increasing NaCl concentration. The levels of both PG and lysyl-PG decreased while a glycolipid, present in the membrane, remained at a constant level. Komaratat and Kates (38) observed that a halotolerant strain of *Staphylococcus epidermidis* had a comparable composition to that of *S. aureus* 209P. The major phospholipid was PG and there were three glycolipids present: diglycosyl diglyceride, glycerophosphoryl diglucosyl diglyceride (GPDGD) and monoglucosyl diglyceride. The lysine derivative of PG was absent. Upon increasing the NaCl concentration, both CL and GPDGD increased at the expense of PG. Neither of the other two glycolipids were affected by the increased NaCl concentration. The greatest change occurred between 15 and 25% NaCl. Even with the decrease in PG level, the average number of negative charges per molecule of phospholipid increased.

In the moderate halophile *Vibrio costicola* (37), an increase in the level of PG was observed upon shifting the cells from 1 M NaCl medium to a 3 M NaCl medium. During this transition, PG synthesis increased while PE synthesis stopped. There was no degradation of PE. Upon adaptation of the cells to the higher NaCl concentration, PE synthesis resumed, and the rate of PE synthesis increased to almost the same level as that of PG synthesis (37). It was suggested that the rela-
tive increase in anionic phospholipids, was required to balance the excess in cations at the membrane surface and that growth could only continue after the adjusted membrane composition was achieved (37,71).

Ohno et al. (56) observed in *Pseudomonas halosaccharolytica* that the ratio of negatively charged phospholipids to the zwitterionic phospholipid (PE), increased as the NaCl concentration increased. They also noticed that there was an increased substitution of cyclopropanoic fatty acids for mono-unsaturated fatty acids when cells were grown in increasing NaCl concentration. A similar effect was observed when cells in a 2 M NaCl medium were grown at increasing temperatures. The salt effect on cyclopropanoic substitution was observed at all growth phases which was unlike *E. coli* (47), where it occurred only at stationary growth. They suggested that the substitution was due to salt induced cyclopropane synthesis.

The halotolerant bacterium, *Halomonas elongata*, had some additional responses to increased NaCl concentration (85). In addition to an increased anionic lipid level with increasing NaCl levels, the cell became more compact. Vreeland et al. (85) suggested that the ability to exist in high salt concentrations was due to alterations in the cell physiology. These alterations were suggested to be in ways to increase structural integrity, and to make the cell less susceptible to NaCl induced dehydration by reducing the amount of cell associated water.

The variation of phospholipid composition has been shown to influence cell morphology and a number of cellular functions. Op den Kamp et al. (58) observed that when cells of *B. megaterium* were grown at a low pH they had a greater relative amount of lysyl-PG. The protoplasts formed from these cells retained the rod

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shape of the intact cells. In contrast, cells grown at a neutral pH had a higher percentage of PG and formed spherical protoplasts. The protoplasts from cells grown at neutral pH were more susceptible to lysis in a hypotonic solution than protoplasts derived from cells grown at an acidic pH. The temperature sensitive mutant of *E. coli* (63) which caused a cessation of growth due an inhibition of pss, had a high level of CL. These cells stopped dividing and formed long filaments. Their ratio of PE to the anionic lipids changed dramatically. The relative percentage of PE dropped from 66 to 32% while PG and CL increased 34 to 68%. Even when the cells were able to grow, there was some slight elongation of the cells. These cells had a lower amount of PE. It was suggested that the PE level was important for cell division. The stability of *B. stearothermophilus* was shown to be related to the relative concentration of cardiolipin (52).

Phospholipid composition might also influence membrane permeability. Hopper et al. (30) noted that bilayers composed of lysyl-PG were anion selective. Conversely, bilayers containing PG and CL were cation selective. Those membranes consisting of PE and diglucosyl diglyceride were slightly cation selective, suggesting that the charge on the polar head groups played an important role in controlling ion selective permeability. This was confirmed in a study of permeability of whole cells and derived liposomes of *S. aureus* (27). In both liposomes and intact cells, the permeability of the nonelectrolyte erythritol increased as the lysyl-PG to PG ratio increased. Thus it was concluded that the phospholipids determined the properties of the permeability barrier.

Examination of phospholipid requirement of the lactose carrier activity in *E.*
coli (10) showed that activity was maximized in E. coli vesicles and proteoliposomes containing phosphatidylcholine (PC) mixed with PE and PS. In liposomes containing exclusively PG, or PC mixed with PG, CL, or phosphatidic acid (PA), the carrier activity was diminished. Increasing the methylation of PE or modifying it with trinitrobenzene sulfonic acid (TNBS) also caused a progressive reduction in carrier activity. Chen and Wilson (10) suggested that amino containing phospholipids may be required for full lactose carrier activity. A similar response to the phospholipid content was seen in proteoliposomes of Pseudomonas aeruginosa (81). Uratani and Aiyama observed that the sodium-dependent leucine transport system functioned optimally in liposomes containing PE. PG containing proteoliposomes also enhanced transport of leucine but to a lesser extent. Proteoliposomes containing exclusively PC inhibited leucine accumulation. A second aspect of this study showed that not only was leucine transport sensitive to the polar headgroup composition but also to the fatty acid composition. Transport activity was enhanced with a lengthening of the acyl group.

The individual phospholipids may be asymmetrically distributed between the inside and outside layer of the membrane bilayer. The membrane which has been most studied, and convincingly shown to have phospholipid asymmetry, is that of the red blood cell (3,26,59,84). It has been shown by chemical modification (26) and use of phospholipases (84) that PE and PS preferentially reside in the inner leaflet whereas PC and sphingomyelin, as well as the glycolipids and cholesterol, reside mainly in the outer leaflet. In the bacteriophage PM2 (72), the outer leaflet
consists primarily of PG whereas the inner leaflet consists mainly of PE. This was determined by the use of $^{35}$S-sulfanilic diazonium salts and the use of a varying LiCl concentration to regulate penetration of the salts to the inner leaflet. The derivative formed with PG was then reduced with NaB$_3$H$_4$. Rothman et al. (68) showed in the influenza virus that PC and phosphatidylinositol (PI) were located mainly in the outer leaflet, PE and PS resided equally in each leaflet and sphingomyelin existed primarily in the inner leaflet. They hypothesized that the virus acquired the asymmetrical bilayer as a result of budding from the host cell plasma membrane.

Bacteria present many variations on membrane phospholipid topography. In a strain of B. subtilis (4), 60% of PE was observed to be in the outer leaflet. Clostridium butyricum showed a similar pattern. The ratio of PE (predominantly in its plasmologen form) was 2:1 outer:inner leaflet (25). The relative percentage of PE was 92% in the outer monolayer in cells of Clostridium amylobiiquefaciens (61). Barsukov et al. (2) used lipid exchange transfer proteins to determine the asymmetry in Micrococcus lysodeikticus. By transferring PC present in PC vesicles with the externally located phospholipids of M. lysodeikticus, it was observed that 80% of PG was located in the outer leaflet. CL was equally distributed between the two layers and PI was preferentially located in the inner leaflet. The distribution of phospholipids may also vary between strains of a bacteria. Demant et al. (18) found that PE was equally distributed between the two leaflets in B. megaterium MK10D whereas in B. megaterium KM (69), two-thirds of PE was found in the inner leaflet of the membrane.
In the study by Rothman and Kennedy (70), PE synthesis was shown to occur only at the inner monolayer. It was then shown by pulse-chase experiments using \(^{33}\text{P}\) labeled cells pulsed with \(^{32}\text{P}\), that the PE was translocated across the bilayer to the outer monolayer. The translocation was suggested to be facilitated by a specific membrane protein which transferred the PE on the order of 30,000 times faster than transbilayer (flip-flop) exchange of PE in artificial membranes (70). The asymmetry of membranes appears to be independent of a kinetic barrier of transbilayer exchange (43), and also of expenditure of metabolic energy (43). The asymmetrical distribution of the membrane phospholipids is maintained under an equilibrium condition which was suggested to primarily reflect on the differential binding of phospholipids, by proteins and other ligands on each side of the membrane (43). Langley and Kennedy suggested that the action of the uncouplers caused a variation in the ionic environment and possibly the membrane potential and thus altering phospholipid distribution.

Varying the charge distribution at the membrane is considered to effect the membrane potential. Studies of bilayer structures have shown that the effects of the asymmetric membrane potential with respect to surface charge density, on the surface potential and the diffusion potential, compensate for each other (54). Thus the effect on the membrane potential is negligible except when the diffusion potential is negative. This could also affect localized charge distribution on the cell wall through differentiation of electronegativity (77), or in the cell membrane by laterally diffusing phospholipids to specific sites since phospholipids have a high rate of lateral diffusion (39). Rigaud et al. (67) showed by the use of an im-
permeable cationic probe and uncouplers of the proton gradient there was an interdependence between the surface or membrane potential and the pH gradient. Not only would variation in the negative charges on the surface affect the pH gradient, but it would also affect the electrochemical proton gradient (46). This was shown by incorporation of various phospholipids in a reconstituted purple membrane (66). Incorporation of PE did not alter proton uptake at the steady state. However, there was some increase in the initial proton pumping rate. Addition of CL and PA caused an increase in the initial pumping rate. In addition, the efficiency of proton pumping due to an increased amount of negatively charged polar headgroups at the surface of the membrane increased by enhancing the pumping rate while decreasing the amount of proton leakage. This follows from the fact that both the membrane potential and the pH gradient determine the electrochemical proton gradient (17). When the pH of the medium is varied, it influences the magnitude of the pH gradient and membrane potential while leaving the proton gradient constant. However, the type of cation present in the medium, which doesn't affect the membrane potential, will vary the proton gradient by influencing the magnitude of the pH gradient (17).

Recent studies of anionic polymers found at the cell membrane surface have suggested an explanation for some of the structural and biochemical differences between Gram positive and Gram negative bacteria. The gram negative cell envelope consists of the cell membrane which is surrounded by a thin peptidoglycan layer. An outer membrane surrounds the cell membrane and peptidoglycan layer. The periplasmic space is the space between the inner and the outer membrane.
Stock et al. (79) showed that the periplasmic space of *E. coli* and *Salmonella typhimurium* represented 20–40% of the total volume of the cell. In addition, they observed that the cytoplasm and periplasmic space had equivalent osmotic pressures. They hypothesized that the presence of an anionic polymer would accomplish this isoosmotic condition by accumulating mobile cations in the periplasmic space as a result of Donnan equilibrium across the outer membrane. The electrical potential, existing across the outer membrane, was also examined by measuring the distribution of $^{22}\text{Na}^+$ and $^{36}\text{Cl}^-$ between the periplasm and cell exterior. It was found to have a magnitude of 30 mV with the periplasm being negative.

A Donnan equilibrium results from the presence of an anionic polymer, which is impermeable to a membrane and mobile ions which can move across this membrane (21). Since the polymer is trapped within a membrane defined space, an equilibria is established by the asymmetric movement of the individual mobile ions. In most cases, the polymer is anionic so that to obtain a neutral charge within the space, a higher amount of cation will cross the membrane compared to the mobile anion. The relative distribution of the cation on either side of the membrane can be determined by the equation:

$$Y = \frac{-zC_p}{2C} + \sqrt{1 + \left(\frac{zC_p}{2C}\right)^2}$$

where *Y* is the ratio of cation concentration, in the presence of the anionic polymer (of concentration $C_p$ and charge *z*) to the cation concentration in the medium (*C*). When the concentration of the polymer is small compared to the mobile ions, then the equation can be simplified to:
\[ Y = 1 - \frac{zC_p}{2C} \]

The Donnan potential resulting from the asymmetrical distribution of mobile ions is determined by the equation:

\[ \Delta \Phi = \frac{(RT \ln Y)}{z_+ F} \]

where \( R \) is the gas constant, \( T \) is the absolute temperature, \( F \) is the Faraday constant, and \( z_+ \) is the charge of the cation.

Van Golde et al. (82) discovered and characterized anionic polymers which were shown to be derived from phospholipid turnover in \emph{E. coli}. These polymers were termed membrane derived oligosaccharides (MDO). MDO are composed of 6-12 glucose units in a highly branched \( \beta-(1 \rightarrow 2) \) and \( \beta-(1 \rightarrow 6) \) linkage (34). It was shown that they are substituted with \emph{sn}-1-glycerophosphate, phosphoethanolamine (35,73) and \emph{O}-succinyl residues (74) attached to position 6 of certain glucose residues. MDO were found to be localized in the periplasmic space (73) and present in a number of Gram negative bacteria (1,73). From the fact that membrane phospholipid is influenced by the osmotic conditions of the culture medium (53) and MDO is localized in the periplasmic space, Kennedy in 1982 (35) reasoned that MDO might be the anionic polymer that Stock et al. (79) postulated to be responsible for control of the osmotic pressure at the cell membrane. Analysis of cells grown under various osmotic conditions (35) showed that MDO synthesis was osmotically regulated. The amount of MDO increased under low osmotic conditions and decreased in cells grown in medium of high osmolarity.
These same results were shown to occur in soil bacteria (49) containing anionic polymers of similar structures.

For Gram positive bacteria, there is no outer membrane to trap ions in a periplasmic space. The Gram positive cell is made up of the cell membrane which is surrounded by a thick peptidoglycan layer. Nevertheless, a Donnan equilibrium could be established without a membrane defined space. All that is required is that an anionic polymer be fixed (immobile) at the outer membrane surface. In gram positive bacteria, this could be accomplished by the anionic lipids at the membrane surface and the presence of the anionic polymers, lipoteichoic acids (LTA) anchored in the outer surface of the membrane. Little is known about the cellular function of LTA (22,86) but much is known about their biological activities (12,19,55,87). They have been shown to be synthesized from PG and/or CL and to represent about 75% of the phospholipid turnover of PG and/or CL in B. stearothermophilus (9). LTA usually consists of 25-30 residues of sn-glycerophosphate linked in a (1 → 3) phosphodiester bond. The second carbon can be variously substituted with sugars in a glycosidic linkage and also with D-alanine (87). Some gram positive bacteria contain lipomannans (62), which are anionic polymers consisting of 52-70 D-mannose residues, of which approximately 15 are succinylated. Both LTA and lipomannans are anchored in the cell membrane by a hydrophobic group (87). There are some similarities between LTA and MDO. They are both derived from membrane phospholipids (82,87). They are both found at the cell membrane, either fixed to the cell membrane surface (LTA) or trapped in a periplasmic space (MDO). Because of these similarities, it can be hypothesized that LTA and MDO have similar functions.
Our working model (Fig. 1) shows that the presence of the fixed negative charges (i.e. the anionic lipids and/or LTA) at the outer surface of the cell membrane would cause an accumulation of mobile cations, including protons, as a result of a Donnan equilibrium. Therefore, the pH and osmotic pressure at the outer membrane surface would not be the same as that of the culture medium, but would be determined by the relative concentration of fixed anionic charges and of mobile ions in the medium. This provides a mechanism for regulating the potential and osmotic pressure across the membrane surface. If the proposed function is correct, then cells grown in a medium of low pH (high proton concentration) would have a lower concentration of anionic lipids (PG and CL) and LTA than cells grown at a high pH (low proton concentration). Conversely, cells grown in a low solute concentration should have a high PG, CL, and/or LTA concentration, whereas cells grown in high osmolarity conditions will be lower in anionic surface charges. This hypothesis would also explain the presence of different types of lipid polar groups (anionic and zwitterionic) and provide an explanation for the presence of the higher concentration of anionic lipids in gram positive bacteria compared to gram negative bacteria. Since gram negative bacteria have a well defined periplasmic space to trap cations at the surface, they would not require anionic lipids for this function. As previously mentioned, PE represents 70–90% of the total membrane phospholipids in gram negative bacteria. Gram positive bacteria have a PG+CL percentage of 40–90% and lack a well defined periplasm. Thus, they must rely more upon the regulatory function of anionic lipids. Finally, the function of anionic lipids in regulating proton concentration and osmolarity at the outer
surface of the membrane represents a major physiological function of membrane phospholipid, in addition to the structural role in forming the bilayer.

Proposed Objectives

The objective of this study was to investigate the relationship of the pH and osmolarity of the culture medium (environment) on the composition and distribution of the membrane phospholipids.

The specific aims were:

1) Characterization of the lipid composition of cells grown in media of different pH or solute concentration.

2) Quantification of phospholipid distribution based the amount of PE modified at the outer surface of the membrane.
Figure 1: The Structural and Biochemical Makeup of Gram Positive and Gram Negative Bacteria.
MATERIALS AND METHODS

Materials.

Trinitrobenzene sulfonic acid (TNBS), lysozyme, Bovine Serum Albumin (BSA), Tris hydroxy aminomethane (Tris) were supplied by Sigma Chemicals. Trypticase Peptone was purchased from Baltimore Biological Laboratories (BBL) Microbiology Systems and yeast extract was purchased from Difco Laboratories. Perchloric acid was from Mallinkodt Chemical Works.

Growth conditions.

Bacillus megaterium ATCC 9885 was grown in a low osmolarity defined medium as described by Cronan et al. (13), the composition of which is shown in the Appendix in Table 1. The cells were grown in 300 ml or 500 ml side arm baffled flasks in a shaking water bath at 37° C. Growth was monitored by optical density at 650 nm in a Coleman Junior II Spectrophotometer.

Bacillus stearothermophilus NCA 2184 was grown in either complex medium (TYE/2) consisting of 1% Trypticase Peptone, 0.5% yeast extract and 40 mM Trismaleate of the appropriate pH, or in low osmolarity defined medium (LODM). The composition of the LODM is shown in Table 2 in the Appendix. The cells were grown at 60° C and the optical density monitored at 600 nm. Stock solutions of NaCl, KCl, sorbitol, and sucrose were added for the osmolarity studies in the final concentration of 0.15 M, 0.15 M, 0.25 M, and 0.25 M, respectively. The pH was maintained at 5.8, 7.0, and 8.0 by dropwise addition of 0.1 N NH₄OH, or 0.1 N HCl.
Lipid Composition.

Cells for the osmolarity studies were grown in 20 ml of medium under the conditions specified, to an O.D. of 0.4. Ten ml of prewarmed media containing 5 μCi of H₃²PO₄ (ICN Biomedical Inc.) was inoculated with 0.3 ml of exponentially growing cells. Cells were grown for at least four generations or until an O.D. of 0.4 had been reached then 2 ml were transferred to a third flask containing 80 ml of prewarmed media containing 100 μCi of 2⁻³H-glycerol (ICN Biomedical Inc.) and 45 μCi of H₃²PO₄. Again, the cells were grown for at least four generations or to an O.D. of 0.4.

Cells were transferred to pre-cooled plastic 50 ml centrifuge tubes and centrifuged at 10,000 rpm for 10 min. at 4° C in a Sorvall Superspeed RC2-B Refrigerated Centrifuge. The culture fluid was saved and stored at −20° C for later analysis. The pellets were resuspended in 5 ml of ice cold 10 mM Tris-Cl (pH 7.0), 5 mM MgCl₂. The suspended cells were pooled and transferred to pre-cooled Corex 15 ml tubes and centrifuged at 10,000 rpm for 15 min. at 4° C. The supernatant was removed by aspiration and the pellet was resuspended in 1 ml of distilled water. In order to facilitate extraction of the lipids, the suspended cells were frozen overnight at −20° C. The cells were thawed and placed on ice and two aliquots of 0.2 ml each were removed for dry weight determination. For lipoteichoic acid analysis, a 0.3 ml sample was transferred to a screw cap tube and frozen at −20° C. A 0.3 ml sample was transferred to a screw cap tube for lipid analysis. Only two cultures were used for the pH studies. From the 20 ml "starter" culture, 2 ml was transferred to 50 ml containing 100 μCi 2⁻³H-glycerol
and 50 µCi of H$_3$°PO$_4$ and grown to an O.D. of 0.4. The cells were harvested in the same manner as the osmolarity studies.

**Lipid Distribution.**

Cells of *B. megaterium* were transferred from a slant to 20 ml of medium and grown to an O.D. of 0.3. Ten ml of prewarmed media containing 50 µCi H$_3$°PO$_4$ was inoculated with exponentially growing cells to an O.D. of 0.01 and grown to an O.D. of 0.3. Cells were collected by centrifugation and washed once with 5 ml of 0.15 M KCl, 50 mM potassium phosphate (pH 7.9), 1 mM MgCl$_2$, 0.5% glucose (Buffer 1). The pellet was resuspended in 7 ml of the same buffer and transferred to a reaction vessel for triphenylation.

Triphenylated derivatives were formed by a slightly modified Rothman and Kennedy procedure (69). To 7 ml of suspended cells, 0.84 ml of freshly prepared Trinitrobenzene sulfonic acid (TNBS) at a concentration of 8.44 mg/ml in 5% (wt/vol) NaHCO$_3$ (pH 9.0) was added dropwise. The pH was maintained at 8.1 with 0.2 N HCl. In order to maintain nonpermeable conditions, the temperature was maintained at 1° C and regulated by a Haake circulating pump containing ice water. A magnetic stirrer was used to equilibrate the pH and prevent cell sedimentation. The reaction was stopped with the addition of 20 µl BSA (5 mg/ml) and 100 µl of ice cold 30% Trichloroacetic Acid (TCA). The precipitated cells were placed on ice for at least 5 min. then centrifuged on an IEC clinical bench top centrifuge at setting 7 (approximately 3000 rpm) for 5-10 min. The supernatant was aspirated off and the pellet placed on ice until the lipids were extracted.

For *B. stearothermophilus*, the culture volume for labeling was increased to
20 ml for pH studies. The cells for pH study were split into two parts and centrifuged at 10,000 rpm for 10 min. at 4°C. The sample for composition was washed once in 10 ml of 0.15 M KCl, 1 mM MgCl₂, 0.5% glucose containing 50 mM Boric Acid-Borax buffer (pH 7.9) (Buffer 2) and centrifuged for 10 min. at 10,000 rpm at 4°C then placed on ice until the lipids were extracted. The distribution aliquot was resuspended in 7 ml of Buffer 2 and triphenylated, using 1.6 ml of TNBS reagent. Cells for the osmolarity study, grown in 40 ml of culture medium, were pelleted and resuspended in 8 ml of Buffer 2. Two ml were removed and centrifuged and the pellet placed on ice. The remaining 6 ml was transferred to the reaction vessel for triphenylation. All culture fluids were saved and frozen at −20°C.

Extraction of Lipids

Labeled cells were extracted by a modified Bligh and Dyer procedure (5). Cells from a 0.3 ml sample were treated with 40 μl of 10 mM Tris-HCl (pH 7.5) containing 2 mg/ml lysozyme which gave a final concentration of 235 μg/ml. The cells were incubated for 30 min. at room temperature and the reaction stopped with the addition of 1.8 ml of chloroform/methanol/1 N HCl (1:2:0.03 vol/vol). The tubes were allowed to stand at room temperature for 30 min. with occasional shaking. The single phase was centrifuged using the table top centrifuge on setting 6 (approx. 2000 rpm) for 20 min. The supernatant was transferred to a conical tube and the pellet resuspended in 0.4 ml of 0.3% NaCl then 1.5 ml of chloroform/methanol/1 N HCl (1:2:0.03) was added. The tubes were vigorously shaken at 68°C for 10 min. and then allowed to stand at room temperature for 30
min. After centrifugation, the pellet was extracted in a single phase twice more. The extracts were pooled in two conical tubes. A two phase system with chloroform/methanol/water ratios of 2:2:1.7 resulted from the addition of 1 ml of chloroform and 1 ml of 0.3% NaCl. The tubes were vortexed for 2 min. and the phases separated by centrifugation on the table top centrifuge on setting 7 (approx. 3000 rpm) for 10 min. The lower chloroform phases were removed and transferred to a clean conical tube and the upper phase was re-extracted twice with 1 ml of chloroform. The chloroform phases were pooled and dried on a Buchler Evapo-Mix at 35° C. The lipid residue was resuspended in 300 μl of chloroform/methanol (2:1 vol/vol).

Distribution samples were extracted by the same procedure with a few modifications. The precipitated cells were resuspended in 0.3 ml of 10 mM Tris-HCl (ph 7.5) and 0.1 ml of Tris-HCl (pH 7.5) containing 200 μg of lysozyme was added. The tubes were incubated at room temperature for 15 min. then were stored at -20° C overnight. The cells were thawed then 1.5 ml of chloroform/methanol/1 N HCl (1:2:03) and 20 μl of carrier lipid (14 nmol/ml) were added. The tubes were then vigorously shaken at 68° C for 10 min., allowed to cool and followed by 45 min. at room temperature with occasional shaking. The tubes were centrifuged for 20 min. and the extract collected. The residues were extracted three more times in the single phase.

The extraction of B. megaterium lipids was by a modified Bligh and Dyer procedure (5) as described by Rothman and Kennedy (69). The precipitated cells were resuspended in 0.4 ml of 0.1 M KCl, 0.1 N HCl followed by the addition of 1.5 ml of...
chloroform/methanol/1 N HCl (1:2:0.03) followed by 20 μl of carrier lipid. After the tubes were mixed, 0.5 ml of chloroform and 0.5 ml of 2 M KCl-0.01 N HCl were added with vortexing after each addition. The tubes were centrifuged for 10 min. and the lower phase carefully drawn off. The upper phase was extracted twice with 0.5 ml chloroform and the chloroform phases pooled. The chloroform phases were separated, the chloroform layer was dried and the residue resuspended in 40 μl chloroform/methanol (2:1).

Lipids were extracted from the culture fluids by a modified Bligh and Dyer procedure (5). Three ml of chloroform/methanol/1 N HCl (1:2:0.03) was added to a screw cap tube containing 1.8 ml of culture fluid. To this mixture, 20 μl of carrier lipid was added and the tube was capped and vortexed for 1 min. One ml of chloroform was added resulting in a two phase system and the tube was vortexed for two min. After centrifugation on setting 6 (approx. 2000 rpm) in a table top centrifuge, the chloroform phase was transferred to a conical tube and the upper phase extracted twice more with 1 ml of chloroform. The chloroform phases were pooled and taken to dryness. The residue was resuspended in 150 μl of chloroform/methanol (2:1). For larger volumes, a separatory funnel was used and the chloroform layer dried down in a Buchli Rotavapor rotary evaporator and the residue resuspended in 4 ml of chloroform. After being transferred to a conical tube, the solvent was removed and the residue resuspended in 300 μl of chloroform/methanol (2:1) for analysis.

Lipid analysis.

Lipids extracted from B. megaterium cells were quantified by chromatography
in one dimension on Whatman SG-81 papers. The chromatography system used for SG-81 papers in one dimension was: chloroform/methanol/diisobutyl ketone/pyridine/0.5 M NH₄Cl pH 9.8 in the ratios 30:17.5:25:35:6 vol/vol. Lipids extracted from *B. stearothermophilus* cells were quantified by chromatography in one dimension on Whatman LK6 Linear K Silica Gel thin layer plates or in two dimensions on Whatman SG-81 papers. The solvent system used for chromatography on LK6 plates was chloroform/methanol/acetic acid (65:25:8 vol/vol). The chromatography solvent systems for two dimensions were those described by Wuthier (88). The chromatography systems used for SG-81 papers in two dimensions were: chloroform/methanol/diisobutyl ketone/acetic acid/water in the ratio 23:10:45:25:4 in the first dimension and chloroform/methanol/diisobutyl ketone/pyridine/0.5 M NH₄Cl pH 9.8 in the ratio 30:17.5:25:35:6 in the second dimension.

Autoradiography was performed using Kodak XAR-5 film on regular intensifying screens. Exposure was for 10–16 hrs. at −70°C to show the location of each lipid or lipid derivative. The spots were scraped off the thin layer plates or cut out of the papers into scintillation vials and counted in 5 ml of Aquasol (New England Nuclear Corp) or Ecolite (West Chem) as the scintillation fluor. Counts were made in a Beckman LS-7500 programmed for dual 3H–32P labeling.

*Preparation of Inverted Vesicles.*

Washed membranes were prepared by modification of the procedure by Sone et al. (76). Labeled *B. megaterium* cells from a 10 ml sample were collected by centrifugation at 10,000 rpm for 10 min. at 4°C and resuspended in 10 ml of 0.15
M NaCl, 50 mM Tris-HCl (pH 7.0), 5 mM MgCl₂. The suspension was divided into two parts, centrifuged at 10,000 rpm for 10 min. and the pellets stored at -20°C. One pellet consisting of 5 ml of cells was resuspended in 5 ml of 0.15 M NaCl, 50 mM Tris-HCl (pH 7.0), 5 mM MgCl₂ (Buffer 3). The suspension was treated for 10 min. at room temperature with 0.2 ml of lysozyme (5 mg/ml) in 0.15 M NaCl, 50 mM Tris-HCl (pH 7.0), 5 mM MgCl₂ (lysozyme buffer). A drop of cells was removed and examined under phase contrast to determine the presence of spheroplasts, then 0.2 ml of lysozyme buffer was added and the cells incubated for 10 min. The cells were incubated for 15 min. with 225 μl of lysozyme buffer. A drop was removed to determine the degree of spheroplast formation and 0.25 ml lysozyme was added. After 15 min., the cells were examined for spheroplasts and 0.25 ml was added. The cells were incubated for 1 hr. and then centrifuged at 10,000 rpm for 10 min. at 4°C. The pellet was resuspended in 2.5 ml of 0.15 M KCl, 50 mM potassium phosphate (pH 7.9), 1 mM MgCl₂ and then sonicated.

Sonication was performed by the procedure of Futai (24) as described by Rothman and Kennedy (69). Cells were sonicated for 5 min. at 1 min. intervals with four cooling periods. The first two periods were for 30 seconds and the last two for 1 min. A Branson Sonifier Cell Disruptor W-350 was used at 40% duty cycle and an output of 4. The temperature was maintained at 4°C in an ice bath. The suspension was centrifuged at 8000xg for 10 min. at 4°C to remove cell fragments and fragments from the sonicator probe. The resulting pellet was resuspended in 5 ml of 0.15 M KCl, 50 mM potassium phosphate (pH 7.9), 1 mM MgCl₂ and counted for ³²P. The supernatant was adjusted to a volume of 7 ml and then triphenylated.
**Phosphorus Assay.**

Samples were transferred to 13x100 mm test tubes and the solvent removed under a stream of air. The residue was resuspended in 0.5 ml of 70% perchloric acid and digested for 4–6 hrs. at 160° C. After the tubes cooled, 2.5 ml of distilled deionized water, 0.25 ml of 2.5% (wt./vol.) ammonium molybdate, and 0.25 ml of Fiske-Subbarow reagent (23) were added in that order. The tubes were mixed and heated at 90–95° C for 7.5 min. After cooling, the absorbances were read in a Coleman Junior II Spectrophotometer at 820 nm against a blank containing all reagents. The phosphorus concentration was determined by comparison to a standard curve. Relative specific activities (disintegrations per min. per nmole phosphorus) were determined.

Samples were transferred to scintillation vials, the solvent removed under a stream of air and the residues resuspended in 5 ml of scintillation fluor and counted.
RESULTS

The pH growth range for Bacillus stearothermophilus.

*B. stearothermophilus* grew over a broad range in Trypticase–yeast extract (TYE) medium. The cell doubling times for the cultures shown in Fig. 2A were 35, 22, 22, 28, 38, and 125 minutes for cells grown at pHs of 5.5, 6.0, 7.0, 7.5, 8.0, and 8.5, respectively. The corresponding specific growth rate constants (k) are shown in Fig. 2B. No growth occurred below pH 5.5 or above pH 8.5 (data not shown). In the experiments described below, cells were grown at pH 5.8, 7.0, and 8.0, which represents the lower, middle, and upper range of the pH growth curve.

Anionic phospholipid concentrations increase as the pH of the growth medium is increased.

*B. stearothermophilus* was grown in TYE/2 medium buffered at pH 5.8, 7.0, and 8.0. The pH was monitored and maintained throughout growth. As shown in Table 1, the relative percentage of PE decreased with a concomitant increase in PG as the pH of the medium increased. CL remained essentially constant at 24–26% of the total phospholipid. As shown in Table 2, the change in the relative phospholipid concentrations resulted primarily from an increase in anionic lipids (especially PG) rather than a decrease in PE.

The amount of total phospholipid, cellular phospholipid, and extracellular phospholipid is influenced by the pH of the culture medium.

Previous studies of phospholipid turnover have shown that a portion of the
Figure 2: pH Range for Growth of *B. stearothermophilus*.

Fig. 2A. Growth curves of *B. stearothermophilus* grown at pH 5.5, 6.0, 7.0, 7.5, 8.0, and 8.5 in TYE containing 20 mM Tris-maleate. ♦, pH 5.5; ■■■■, pH 6.0; ▲▲▲▲, pH 7.0; ♦♦♦♦, pH 7.5; ▼▼▼▼, pH 8.0; ●●●●, pH 8.5.

Fig. 2B. Growth Rate Constants (k).

\[
\log_{10} N_2 - \log_{10} N_1 = \frac{k(t_2 - t_1)}{2.303}
\]

\(N_2\) = Absorbance at time \(t_2\)

\(N_1\) = Absorbance at time \(t_1\)

at \(N_2 = 2N_1\), \(t_2 - t_1 = g\) (generation time)

\[
\log_{10} 2N_1 - \log_{10} N_1 = \frac{kg}{2.303}
\]

\(kg = 2.303 \log_{10} 2 = 0.693\)

\(k = 0.693/g\)
Fig. 2

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label lost from the cellular phospholipid can be accounted for as lipid released from the cell during steady-state exponential growth (8). The higher concentrations of PG in cells grown at pH 8.0 compared to pH 5.8 might, therefore, have resulted from a decrease in the amount of PG released from the cells at the higher pH. As shown in Table 3, this was not the case. As the pH of the growth medium was increased, the total amount of both cellular and extracellular lipid increased. There was slightly greater than a four-fold increase in the amount of lipid released from the cells grown at pH 8.0 compared to those grown at pH 5.8. The nature of the extracellular lipid is unknown: however, as can be seen by comparing the results shown in Table 4 with those in Table 1, the relative amount of anionic (PG and CL) lipid was higher in the extracellular lipid than the cellular lipid for cells at each growth condition. Based on the relative distribution of PE and the anionic lipids (discussed below), this suggests that extracellular lipid was derived primarily from the outer layer of the membrane lipid bilayer.

Effect of osmolarity and ionic strength on growth at different pH.

In addition to influencing the relative proton concentration at the membrane surface, the concentration of fixed anions (PG and CL) would also influence the concentration of other mobile ions and therefore the ionic strength and osmolarity at the membrane surface. If regulation of anionic lipid concentrations provides for adapting to growth in media of different osmolarities and/or ionic strengths, then it would be expected that as the ionic strength and/or osmolarity of the medium is increased there should be a decrease in anionic lipid. If cells adapt to growth at higher pH (lower proton concentration) by increasing anionic lipid, and increasing
Table 1: Effect of pH on the Relative Percentage of Lipid Composition.

<table>
<thead>
<tr>
<th>Culture pH</th>
<th>PE (±)</th>
<th>PG (±)</th>
<th>CL (±)</th>
<th>Ratio&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.8</td>
<td>49.6± 6.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.8± 3.5</td>
<td>24.6± 8.5</td>
<td>1.02</td>
</tr>
<tr>
<td>7.0</td>
<td>36.0± 2.4</td>
<td>39.7± 2.6</td>
<td>24.3± 3.7</td>
<td>1.78</td>
</tr>
<tr>
<td>8.0</td>
<td>32.5± 2.2</td>
<td>41.4± 2.5</td>
<td>26.3± 2.6</td>
<td>2.08</td>
</tr>
</tbody>
</table>

<sup>b</sup>Ratio of anionic (PG+CL) to zwitterionic (PE) lipid.

Table 2: Effect of pH on Lipid Concentration.

<table>
<thead>
<tr>
<th>Culture pH</th>
<th>Total (±)</th>
<th>PE (±)</th>
<th>PG (±)</th>
<th>CL (±)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.8</td>
<td>34.5± 5.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.1± 2.5</td>
<td>8.9± 0.9</td>
<td>8.5± 2.5</td>
</tr>
<tr>
<td>7.0</td>
<td>42.2± 13.2</td>
<td>15.2± 4.1</td>
<td>16.8± 5.7</td>
<td>10.3± 2.6</td>
</tr>
<tr>
<td>8.0</td>
<td>48.0± 7.5</td>
<td>15.6± 2.9</td>
<td>19.8± 3.5</td>
<td>12.6± 2.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean ± Standard Deviation.
Table 3: Relationship of pH to Release of Extracellular Lipid.

Lipid Concentration  
(NMOLS/MG. DRY WT.)

<table>
<thead>
<tr>
<th>Culture pH</th>
<th>Cellular Lipid</th>
<th>Extracellulara</th>
<th>Total Lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.8</td>
<td>34.5 ± 5.6c</td>
<td>1.56</td>
<td>36.06</td>
</tr>
<tr>
<td>7.0</td>
<td>42.2 ± 13.2</td>
<td>4.38</td>
<td>46.58</td>
</tr>
<tr>
<td>8.0</td>
<td>48.0 ± 7.5</td>
<td>6.29</td>
<td>54.29</td>
</tr>
</tbody>
</table>

a Determined by dividing total counts of extracted lipid, adjusted for $^{32}$P decay, by the specific activity of $^{32}$P of cell extract. This value was then multiplied by the ratio of total volume of culture fluid to the volume extracted.

Table 4: Effect of pH on the Relative Composition of Extracellular Phospholipid.

<table>
<thead>
<tr>
<th>Culture pH</th>
<th>PE</th>
<th>PG</th>
<th>CL</th>
<th>Ratiob</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.8</td>
<td>41.0</td>
<td>49.6</td>
<td>9.5</td>
<td>1.44</td>
</tr>
<tr>
<td>7.0</td>
<td>24.7 ± 3.8c</td>
<td>61.6 ± 5.1</td>
<td>13.8 ± 1.9</td>
<td>3.05</td>
</tr>
<tr>
<td>8.0</td>
<td>23.1 ± 4.7</td>
<td>64.2 ± 6.5</td>
<td>12.8 ± 4.6</td>
<td>3.33</td>
</tr>
</tbody>
</table>

c Mean ± Standard Deviation

b Ratio of anionic (PG+CL) to zwitterionic (PE) lipid.
the osmolarity or ionic strength causes a decrease in anionic lipid, then cells growing at a higher pH might be more sensitive to increases in the ionic strength or osmolarity of the culture medium than cells growing at a lower pH. As shown in Fig. 3 and 4, this pattern of growth was observed. Cell doubling times for cultures shown in Fig. 3A were 23, 25, 27, and 37 minutes for cells grown at NaCl concentrations of 0.025, 0.05, 0.075, and 0.1 M at pH 6.0, respectively. For cells grown at pH 7.0, the cell doubling times for the same NaCl concentrations, were 25, 27, 32, and 230 minutes, respectively. At pH 8.0, the generation times were 32, 32, 125, and 375 minutes for 0, 0.025, 0.05, and 0.075 M NaCl. There was no growth at 0.1 M NaCl at pH 8.0. Fig. 3D shows the corresponding growth rate constants (k). As can be seen, the decline in the growth rate constants is greatest in cells grown at pH 8.0 followed by cells at pH 7.0. There is an acute decrease at pH 7.0 when the NaCl concentration is increased from 0.075 to 0.1 M. Similar results are shown in Fig. 4A when the sorbitol concentration is raised at pH 5.5 compared to the increase at pH 7.5. At pH 5.5, the generation times for 0.1, 0.2, 0.3, and 0.4 M sorbitol were 30, 36, 42, and 45 minutes, respectively. The cell doubling times at pH 7.5 for the same sorbitol concentrations were 40, 65, and 140 minutes. Cells did not grow at pH 7.5 when 0.4 M sorbitol was added to the media. Fig. 4C shows the corresponding growth rate constants, confirming what was expected to be seen. At pH 7.5, there was a greater decline in the growth rate constants with increased sorbitol concentrations than at pH 5.5.

The effect of osmolarity on lipid composition.

The addition of electrolyte (NaCl or KCl) to the medium at pH 5.8 resulted in
Figure 3: Effect of pH on Cell Growth in Media Containing Various NaCl Concentrations.

Fig. 3A. Cells were grown at NaCl concentrations of 0.025 M, 0.05 M, 0.075 M, and 0.1 M in TYE at pH 6.0. The growth was monitored at 600 nm at 30 minute intervals.

B. Cells were grown in TYE at the same NaCl concentrations at pH 7.0. Symbols represent the same concentrations as in A.
Cells were grown in TYE at pH 8.0 with various NaCl concentrations. The 0.1 M flask was replaced by one containing no NaCl.

C. Specific Growth Rates (k) for NaCl grown cells. Rates calculated in same manner as in Fig. 2B.
Fig. 4A and B.

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Figure 4: Effect of pH on Growth of Cells in Media Containing Various Concentrations of Sorbitol.

Fig. 4A. Cells grown in TYE with 20 mM Tris-maleate containing 0.1 M, 0.2 M, 0.3 M, and 0.4 M sorbitol. Cells monitored at 600 nm and O.D. recorded at 30 minute intervals at pH 5.5.

- \( \downarrow \), 0.1 M; \( \triangle \), 0.2 M; \( \blacksquare \), 0.3 M; \( \bullet \), 0.4 M sorbitol.

B. Cells grown at pH 7.5 at same sorbitol concentrations. Symbols represent same sorbitol concentrations.

C. Growth Rate Constants (k), were calculated in same way as in Fig. 2B.
a decrease in the total lipid concentration (Table 6). This was primarily due to a
decrease in PE, which resulted in an increase in the molar ratio of anionic to zwit­
terionic (PE) lipid from 1.02 to 1.86 and 1.68, for NaCl and KCl, respectively. There
was also an increase in CL with a corresponding decrease in PG. The addition of
nonelectrolyte (sorbitol or sucrose) resulted in an increase in the total lipid con­
centration. The slight increase with sorbitol was due to a slight increase in CL.
The doubling of the total lipid concentration with sucrose was due primarily to an
increase in PE. As a result of the PE increase, the anionic to zwitterionic lipid ratio
dropped from 1.02 to 0.65.

The addition of either NaCl or the nonelectrolytes to the medium at pH 7.0,
resulted in an increase in total lipid but no noticeable changes in the relative lipid
composition or molar ratio of PG+CL to PE (Table 7 and 8). There was no growth
at pH 8.0 with the addition of the electrolytes (Table 9). The total lipid concentra­
tion decreased with sorbitol addition (Table 10). However, the molar ratio of
PG+CL to PE remained the same. There was an increase in CL with a comparable
decrease in PG. When sucrose was added to the medium, there was a slight
decrease in total lipid due primarily to a decrease in PG. As a result of a rise in
PE, the molar ratio of PG+CL to PE decreased slightly.

Changes in osmolarity influences the amount of total, cellular, and extracellular
lipids.

The increase in total cellular lipid observed when cells were grown at higher
pH or with the addition of nonelectrolytes could have resulted from an increase in
synthesis or a decrease in the amount of lipid released from the cells. As shown
Table 5: Effect of Osmolarity on the Relative Lipid Composition of Cells Grown at pH 5.8.

<table>
<thead>
<tr>
<th>Additions</th>
<th>PE (%)</th>
<th>PG (%)</th>
<th>CL (%)</th>
<th>Ratio(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>----------</td>
<td>49.6(^\pm) 6.2(^c)</td>
<td>25.8(^\pm) 3.5</td>
<td>24.6(^\pm) 8.5</td>
<td>1.02</td>
</tr>
<tr>
<td>NaCl(^a)</td>
<td>34.9</td>
<td>19.6</td>
<td>45.5</td>
<td>1.86</td>
</tr>
<tr>
<td>KCl(^b)</td>
<td>37.3</td>
<td>15.9</td>
<td>46.8</td>
<td>1.68</td>
</tr>
<tr>
<td>Sorbitol (^b)</td>
<td>42.8</td>
<td>22.3</td>
<td>34.9</td>
<td>1.34</td>
</tr>
<tr>
<td>Sucrose (^b)</td>
<td>60.7</td>
<td>15.7</td>
<td>23.6</td>
<td>0.65</td>
</tr>
</tbody>
</table>

\(^d\) Ratio of anionic to zwitterionic lipid

Table 6: Effect of Osmolarity on the Lipid Concentration of Cells Grown at pH 5.8.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Total (NMOLS/MG. DRY WT.)</th>
<th>PE</th>
<th>PG</th>
<th>CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>----------</td>
<td>34.5(^\pm) 5.6(^c)</td>
<td>17.1(^\pm) 2.5</td>
<td>8.9(^\pm) 0.9</td>
<td>8.5(^\pm) 2.5</td>
</tr>
<tr>
<td>NaCl(^a)</td>
<td>28.8</td>
<td>10.0</td>
<td>5.6</td>
<td>13.1</td>
</tr>
<tr>
<td>KCl(^a)</td>
<td>23.2</td>
<td>8.6</td>
<td>3.7</td>
<td>10.8</td>
</tr>
<tr>
<td>Sorbitol (^b)</td>
<td>35.6</td>
<td>15.3</td>
<td>7.9</td>
<td>12.4</td>
</tr>
<tr>
<td>Sucrose (^b)</td>
<td>76.1</td>
<td>46.2</td>
<td>11.9</td>
<td>18.0</td>
</tr>
</tbody>
</table>

\(^a\) at final concentration of 0.15 M
\(^b\) at final concentration of 0.25 M
\(^c\) Mean \(^\pm\) Standard Deviation

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Table 7: Effect of Osmolarity on the Relative Lipid Composition of Cells Grown at pH 7.0.

<table>
<thead>
<tr>
<th>Additions</th>
<th>PE (%)</th>
<th>PG (%)</th>
<th>CL (%)</th>
<th>Ratio of anionic to zwitterionic lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl†</td>
<td>36.5</td>
<td>31.1</td>
<td>32.4</td>
<td>1.74</td>
</tr>
<tr>
<td>KCl†</td>
<td>38.6</td>
<td>24.0</td>
<td>37.4</td>
<td>1.59</td>
</tr>
<tr>
<td>Sorbitol†</td>
<td>37.0</td>
<td>32.4</td>
<td>30.6</td>
<td>1.70</td>
</tr>
<tr>
<td>Sucrose‡</td>
<td>38.2</td>
<td>32.2</td>
<td>29.6</td>
<td>1.61</td>
</tr>
</tbody>
</table>

Table 8: Effect of Osmolarity on the Lipid Concentrations of Cells Grown at pH 7.0.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Total (NMOLS/MG. DRY WT.)</th>
<th>PE</th>
<th>PG</th>
<th>CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl†</td>
<td>55.6</td>
<td>20.3</td>
<td>17.3</td>
<td>18.0</td>
</tr>
<tr>
<td>KCl†</td>
<td>43.4</td>
<td>16.7</td>
<td>10.4</td>
<td>16.2</td>
</tr>
<tr>
<td>Sorbitol‡</td>
<td>56.0</td>
<td>20.7</td>
<td>18.1</td>
<td>17.1</td>
</tr>
<tr>
<td>Sucrose‡</td>
<td>57.5</td>
<td>22.0</td>
<td>18.5</td>
<td>17.0</td>
</tr>
</tbody>
</table>

*a at final concentration of 0.15 M

*b at final concentration of 0.25 M

c Mean ± Standard Deviation

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Table 9: Effect of Osmolarity on the Relative Lipid Composition of Cells Grown at pH 8.0.

<table>
<thead>
<tr>
<th>Additions</th>
<th>PE (%)</th>
<th>PG (%)</th>
<th>CL (%)</th>
<th>Ratio^d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>32.5± 2.2</td>
<td>41.4± 2.5</td>
<td>26.3± 2.6</td>
<td>2.08</td>
</tr>
<tr>
<td>Potassium</td>
<td>NG</td>
<td>NG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sorbitol</td>
<td>33.4 ± 2.8</td>
<td>33.7 ± 2.8</td>
<td>32.9 ± 2.8</td>
<td>1.99</td>
</tr>
<tr>
<td>Sucrose</td>
<td>36.3 ± 2.9</td>
<td>34.4 ± 2.9</td>
<td>29.3 ± 2.9</td>
<td>1.75</td>
</tr>
</tbody>
</table>

^dRatio of anionic to zwitterionic lipid
NG- no growth

Table 10: Effect of Osmolarity on the Lipid Concentrations of Cells Grown at pH 8.0.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Total (NMOLS/MG. DRY WT.)</th>
<th>PE (NMOLS/MG. DRY WT.)</th>
<th>PG (NMOLS/MG. DRY WT.)</th>
<th>CL (NMOLS/MG. DRY WT.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>48.0± 7.5c</td>
<td>15.6± 2.9</td>
<td>19.9± 3.5</td>
<td>12.6± 2.3</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>26.4</td>
<td>8.8</td>
<td>8.9</td>
<td>8.7</td>
</tr>
<tr>
<td>Sucrose</td>
<td>45.0</td>
<td>16.3</td>
<td>15.5</td>
<td>13.2</td>
</tr>
</tbody>
</table>

^aat final concentration of 0.15 M
^b at final concentration of 0.25 M
^cMean ± Standard Deviation
in Tables 11-15, almost all conditions which resulted in an increase in cellular lipid also resulted in an increase in extracellular lipid. It should also be noted (Table 13 and 15) that the relative percentages of anionic lipids (PG+CL) was higher in the extracellular lipid fraction than in the cellular lipid fraction.

The addition of electrolytes to medium at pH 5.8 resulted in a decrease in total lipid due to a reduction in both cellular and extracellular lipid levels. Sorbitol addition resulted in a slight increase in the total lipid level. Sucrose caused a doubling in the total lipid primarily due to the doubling of cellular lipid.

KCl resulted in only a slight increase in total lipid level reflected by the small rise in cellular lipid (Table 12). The addition of sucrose and the electrolytes resulted in an increase in the extracellular lipid level. The relative percentage of anionic lipids (PG+CL) decreased under all conditions (Table 13).

The addition of the nonelectrolytes to media at pH 8.0 resulted in the decrease in both cellular and extracellular lipid levels. The extracellular lipid composition was also different for the two nonelectrolytes. Sorbitol caused a decrease in the ratio of anionic lipids to zwitterionic lipid (PE) from 3.33 to 2.95. Sucrose caused the relative percentage of anionic lipids to increase by approximately the same magnitude.

The pH growth range for cells grown in low osmolarity medium.

The growth of cells in low osmolarity defined medium (LODM) at pH 5.8, 7.0, and 8.0, is displayed in Fig. 5A. The generation times were 48, 37, and 55 minutes for pH 5.8, 7.0, and 8.0, respectively. Fig. 5B shows the corresponding specific growth rate constants (k).
Table 11: Relationship of Osmolarity to the Release of Extracellular Lipid from Cells Grown at pH 5.8.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Cellular Lipid</th>
<th>Extracellular Lipid$^d$</th>
<th>Total Lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>----</td>
<td>34.5 ± 5.6$^c$</td>
<td>1.56</td>
<td>36.1</td>
</tr>
<tr>
<td>NaCl$^a$</td>
<td>28.8</td>
<td>0.58</td>
<td>29.4</td>
</tr>
<tr>
<td>KCl$^a$</td>
<td>23.2</td>
<td>0.75</td>
<td>49.6</td>
</tr>
<tr>
<td>Sorbitol$^b$</td>
<td>35.3</td>
<td>2.6</td>
<td>37.9</td>
</tr>
<tr>
<td>Sucrose$^b$</td>
<td>76.1</td>
<td>2.1</td>
<td>78.2</td>
</tr>
</tbody>
</table>

$^a$ at final concentration of 0.15 M  
$^b$ at final concentration of 0.25 M  
$^c$ Mean ± Standard Deviation  
$^d$ Calculated in same manner as in Table 3.
Table 12: Relationship of Osmolarity to the Release of Extracellular Lipid from Cells Grown at pH 7.0.

Lipid Concentration (NMOLS/MG. DRY WT.)

<table>
<thead>
<tr>
<th>Additions</th>
<th>Cellular Lipid</th>
<th>Extracellular Lipid</th>
<th>Total Lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>----</td>
<td>42.2± 13.2c</td>
<td>4.4</td>
<td>46.6</td>
</tr>
<tr>
<td>NaCl⁴</td>
<td>55.7</td>
<td>5.9</td>
<td>61.6</td>
</tr>
<tr>
<td>KCl⁴</td>
<td>43.1</td>
<td>6.8</td>
<td>49.9</td>
</tr>
<tr>
<td>Sorbitol⁵</td>
<td>56.0</td>
<td>3.7</td>
<td>59.7</td>
</tr>
<tr>
<td>Sucrose⁵</td>
<td>57.5</td>
<td>5.8</td>
<td>63.3</td>
</tr>
</tbody>
</table>

dCalculated in same manner as in Table 3.

Table 13: Effect of Osmolarity on the Relative Percentage of Extracellular Lipid Released from Cells Grown at pH 7.0.

Lipid Percentage

<table>
<thead>
<tr>
<th>Additions</th>
<th>PE (%)</th>
<th>PG (%)</th>
<th>CL (%)</th>
<th>Ratioe</th>
</tr>
</thead>
<tbody>
<tr>
<td>----</td>
<td>24.7± 3.8c</td>
<td>61.6± 5.1</td>
<td>13.8± 1.9</td>
<td>3.05</td>
</tr>
<tr>
<td>NaCl⁴</td>
<td>30.3</td>
<td>35.2</td>
<td>34.5</td>
<td>2.30</td>
</tr>
<tr>
<td>KCl⁴</td>
<td>31.0</td>
<td>42.9</td>
<td>26.1</td>
<td>2.23</td>
</tr>
<tr>
<td>Sorbitol⁵</td>
<td>26.8</td>
<td>54.6</td>
<td>18.6</td>
<td>2.73</td>
</tr>
<tr>
<td>Sucrose⁵</td>
<td>30.5</td>
<td>43.8</td>
<td>25.8</td>
<td>2.28</td>
</tr>
</tbody>
</table>

⁴at a final concentration of 0.15 M
⁵at a final concentration of 0.25 M
³Mean ± Standard Deviation
²Ratio of anionic to zwitterionic lipid.
Table 14: Relationship of Osmolarity to Release of Extracellular Lipid from Cells Grown at pH 8.0.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Cellular Lipid</th>
<th>Extracellular Lipid</th>
<th>Total Lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lipid Concentration (NMOLS/MG. DRY WT.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----</td>
<td>48.0± 7.5^c</td>
<td>6.3</td>
<td>54.3</td>
</tr>
<tr>
<td>Sorbitol^a</td>
<td>26.3</td>
<td>3.4</td>
<td>29.7</td>
</tr>
<tr>
<td>Sucrose^a</td>
<td>45.0</td>
<td>4.9</td>
<td>49.9</td>
</tr>
</tbody>
</table>

^Calculated in same manner as in Table 3.

Table 15: Effect of Osmolarity on the Relative Composition of Extracellular Lipid Released from Cells Grown at pH 8.0.

<table>
<thead>
<tr>
<th>Additions</th>
<th>PE (%)</th>
<th>PG (%)</th>
<th>CL (%)</th>
<th>Ratio^d</th>
</tr>
</thead>
<tbody>
<tr>
<td>-----</td>
<td>23.1± 4.7^c</td>
<td>64.2± 6.5</td>
<td>12.8± 4.6</td>
<td>3.33</td>
</tr>
<tr>
<td>Sorbitol^a</td>
<td>25.3</td>
<td>56.1</td>
<td>18.6</td>
<td>2.95</td>
</tr>
<tr>
<td>Sucrose^a</td>
<td>21.4</td>
<td>43.0</td>
<td>35.6</td>
<td>3.67</td>
</tr>
</tbody>
</table>

^at final concentration of 0.25 M
^c Mean ± Standard Deviation
^d Ratio of anionic to zwitterionic lipid
Cells grown in LODM showed a marked difference in pH effect to that of cells grown in TYE.

The total lipid concentration was higher in LODM grown cells than cells grown in TYE at all pH values. The amount of lipid (per mg. cell dry wt.) was almost double at pH 5.8 and 7.0 than that of cells grown in TYE at pH 5.8 and 7.0. In contrast to cells in the complex media (TYE), the total lipid level decreased with increasing pH (Table 16). The increase in pH resulted in a rise in the molar ratio of (PG+CL) to PE (Table 17). By increasing the pH, both cellular and extracellular lipid levels decreased, causing the total lipid level to drop.
The distribution of phospholipid between the inner and outer membrane surface is asymmetric.

The net concentration of fixed anions at the cell surface would be influenced by both the relative concentration of anionic and zwitterionic lipid in the membrane and also the distribution between the inner and outer membrane leaflets. The distribution of PE between the two sides of the membrane was analyzed by determining the relative percentage of PE which was accessible to react with trinitrobenzene sulfonic acid (TNBS) under conditions where the membrane was impermeable to TNBS.

To determine the effectiveness of TNBS treatment, cells of B. megaterium, either as whole cells or inverted vesicles, were treated with TNBS. As shown in Fig. 6, a low fraction, 20%, of PE in whole cells was modified to TnpPE. In the case of inverted vesicles, with the inner leaflet exposed to the reagent, 80% of the PE was modified, indicating only the outside leaflet was modified and little of the
Figure 5: Effect of pH on the Growth of Cells Grown in Low Osmolarity Defined Medium (LODM).

Fig. 5A. Cells grown in low osmolarity defined medium (LODM) at pH 5.8, 7.0, and 8.0. Growth was monitored at 600 nm. • , pH 5.8; ■ , pH 7.0; ▲ , pH 8.0.

5B. Specific Growth Rates (k) were calculated in same manner as in Fig. 2B.
Table 16: Effect of pH on the Relative Lipid Composition of Cells Grown in Low Osmolarity Defined Medium (LODM).

<table>
<thead>
<tr>
<th>Culture pH</th>
<th>PE (%)</th>
<th>PG (%)</th>
<th>CL (%)</th>
<th>Ratioa</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.8</td>
<td>53.1</td>
<td>24.6</td>
<td>22.0</td>
<td>0.88</td>
</tr>
<tr>
<td>7.0</td>
<td>35.9</td>
<td>47.7</td>
<td>16.4</td>
<td>1.79</td>
</tr>
<tr>
<td>8.0</td>
<td>41.7</td>
<td>40.3</td>
<td>18.0</td>
<td>1.40</td>
</tr>
</tbody>
</table>

*aRatio of anionic to zwitterionic lipid.

Table 17: Effect of pH on the Lipid Concentrations of Cells Grown in LODM.

<table>
<thead>
<tr>
<th>Culture pH</th>
<th>Total (NMOLS/MG. DRY WT.)</th>
<th>PE</th>
<th>PG</th>
<th>CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.8</td>
<td>89.7</td>
<td>47.6</td>
<td>22.1</td>
<td>19.7</td>
</tr>
<tr>
<td>7.0</td>
<td>81.8</td>
<td>29.4</td>
<td>39.0</td>
<td>13.4</td>
</tr>
<tr>
<td>8.0</td>
<td>57.0</td>
<td>23.8</td>
<td>23.0</td>
<td>10.3</td>
</tr>
</tbody>
</table>
Table 18: Relationship of pH to the Release of Extracellular lipid from Cells Grown in LODM.

<table>
<thead>
<tr>
<th>Culture pH</th>
<th>Cellular Lipid (NMOLS/MG. DRY WT.)</th>
<th>Extracellular Lipid^a (NMOLS/MG. DRY WT.)</th>
<th>Total Lipid (NMOLS/MG. DRY WT.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.8</td>
<td>89.7</td>
<td>15.1</td>
<td>104.8</td>
</tr>
<tr>
<td>7.0</td>
<td>81.8</td>
<td>9.5</td>
<td>91.3</td>
</tr>
<tr>
<td>8.0</td>
<td>57.0</td>
<td>2.9</td>
<td>59.9</td>
</tr>
</tbody>
</table>

^aCalculated in same manner as in Table 3.
reagent crossed the membrane. These observations were essentially identical to previous studies with *B. megaterium* (69).

The time-course reactions with TNBS to determine PE distribution, of *B. stearothermophilus* cells grown at pH 7.0 and 8.0, are displayed in Fig. 7. A low percentage of PE was modified by TNBS in cells grown at both pH values. This indicates that primary distribution of PE is in the inner monolayer. Also shown is the increase in the percentage of anionic lipids (PG+CL), as the pH of the media increases, which follows the results seen in Table 1.

Fig. 8 shows the time-course reactions using cells grown at pH 5.75 and 8.0. The percentage of PE modified increased slightly in cells grown at pH 5.75 compared to cells grown at pH 8.0. Also seen was a greater increase in anionic lipid percentage proportional to the pH increase. The relative percentage of anionic and zwitterionic lipids appears to fluctuate as the TNBS treatment proceeds. There is stabilization of PE modification between 60 and 90 min. Fig. 8 also corroborated the results shown in Table 1.

The next step was to examine the possibility of changes in PE distribution in cells grown under various osmotic conditions. Fig. 9 shows the distribution of PE in LODM at pH 5.8, 7.0, and 8.0. At all pH values, the percentage of PE modified to TnpPE was low. As the pH increased, the total percentage of anionic lipids increased, from approximately 45% at pH 5.8 to 58% at pH 7.0 and 8.0. The modification doesn’t stabilize at any of the pH values in LODM as it does in the complex media shown in Fig. 7 and 8. Even so, a low percentage of TnpPE is formed at each pH. Fig. 10 shows the time–course reaction of TNBS with cells
grown at pH 7.0 in TYE/2 containing NaCl or sucrose. A similar response was
seen with a low percentage of the derivative formed. It appeared from these
results that PE was located primarily on the inner membrane layer under all con-
ditions used in this study.
Figure 6: Time Course Reaction for Triphenylation of Whole Cells and Inverted Vesicles of *B. megaterium* at 1° C.

**Fig. 6A.** Cells labeled with $^{32}$P were incubated at 1° C and triphenylated with 0.8 ml of 8.4 mg/ml TNBS in 5% NaHCO$_3$ (pH 9.0). Samples were removed and reactions stopped with 20 μl of BSA (5 mg/ml) and 100 μl TCA and stored on ice. Cells were pelleted and extracted by a modified Bligh and Dyer procedure (5) as described by Rothman and Kennedy (70). Total lipid fractions were chromatographed in one dimension on SG-81 paper using the solvent system: chloroform/methanol/diisobutyl ketone/pyridine/0.5 M NH$_4$Cl pH 9.8 in the ratio 30:17.5:25:35:6 vol/vol. Autoradiography revealed lipid location, which were cut out and counted in a Beckman LS-7500 using Aquasol as the scintillation fluor.

**Fig. 6B.** Inverted vesicles were made from lysozyme treated cells which were sonicated for 5 min. and centrifuged at 8000xg for 10 min. The pellet was resuspended and incubated with TNBS as described for whole cells.
Figure 7: Distribution of Phosphatidylethanolamine in Cells Grown at pH 7.0 and 8.0.

Cells grown at pH 7.0 and 8.0 at 60°C were continuously labeled with $^{32}$P until an O.D. of 0.3 was reached. Cells were split and harvested by centrifugation at 10,000 rpm for 10 min. at 4°C. Both samples were resuspended in 7 ml of 0.15 M KCl, 50 mM Boric Acid–Borax (pH 7.9), 1 mM MgCl$_2$, 0.5% glucose. One sample was pelleted at 10,000 rpm at 4°C. The other sample was incubated in 1.5 ml of TNBS solution in a time-course study with samples removed at 30, 60, and 90 min. The reaction was stopped in the same manner as Fig. 6. Lipids were extracted by a modified Bligh and Dyer procedure (5). Total lipid fractions were chromatographed on LK6 thin layer plates using the solvent system chloroform/methanol/acetic acid in the ratio of 65:25:8 vol/vol. Lipids were located by autoradiography and the scraped off spots counted using Ecolite as scintillation fluor.
Figure 8: Distribution of PE in Cells Grown at pH 5.75 and 8.0.

Cells grown at pH 5.75 and 8.0 in TYE/2 were split and harvested in same manner as in Fig. 7. Cells were also incubated at 1° C in the same manner as those of Fig. 7. All extractions, chromatography, and counts were performed in same manner as those of pH 7.0 and 8.0 from Fig. 7.
Figure 9: Distribution of PE in Low Osmotic Conditions.

Cells grown at a) pH 5.8, b) pH 7.0, and c) pH 8.0 were continuously labeled with $^{32}$P in 40 ml of low osmolarity defined medium (LODM). Cells were split and harvested in same manner as in Fig. 7. Cells were also incubated in same manner as those of Fig. 7. All extractions, chromatography and counts were performed in same manner as those of pH 7.0 and 8.0 from Fig. 7.
Figure 10: Distribution of PE in Cells Grown in the Presence of an Electrolyte (NaCl) and Nonelectrolyte (sucrose).

Cells grown in TYE/2 containing either NaCl or sucrose were continuously labeled with $^{32}$P in 40 ml at pH 7.0. Both sets of cells were harvested, extracted and analyzed in same manner as cells displayed in Fig. 7.
DISCUSSION

This study was guided by the hypothesis that changes in anionic membrane lipid content contributes to osmoregulation in Gram positive bacteria. The rationale was that the relative concentration of mobile ions (therefore the proton concentration (pH), ionic strength, and osmolarity) at the membrane surface would be influenced by the relative concentration of fixed anions at the outer membrane surface as a result of Donnan equilibrium between the outer membrane surface and the medium. If the hypothesis is correct, then the relative concentration and/or distribution of anionic membrane lipid should be influenced by the proton concentration (pH), ionic strength, and osmolarity of the culture medium. As discussed below, the changes in PG and CL concentrations in response to the pH of the culture medium followed the patterns predicted by the model. The changes in PG and CL in response to the changes in the ionic strength and osmolarity of the culture medium, however, were more complex and difficult to interpret.

As predicted, the pH of the culture medium had a marked effect on the relative concentrations of PE and the anionic lipids (PG and CL). As the pH of the culture medium was increased, the relative percentages of both PG and CL increased and PE decreased (Table 1). The change in composition resulted primarily from an increase in PG (Table 2).

Previous studies of lipid metabolism in B. stearothermophilus have shown that PG and CL are metabolically active with high turnover rates during exponential
growth, whereas PE is metabolically stable (8). Portions of both the anionic and zwitterionic lipids are released from the cell during exponential growth (9). The change in composition could have resulted from an increase in the rate of synthesis, a decrease in the rate of PG degradation, or a decrease in the amount of lipid released from the cell when grown at the higher pH. As shown in Table 4, the amount of total cellular lipid and total extracellular lipid increased as the pH of the growth medium increased. It appeared, therefore, that the pH of the medium influenced the rate of PG and CL synthesis and possibly the rate of PG turnover.

Several workers have reported that the pH of the culture medium influenced the ratio of zwitterionic to anionic lipids in gram positive bacteria (31,45,51,57). In B. stearothermophilus B65, the relative percentage of PE decreased with an increase in pH (45). In other studies, the change was primarily in the relative percentages of amino containing derivatives of PG (31,51,57). Changes in lipid composition similar to those observed in this study were recently reported for alkalophilic bacteria (11). Alkalophilic bacteria grow at high pH. Facultative alkalophiles were shown to decrease in the ratio of anionic lipids to zwitterionic lipids when the pH was decreased from 10.5 to 7.5 (11). In contrast, E. coli showed no change in the phospholipid composition when the pH of the medium was changed (31).

Another aspect of the membrane which may be important in regulating the proton concentration at the membrane surface is the phospholipid asymmetry of the membrane. As shown in Figures 7 and 8, the distribution of PE doesn't change with increasing pH. The distribution profile of PE in this study is similar to B.
megaterium KM (69) and the strain of B. megaterium used in this study, in which 66% and 80% respectively, of the total PE was localized in the inner leaflet. However, lipid topography based on PE distribution has been shown in past studies not to be the same in all membranes (4,18,25,61,69). Therefore, the significance of the lipid asymmetry should be dealt with based on the specific bacteria studied. If we assume that 50% of the lipids are in the inner leaflet and 50% are in the outer leaflet, then PG and CL should reside primarily in the outer leaflet, since most of the PE is in the inner leaflet. Thus the outer leaflet is primarily filled with negatively charged lipids and thus creates a charge asymmetry across the membrane bilayer and therefore a membrane potential across the bilayer. As the pH increases, the level of the anionic lipids increases. If the additional PG and CL are distributed evenly between the two leaflets, then there would be no change in the potential since the PE asymmetry is unaltered. Also, the additional negative charges due to the increased anionic lipid on the inner leaflet would be counteracted by the additional anionic lipid on the outer leaflet. However, the surface charge would become increasingly negative. Thus, the higher degree of negative charges on the membrane surface should accumulate protons and other cations at the surface by a Donnan equilibrium. The result of the asymmetrical distribution of mobile cations, other than protons, would generate a Donnan potential between the outer surface of the cell membrane and the culture medium.

If on the other hand, the outer leaflet contains a greater amount of lipids compared to the inner leaflet, and the added anionic lipids are asymmetrically added to the outer leaflet, then two things should occur. One is that a greater
amount of protons would be accumulated, increasing the Donnan potential. The
other thing is that the increased charge asymmetry would result in a larger poten-
tial across the lipid bilayer with the inner leaflet becoming increasingly positive
with respect to the outer leaflet. With the increased accumulation of protons at
the surface, the pH would be lower compared to the bulk medium. In order to as­
certain what effect varying the medium pH would have on the charge at the
membrane surface, the distribution of both PG and CL should be examined. One
method that would show the membrane topology of PG and CL is the use of ex­
change proteins (2). Here the cell wall is removed and the exchange of PC in
vesicles, and PG and CL in protoplasts, would be mediated by rat liver homogenate
containing exchange proteins. Another procedure was used by Schäfer et al. (72)
in studying the asymmetry of PM2 virus using diazonium $^{35}$S–sulfanilic salt
modifying permeability with LiCl. PG was then reduced with NaB$_3$H$_4$. For CL,
labeled antibodies (59) could be used. Therefore, all the lipid locations would be
mapped out and the charge asymmetry could be determined.

The next question is why should phospholipid content and asymmetry be im­
portant in regulating proton concentration at the membrane surface. As the pH
changes, the amount of protons present changes by a factor of ten with each pH
unit. Adaptation is needed for cell viability through protection of cellular functions;
metabolism, protein requirements for optimum function and possibly maintenance
of energy transducing functions. As discussed by Koch (36), the pH at the
membrane surface will be dependent on the potential resulting from the chemios­
motic translocation of protons and the Gouy–Chapman potential due to the fixed
negative charges of the phospholipid. Although we do not have sufficient data to
determine the actual values for surface potential for \textit{B. stearothermophilus}, some
general statements can be made.

First, the potential at the membrane surface due to fixed negative charges
(i.e. the Gouy-Chapman potential, ref. 36) is directly related to charge density and
is described by the following equation:

\[ \sigma = \frac{DK\Psi_0}{4\pi} \]

where \( \sigma \) is the charge density (e/ unit area), \( D \) is the dielectric constant of the
medium, \( K \) the thickness of the layer and \( \Psi_0 \) is the potential at the membrane sur-
face. The charge density (\( \sigma \)) would be determined by the relative concentration of
anionic lipid (PG+CL) compared to total lipid, the packing patterns, and the relative
amount of lipid and protein per unit area of membrane. The effect of membrane
proteins on the charge density would depend on their net charge and the degree
in which the anionic lipids are masked by peripheral proteins. In this discussion,
the main concern is what effect changes in anionic lipid concentration might have
on the charge density. As determined by Trauble and Overath in their study of the
\textit{E. coli} membrane (80), there are 312 lipid molecules per unit area (20,425 sq. \( \text{Å} \)).
Eighty percent of these, or 249, are involved in the formation of the bilayer, while
20\% (63 molecules) are associated with the integral protein (Fig. 12 of ref. 80).
The presence of peripheral proteins leaves only 42\% of the lipid molecules ex-
posed to the aqueous phase. Of the 312, 80\% or 250 molecules are anionic lipids
at pH 5.8. As the pH increases, the number increases to 278 or 90\% are anionic
lipid. This depends on the assumption that 50% of the lipids are present in the outer monolayer and the number of lipid molecules per unit area doesn't change. If the packing of lipids per unit area or if the charge density would increase, the effect of anionic lipid charge would increase, as previously mentioned.

Second, the proton concentration at the surface is exponentially related to the Gouy-Chapman potential, according to the following equation:

\[ C = C_\infty e^{-e\Psi(x)/kT} \]

where \( C \) is the concentration at the surface and \( C_\infty \) is the concentration in the bulk medium. Koch (36) has expressed this relationship in terms of pH as follows:

\[ \text{pH} = \text{pH}_\infty + \frac{F\Psi(x)}{2.303RT} \]

or at 25°C where \( 2.303RT/F = 60 \text{ mVolts} \)

\[ \text{pH} = \text{pH}_\infty + \frac{\Psi(x)}{60 \text{ mVolts}} \]

where \( \text{pH}_\infty \) is the pH of the external medium, \( \Psi(x) \) is the potential at distance \( x \) from the membrane surface. Koch (36) has calculated values for a typical membrane and shown that the potential is very short-ranged (decreasing exponentially with distance from the membrane surface; Fig. 3 of ref. 36). Ignoring lipid asymmetry, Koch (36) has calculated a potential at the bilayer surface (\( \Psi_o \)) of about -160 mV, which would give a difference of 2.6 pH units between the medium and the membrane surface. There was a two-fold difference in the amount of anionic
lipid between cells grown at pH 5.8 and 7.0. If this resulted in a doubling of the charge density at the membrane surface, then the surface pH of cells grown at pH 5.8 would be similar to the surface pH of cells grown at pH 7.0.

Third, the increase in anionic lipid with increasing pH of the growth medium could increase the buffering capacity of the membrane. In this respect, it is interesting to note that Krulwich et al. (40) have shown that the buffering capacity of the membrane represents a greater percentage of the total cellular buffering capacity as the pH of the growth medium is increased. Hopfer et al. (30) have shown that the surface charge ratio would affect the ion permeability where the membrane selectivity for cations becomes greater as the negativity of the membrane increases. Erythritol transport increases as the level of anionic lipids in the membrane increases (27). As the pH of the medium increases, the cytoplasmic pH also increases in order to maintain the same magnitude of the pH gradient. However, the pH range for the cytoplasm for neutralophiles, such as B. stearothermophilus, is 7.5 to 8.0 (6). The optimum metabolic rate occurs when the internal pH is higher than that of the bulk medium. As discussed earlier, the pH at the membrane surface would be drastically different than that of the cytoplasm and the bulk medium (36).

Another aspect to consider is the effect on the membrane potential, which, along with the pH gradient, determines the proton-motive force. Due to the accumulation of protons, the charge at the membrane surface may be lower than in the bulk media. The charge in the cytoplasm is negative with respect to the outer surface, thus a positive potential is produced. It should increase in magnitude as
the pH increases since the cytoplasmic pH increases proportionately while the pH at the surface stays relatively the same due to proton accumulation. This would affect the magnitude of the proton-motive force.

The second part of the proposed function for phospholipids is the regulation of cation concentration or ionic strength at the membrane surface. It was thought that by increasing the osmolarity of the medium, the ratio of zwitterionic to anionic lipids would increase. Raising the pH would therefore have the opposite effect of raising the osmolarity of the medium. Indeed, by examining Figures 3 and 4, we see that raising the pH in cultures containing increasing concentrations of either NaCl or sorbitol caused a reduction in the growth rates. In fact, if we were to superimpose Fig. 3B over 3C, we would see a lower growth rate for pH 8.0 grown cells containing no NaCl. Thus it is independent of the type of solute present, by raising the pH of the medium, the growth rates are reduced.

During this study, the media was initially adjusted to the required pH level prior to autoclaving. Early on, this adjustment was made at room temperature (Media 1). Later, the media was adjusted at 50° C (Media 2). The pH fell from 6.8 to 6.5 when the temperature was increased. For media buffered at pH 5.8, less HCl was required to adjust the pH at 50° C than at room temperature. Thus, the initial ionic strength is lower. Conversely, the ionic strength of pH 7.0 and 8.0 media would be higher, due to an increased amount of NaOH added to the medium, in order to achieve the initial pH level at 50° C. An interesting effect occurred when comparing the generation times in the two media types upon addition of solute. The generation times were higher in Media 2 than in the first media type. For ex-
ample, the generation time for the control cells at pH 7.0 in Media 1 was 54 minutes while it was 62 minutes in the second media (data not shown). This occurred as well when solutes were added to the media. The growth rates, and thus the generation times, for most of the culture conditions could be determined when cells were grown in Media 1, while many of these same culture conditions required overnight growth in the gyratory shaker to reach an acceptable optical density. This was especially noticeable in the cases of sorbitol and sucrose at pH 8.0. Thus, the change in initial ionic strength affected the growth rates more for nonelectrolytes than for the electrolytes.

The major thrust of the osmolarity studies dealt with the effects on phospholipid composition when the osmotic conditions were changed. If we examine Tables 5 through 10, the results are not very conclusive, which could mean that the change in phospholipid is dependent upon what solute is present in the media. However, when the results are matched to increasing pH, which is known to increase anionic lipid level, the results become more clear. The general trend is that the electrolytes elicit a decrease in the ratio of anionic to zwitterionic lipids while the nonelectrolytes caused the ratio to increase. This suggests that the influence of changing osmotic conditions upon the phospholipid composition is dependent on the ionic strength of the medium and not the solute concentration. The results of the nonelectrolytes in this study were similar to previous studies involving halotolerant bacteria (33,38), where increasing the NaCl caused the CL content to increase. When Miller (50) studied the effect of monovalent and divalent salts on the phospholipid compositions, it was found that the ratio of anionic to zwit-
Terionic lipids increased and thus the degree of negative charges increased. This was also true for the moderate halophiles *Vibrio costicola* (37) and *Pseudomonas halosaccharolytica* (29), in which the anionic lipid content increased compared to the zwitterionic lipid PE without degradation of PE (37). The study of non-halophiles have been concerned mainly with "compatible solute" concentrations as influence by increased osmotic pressures (32,48,60). Another concern has been with the presence of an anionic polymer and its relationship to osmoregulation in the periplasmic space of gram negative bacteria (1,34,35,49,73,82). About the time that it was found that the osmotic conditions affected the phospholipid metabolism of *E. coli* (53), a novel class of oligosaccharides (MDO) were found present in *E. coli* (81). In their study of the periplasmic space and its equivalence in osmotic pressure to the cytoplasm under low osmotic conditions, Stock et al. (79) hypothesized that the presence of an anionic polymer would accomplish this isoosmotic condition by generation of a Donnan equilibrium. MDO was found to be present in the periplasmic space of *E. coli* (73) and other gram negative bacteria (1,73) and its concentration in the periplasmic space osmoregulated (35,49), with a low osmotic condition causing the concentration to increase (35).

The extracellular lipid also showed little trend in comparing electrolytes and nonelectrolytes. This is also true when the pH effect is examined. Both electrolytes show the same trend with increase in cellular and extracellular lipid levels. One explanation is that transport of solutes across the membrane occurs, which affects the influence that increasing the osmotic strength might have. It is known that NaCl and KCl are important in transport mechanisms (6). Also, sucrose
appears to be transported and is utilized. If so, the effect of sucrose is reduced since it is transported into the cell. In the case of sorbitol, it is unknown whether it is transported into the cell.

LODM causes the cellular lipid level to be higher in cells grown in this medium at comparable pH values for TYE grown cells, especially at pH 5.8 and 7.0, which had almost double the cellular lipid level of TYE grown cells. The cellular lipid level decreased when the pH of the medium increased in LODM grown cells. Some other factors may be involved, which cause the opposite effect on lipid concentrations between LODM and TYE, perhaps something in TYE, since it is not defined. The osmolarity of LODM was slightly greater than one-half of TYE/2. The studies of *E. coli* (35) and other gram negative bacteria (1,50,75) and the effect of osmolarity on MDO concentration was performed using a low osmolarity defined medium where solutes were added, and the effect examined. In this study, the complex medium (TYE) was used in studying the effect osmolarity had on phospholipid composition. If we examine the effects of a change in osmolarity by addition of solutes to LODM on phospholipid composition, we may see different results. One problem in using LODM would occur though. LODM has a lower buffering capacity than TYE and would require more acid or base to maintain the pH and thus the ionic strength would be affected. One solution to this would be to use agar which is made up using LODM and adding liquid LODM over the top. Thus, it would essentially increase the buffering capacity of the medium, as if the culture was being grown completely in liquid medium. The other concern is that the solutes used to study the osmotic effect on phospholipid composition should
not be transported or utilized. Thus, the full effect of increased osmolarity could be examined.

These results show that the pH does influence the anionic lipid to zwitterionic lipid ratio by increasing the anionic lipid levels. Electrolytes also appeared to follow the proposed effect, with an increase in PE compared to PG and CL. However, the nonelectrolyte effect was less substantial, and this may be due to the choice of media and solutes which were added to the media. Even so, the change in the charges in the membrane should influence, via the Donnan equilibrium, the pH and ionic strength at the membrane surface.
SUMMARY

Based upon the hypothesis that the anionic membrane lipids are part of the cellular mechanism of osmoregulation, cells were grown under varying culture conditions in complex media (TYE) and defined media (LODM). The cells were examined for lipid composition and membrane lipid distribution in this study. The results from this study can be summarized as follows:

1. Increasing the pH of the culture medium resulted in an increase in the total cellular lipid level, which was primarily due to an increase in anionic lipid concentrations (especially phosphatidylglycerol, PG).

2. The level of lipid being released rose as the pH of the culture medium was increased.

3. The ratio of anionic lipid to zwitterionic lipid (PE) was higher in the extracellular lipid fraction than in the cellular lipid. This suggested that the lipid being released was released from the outer membrane surface.

4. Addition of solutes to media at different pH values resulted in a decrease in growth rates. The decline in growth rates, due to increased solute concentrations, increased with increasing pH.

5. Addition of electrolytes to TYE/2 resulted in a decrease in total cellular lipid concentration. There was no growth at pH 8.0. The ratio of anionic to zwitterionic lipid was again higher in the extracellular lipid fraction than in the cellular lipid.

6. When nonelectrolytes were added to the culture medium, the total lipid concentration in the cell increased. The anionic to zwitterionic lipid ratio decreased.

7. Growth in LODM resulted in a increase in total lipid concentration compared to cells grown in TYE/2. The lipid concentrations of LODM
grown cells decreased with increasing pH. It is suggestive of the fact that the upper limit of the cellular lipid concentration was reached and could only decrease with increasing pH.

8. Cells grown in TYE, TYE containing either NaCl or sucrose, or in LODM were analyzed for lipid distribution using TNBS. The relative distribution of lipids remained essentially constant under all culture conditions.

9. The data presented shows that the membrane lipids have a role in regulating the proton concentration (pH), ionic strength, and osmolarity at the cell membrane surface as a result of a Donnan equilibrium.
APPENDIX

1. Table 1: Composition of Defined Medium for B. megaterium

0.1 M Tris-HCl (pH 7.2)
0.01 M KCl
0.3 mM KH$_2$PO$_4$
7.6 mM (NH$_4$)$_2$SO$_4$
9x10$^{-7}$ M FeSO$_4$ • 7H$_2$O
4x10$^{-4}$ M MgSO$_4$ • 7H$_2$O
2.0% Glucose
0.4% Casamino Acids
3.4x10$^{-5}$ M CaCl$_2$ • 2H$_2$O
1x10$^{-5}$ M KNO$_3$
2. Table 2: Composition of Low Osmolarity Defined Medium (LODM)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized distilled water</td>
<td>900 ml/Liter</td>
</tr>
<tr>
<td>0.9 mM NaCl</td>
<td></td>
</tr>
<tr>
<td>0.4 mM (NH₄)₂SO₄</td>
<td></td>
</tr>
<tr>
<td>20 mM Tris-maleate (pH 5.8, 7.0, or 8.0)</td>
<td>(above autoclaved separately)</td>
</tr>
<tr>
<td>4 mM Amino Acid Mix</td>
<td></td>
</tr>
<tr>
<td>&gt;1 mM Vitamin Mix</td>
<td></td>
</tr>
<tr>
<td>4.5x10⁻⁵ M CaCl₂</td>
<td></td>
</tr>
<tr>
<td>1.85x10⁻⁸ mM FeCl₃</td>
<td></td>
</tr>
<tr>
<td>2.9x10⁻⁵ mM ZnSO₄</td>
<td></td>
</tr>
<tr>
<td>1 mM MnCl₂</td>
<td></td>
</tr>
<tr>
<td>1 mM MgCl₂</td>
<td></td>
</tr>
<tr>
<td>0.1% Glucose</td>
<td></td>
</tr>
<tr>
<td>1 mM KH₂PO₄</td>
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</table>
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