Regulation of Cytoplasmic pH in Bacteria

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INTRODUCTION ................................................................. 359

NATURE OF THE PROBLEM .............................................. 360
Metabolism and Control of pH ........................................... 360
Growth and Intracellular pH .............................................. 360

ASSESSMENT OF HOMEOSTASIS ......................................... 361
Measurement of Cytoplasmic pH ......................................... 361
Weak acids ........................................................................ 361
Weak bases ......................................................................... 361
Fluorescent probes ............................................................. 362
Perturbation of pH ............................................................. 362
Cytoplasmic buffers ............................................................ 363
Production of acids and bases .............................................. 363
Active transport of H+ (or its equivalent OH−) .......................... 363
Components of the pH Homeostatic System ............................ 363
Sites of pH Control ............................................................. 364

ACIDOPHILES ................................................................. 364
Generation of the pH Gradient in Acidophiles ......................... 365
ALKALOPHILES ............................................................... 366
Sodium and the Growth of Alkalophiles ................................. 366
Sodium and the Generation of the pH Gradient ....................... 366
Mechanism of pH Homeostasis in Alkalophiles ...................... 367
Mechanism of Na+ Entry into Cells ....................................... 368
Site of pH Control ............................................................. 369
Genetics of Alkalophilic Growth ......................................... 369

NEUTROPHILIC BACTERIA ................................................. 370
Streptococcus faecalis .......................................................... 371
Escherichia coli and Similar Organisms .................................. 371
Acidification of the Cytoplasm .............................................. 372
Potassium-proton antiport .................................................... 372
Sodium-proton antiport ........................................................ 373
Site of pH Control in E. coli .................................................. 375

CATION TRANSPORT AND pH HOMEOSTASIS: A SUMMARY .................. 375
CONCLUDING REMARKS .................................................. 375
REFERENCES ............................................................... 375
LITERATURE CITED .......................................................... 375

INTRODUCTION

In the last 30 years, bacteria have provided experimentalists with an easily manipulable system in which to study regulatory phenomena. This has led to a relatively clear understanding of the regulatory mechanisms which operate to control gene expression (35). However, with a few exceptions (e.g., chemotaxis, the control of enzyme activity) regulatory phenomena which primarily involve control of extant biochemical systems have fared less well. In particular the regulation of transport systems involved in cellular homeostasis is still poorly understood despite the early recognition of its importance (28). In recent years cytoplasmic pH has come to be recognized as an important aspect of bacterial cell physiology over which the cell exerts relatively tight regulation. In essence, regulation of cytoplasmic pH implies control over the permeability of the cell membrane to protons. It is generally accepted that this is achieved by control of the activity of ion transport systems which facilitate proton entry. The aim of this review of pH homeostasis in bacteria is to assess the role of transport systems in the regulation of cytoplasmic pH and to consider the principal questions which remain to be answered.

As a result of work on a wide range of organisms, a number of generalizations can be made about bacterial pH homeostasis. First, it is clear that there is no magic value of internal pH (pHi) which all organisms aim to achieve. Thus it is recognized that acidophiles exhibit pHi values in the range of pHi 6.5 to 7.0, neutrophiles have pHi values of 7.5 to 8.0, and alkalophiles have pHi values of 8.4 to 9.0 (3, 37, 40, 41, 43, 49, 59, 80, 82, 108; D. McLaughlin, Ph.D. thesis, University of East Anglia, Norwich, England, 1984). This variance in pHi values reflects a remarkable capacity to evolve enzyme systems which operate in these ranges. Second, it is clear that organisms exhibit different capacities to regulate their cytoplasmic pH. In many organisms, the pHi varies by only 0.1 units per pH unit change in external pH, whereas in others much larger changes in pHi have been observed (3, 41). One caveat to this generalization is that only rarely has the cytoplasmic pH been measured under growth conditions where cells might be expected to show optimum regulatory capacity. Third, fermentative organisms
exhibit a greater range of values of $pH_i$ over which growth will occur than do organisms which have a respiratory mode of metabolism (10, 43, 61, 108). Fourth, in one respect alkalophiles differ from acidophiles and neutrophiles, namely, the regulated value of $pH_i$ is lower than the external pH (37, 75). In these organisms it appears to be generally true that the generation of a transmembrane pH gradient, interior acid, is dependent upon the cycling of sodium ions across the cell membrane (66a, 68, 75; McGladden, Ph.D. thesis). Finally, in no group of organisms do we have satisfactory description of the mechanism of control of cytoplasmic pH.

**NATURE OF THE PROBLEM**

Before considering how cells regulate their cytoplasmic pH, it is valuable to consider what factors act to displace $pH_i$ and how tolerant microbial cells are to variations in $pH_i$.

**Metabolism and Control of $pH_i$**

The problem of metabolism and control of $pH_i$ has been considered in depth for plant cells (88), and the lessons from such systems have been applied to microorganisms (89). Raven and Smith proposed that the two principal factors causing perturbation of $pH_i$ are likely to be passive movement of protons across the cytoplasmic membrane and the production of acids and bases in the cytoplasm.

Most bacteria possess membrane-bound proton pumps which extrude protons from the cytoplasm to generate a transmembrane electrochemical gradient of protons, the proton motive force (78). The recorded values of the proton motive force are around 200 mV in respiring organisms and somewhat less in fermenting organisms (E. R. Kashket, Annu. Rev. Microbiol., in press; although see references 37 and 67 for exceptions). The passive influx of protons in response to the proton motive force could be a major problem for cells attempting to regulate their cytoplasmic pH. However, biological membranes exhibit a low proton permeability due to the intrinsic impermeability of the lipid bilayer and very specific control of ion flux through the protein complexes inserted in the membrane. Thus the proton permeability of biological membranes is usually only twofold to threefold higher than the permeability of pure lipid liposomes (87). Consequently, the recorded perturbations of $pH_i$ caused by large shifts in external pH ($pH_e$) are generally small (106; although see references 122 and 123 for an exception). Of course the observed change in $pH_i$ is the balance of perturbation and recovery. Notwithstanding this complication, the fact that the observed $pH_i$ changes are small indicates that the passive influx of protons is readily countered by the pH homeostatic capacity of the organism. Indeed the ability of the cell to cope with changes in external pH in the presence and absence of various ions has been used to determine the essential features of the homeostatic system (see below).

The other major sources of cytoplasmic pH perturbation are essential metabolic processes associated with growth and metabolism. We have referred to this as the stress or “load” against which the pH-regulating systems must act (15). Raven and Smith identified acid production consequent on ammonia assimilation as a major problem for cells attempting to maintain a constant intracellular pH during growth on neutral molecules such as glucose and carbon dioxide (89). In a recent analysis of the acid production during growth of *Penicillium cyclopium* it was shown that net acid production was equimolar with ammonia assimilation (93). Similarly it has been proposed that the major perturbation of intracellular pH in *Neurospora crassa* is the production of acid from metabolism (97). On the other hand, growth of *Escherichia coli* on acidic substrates such as succinate leads to alkalization of the medium, suggesting that metabolism of this carbon source results in the consumption of protons (3). In fermentative organisms the production of acidic fermentation products and their consequent accumulation by cells leads to the failure of pH homeostasis (10). This problem may be so acute that some organisms have evolved mechanisms which convert acidic products to neutral compounds to avert the toxic effect of high concentrations of weak acids in the medium.

The observation that the pH of the growth medium could affect the metabolic properties of a bacterium was first made 40 years ago (30). There are now many such observations, although our understanding of them is still very imprecise. It is a reasonable generalization that cells produce acidic fermentation products when growing at alkaline pH and neutral products at acidic pH. In many instances a neutral product is synthesized from a preexisting acidic product, thus acting to detoxify the environment. Two examples of this phenomenon which have been well studied are the production of butanol from butyrate by *Clostridium acetobutylicum* and of butanediol from acetate by *Klebsiella aerogenes* (4, 32, 46, 105, 110, 121). In the latter instance it has been shown that the induction of the new metabolic pathway is not the result of perturbation of $pH_i$ (110). Rather, in both examples the basis of this phenomenon appears to be that the buildup of acidic fermentation products in the medium caused a fall in $pH_e$, and the resulting transmembrane pH gradient causes the accumulation of the acid in the cytoplasm. High intracellular concentrations of the acid induce the enzymes for production of neutral products (34, 46, 105, 110). Thus *C. acetobutylicum* will synthesize the enzymes for butanol production at $pH_e$ 7 if the butyrate concentration in the medium is raised (46). Similarly in *K. aerogenes* synthesis of the enzymes of the butanediol pathway can be induced at pH 7 by the addition of high concentrations of acetate (105, 110). Thus the role of external pH is to create a larger pH gradient such that induction can take place before the external concentration of acid becomes high enough to cause growth inhibition.

In addition to fermentation product formation, Gale and Epps (30) have demonstrated that in *E. coli* many decarboxylases and deaminases are induced in response to low and high pH, respectively. This phenomenon, which may be restricted to complex media, results in a drift of $pH_e$ toward neutrality. The synthesis of the enzymes is subject to catabolite repression and thus is unlikely to be a general response to $pH_i$ shifts imposed by the external pH. No molecular mechanism for this pH dependence of enzyme synthesis has been proposed.

**Growth and Intracellular pH**

In a simple and elegant experiment, Harold and van Brunt established that rapid growth of *Streptococcus faecalis* was dependent upon maintenance of an alkaline intracellular pH value (44). In similar experiments the growth rate was still above 50% of the maximum when the intracellular pH was reduced to $pH_i$ 6.6, and growth was still possible below $pH_i$ 6 (61; Fig. 1). These experiments indicate that, although alkaline internal pH values are optimal for growth, the organism has a marked tolerance of reductions in $pH_i$ of up to 1 unit. Substantial confirmation of this tolerance of
changes in $pH_i$ has emerged from measurements of internal $pH$ in growing cultures of other fermentative organisms (10, 92, 108). Thus in Clostridium pasteurianum, Clostridium thermoaceticum, and Streptococcus cremoris the $pH$ of the medium falls during growth due to the production of acidic fermentation products.

Initially, the growing cells have a $pH$ gradient of 0.8 to 1.0 unit, alkaline inside, which causes the acids to accumulate intracellularly and reduce $pH_i$. In this way $pH_i$ falls during growth (10, 92, 108). With this type of experiment it was routinely observed that cells continued to grow with internal $pH$ values below $pH_i$ 6.0 and 6.5 and in some cases with values below $pH_i$ 6. Thus these obligately fermentative organisms have adapted to the consequences of their lifestyle.

Similar experiments with E. coli have indicated that this organism is more sensitive to internal $pH$ variation. Titration of the internal $pH$ with the weak acid dimethylxaloxalondinenedione produced 50% growth inhibition at $pH_i$ 7.2 and almost complete inhibition at $pH_i$ 6.6 to 6.8 (C. M. Stirk, C. G. Lorimer, R. G. Kroll, and I. R. Booth, unpublished data). Similar data have been obtained by using acetate to lower $pH_i$ in E. coli (E. P. Bakker, personal communication). In the alkaline range E. coli has been reported to be very intolerant of perturbation of $pH_i$ (122, 123). Unfortunately no comparable data are available on the tolerance of obligate alkalophiles to perturbation of $pH_i$.

The requirement for a high internal $pH$ for growth has been used for many years in the preservation of food from microbial spoilage (17). The effect on $pH_i$ of weakly acidic food preservatives has recently been compared with that of other acids of similar structure and $pK$ (95). From this study it was concluded that food preservatives are more effective at lowering $pH_i$ than other acids of comparable $pK$ (95). This appears to be a consequence of the inhibition of metabolism by the undissociated acid acting synergistically with the accumulation of the anion (26, 95). Thus, although the food preservatives have specific effects on metabolism, their inhibition of growth is exerted in part by their effect on $pH_i$.

In conclusion, the tolerance of $pH_i$ perturbation of organisms is likely to be species dependent. At the present time there is a paucity of data relating the observed values of $pH_i$ to the range of $pH_i$ values which will sustain growth.

**ASSESSMENT OF HOMEOSTASIS**

Although it is generally accepted that bacteria regulate their cytoplasmic $pH$, the evidence for many organisms rests on steady-state measurements of $pH_i$. Although such data are suggestive of homeostasis, they may also simply indicate that changes in external $pH$ are not the major factor perturbing $pH_i$. For a system to be considered truly homeostatic, it is desirable to find ways of perturbing $pH_i$ which elicit a response restoring the system to close to the preperturbation value of $pH_i$. This response is now well documented for E. coli, Exiguobacterium aurantium, and Bacillus firmus (65, 66a, 75, 100). Perturbation of $pH_i$ affords the only direct way of analyzing the control of intracellular $pH$. Thus although one can measure the activity of different putative components of the regulatory system and assess their properties as being consistent with a role in homeostasis, it is important to demonstrate a change in the activity of that system in response to perturbation of $pH_i$.

**Measurement of Cytoplasmic $pH$**

Measurement of cytoplasmic $pH$ has been reviewed recently by others (84; E. R. Keshet, Annu. Rev. Microbiol., in press), and it is not my aim to describe in detail the methods used. Rather, I wish to add to the discussions of earlier authors. There are two types of probes freely available to most workers: weak acids and bases and $pH$-sensitive fluorescent compounds. The use of $^{31}P$ nuclear magnetic resonance to measure $pH_i$ provided a major piece of evidence confirming the validity of weak acids as probes of intracellular $pH$, but the technique is not readily available to all workers (101, 116). The important properties of a molecular probe are as follows: (i) the neutral species of the probe should be permeant, and the charged species should be impermeant; (ii) the probe should not be actively transported, be itself metabolized, or interfere with cellular metabolism; (iii) the probe should exhibit relatively little nonspecific binding to cell components and should not interact with the components of the assay system. Of these three characteristics, (ii) seems to cause most problems with weak acids and bases, whereas (iii) is frequently a problem with fluorescent bases.

**Weak acids.** A simple test for the presence of a transport system for a weak acid (or base) is to determine the accumulation of the molecule when $\Delta pH$ is close to zero. In E. coli 7, we have observed 10-fold accumulation of acetylsalicylic acid and salicylic acid at $pH_6$ 8, suggesting that these acids are accumulated by transport systems (C. V. Hammon and I. R. Booth, unpublished data). Consistent with this, these acids registered higher values of $pH_i$ than either benzoxo or dimethylxaloxalondinenedione, which were not accumulated at $pH_6$ 8. Further, high concentrations of these acids reduce both $\Delta \nu$ and $\Delta pH$ in bacterial cells (2, 100). This may indicate that these acids act to some extent as uncouplers either via an uptake system for the acid or via permeability of the anion.

Acetate may also be actively transported in some E. coli strains, but the evidence is more equivocal than for acetylsalicylate. Certainly this acid can act as a carbon source for growth of this organism, and it may readily be phosphorylated even in cells grown on glucose (9).

The interference of weak acids with metabolism is most marked with benzoate. This acid is well known as a food
preservative due to its bacteriostatic effect (17). Although the mode of action of food preservatives is not fully understood, it is known that the undissociated acid is a potent inhibitor of metabolism (17) and acts synergistically with the accumulation of the anion to reduce pH (95). At the concentrations used to measure ΔpH (<20 μM), there is little or no effect on metabolism (95), but the side effects of this acid increase both with its concentration and with decreasing external pH (17). Thus it is a factor to be borne in mind when using acids to lower pH.

One cause of controversy in the measurement of pH, particularly in studies at acidic pH, is the use of weak acids below their pK value (51, 77; see below). In measurements of this type the neutral species of the acid passes freely across the membrane while the anion is impermeant. Thus the distribution of the anion will reflect the transmembrane difference in proton activity. When the acid is used below its pK, the concentration of the undissociated acid is not negligible and must be corrected for by multiplication of the accumulation ratio of total acid by a factor 10^{pK-pH}. As the value of this factor increases, any error in measurement of the accumulation ratio is magnified. Studies in E. coli have shown that the error arising from this correction is greatest when ΔpH is small (2). Thus, it is always preferable to use acids in the pH range above their pK value.

**Weak bases.** The measurement of the pH gradient when the external pH is more alkaline than the internal pH continues to give cause for concern. The predominant method is the accumulation of the weak base methylamine. However, the number of reports of transport systems for this ammonia analog has continued to grow (33, 56, 57, 102; Kashket, in press). Further, methylamine can be metabolized to N-methylglutamine (33, 56). The genes for transport and metabolism of methylamine appear to be regulated by the availability of free ammonia in the growth medium (56). Growth of cells in such media may be sufficient to suppress methylamine uptake by this route, but it may be advisable to use this probe only in mutants lacking the transport system. In the absence of such precautions the validity of methylamine as a quantitative probe of pH, remains in doubt.

Among radioactively labeled bases, dimethylamine, benzylamine, ethanalamine, and dopamine are potential substitutes for methylamine. However, in E. coli both dopamine and ethanalamine may be transported (Kroll and Booth, unpublished data). Benzylamine has not been extensively studied, but it appears not to be transported (Kashket, in press; P. M. Giffard and I. R. Booth, unpublished data). Dimethylamine has been used to good effect in *Vibrio alginolyticus*, but the possibility of transport of this compound was not tested (81).

**Fluorescent probes.** Fluorescent probes have been used extensively in membrane vesicles, but more rarely in cells (99). In vesicles the use of pyranine, 9-aminoacridine, and related compounds has been successful for qualitative studies of antiproton activity, but has been used less frequently to determine pH, per se (23, 85, 90).

The use of the carboxyfluorescein esters to study pH, has been discussed elsewhere (99, 109). This technique relies upon the presence of esterases in the cytoplasm which split carboxyfluorescein diacetate to release the fluorescent probe, carboxyfluorescein, into the cytoplasm. The fluorescence intensity of carboxyfluorescein is pH dependent; thus, if the membrane is impermeable to the probe, then one has a pH indicator trapped in the cytoplasm (109). The problem with most bacteria is that the cytoplasmic membrane is very permeable to carboxyfluorescein (109). This severely limits the usefulness of this probe because the resultant signal depends upon pH, ΔpH, the pH, dependence of the esterases, and the concentration of the released probe at the instant of measurement (I. R. Booth and E. P. Bakker, unpublished data). It is thus difficult to interpret the signal from such systems.

**Perturbation of pH.**

One of the major problems to be faced by investigators of pH homeostasis is the mechanism of perturbation of pH,. It is remarkably difficult to completely dissipate the transmembrane pH gradient (other than by the addition of uncouplers). The reasons for the stability of pH, are first that the membrane is relatively impermeable to protons (87). This is complemented by a low permeability to cations in general, such that, even when the driving force is large, net influx of protons is limited by the capacity for cation extrusion. This may be of significance in some experiments with acidophiles (see below, Acidophiles).

Coupled with the intrinsic impermeability to protons is the relatively high buffering capacity of the cytoplasm. A number of measurements have pointed to values of around 50 to 100 μmol of H+ per pH unit per mg of cell protein for the buffering capacity of cells at around pH, 7 (Table 1). At both acid and alkaline extremes of pH, buffering capacity increases (21, 65b, 97, 127; McLaggen, Ph.D. thesis). Thus the cytoplasmic buffering capacity itself tends to render pH, more sensitive to perturbation in the neutral range than at extremes of pH,. However, buffering capacity is a limited resource which can limit the variation of pH, but which can eventually be outstripped (see below). Further, recent data suggest that the buffering capacity of the cytoplasm plays little or no role in determining the pH range for the growth of an organism (65b).

Despite these intrinsic barriers to perturbation of pH, methods have been developed which allow intracellular pH to be manipulated and the recovery of pH, to the regulated value to be observed. These methods fall into two categories, depending upon whether they manipulate internal pH directly or indirectly. Into the latter class fall the pH shift

<table>
<thead>
<tr>
<th>Organism</th>
<th>Buffering capacity (nmol of H+/ pH unit per mg of protein)</th>
<th>pH Minimum</th>
<th>pH Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. acidocaldarius</td>
<td>20–400</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>T. acidophilus</td>
<td>97</td>
<td>(5.6)</td>
<td>27</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>400–1,000</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>S. aureus</td>
<td>160–360</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>E. coli</td>
<td>120–480</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>E. coli</td>
<td>80–340</td>
<td>8</td>
<td>5.5</td>
</tr>
<tr>
<td>E. coli</td>
<td>160</td>
<td>(5.7)</td>
<td>100</td>
</tr>
<tr>
<td>B. stearothermophilus</td>
<td>20–400</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>B. alkalophilus</td>
<td>260–800</td>
<td>8</td>
<td>9.5</td>
</tr>
<tr>
<td>B. firmus</td>
<td>260–1,000</td>
<td>8</td>
<td>9.5</td>
</tr>
<tr>
<td>E. aurantiacum</td>
<td>60–140</td>
<td>7.5</td>
<td>9.5</td>
</tr>
</tbody>
</table>

* The cytoplasmic buffering capacity is taken from the reference cited and is indicated as a range when the parameter was measured as a function of pH. In the latter instance, the value of pH at which the maximum and minimum values of buffering capacity were observed are shown.

*When the data represent a single value, then the pH range over which the measurement was made is indicated.
experiments in which the cells are exposed to a rapid large change in external pH (66a, 75, 100, 122). As a consequence, the internal pH is altered in the direction of the pH shift employed. Direct manipulation of pH can be achieved by the addition of weak acids or weak bases to cell suspensions (2, 9, 81, 96, 100). Generally, this technique leads to a permanent change in pH, and it is therefore used to study the response of either whole cells or specific transport systems to changes in pH.

A hybrid of these two approaches has been adopted with *E. coli* and *V. alginolyticus* (7, 64, 65, 80). The intracellular pH of cells which are partially depleted of potassium is sensitive to perturbation by washing with buffers of low pH (64). The intracellular pH of such cells can be manipulated simply by careful choice of the pH of the wash buffer and of the pH of the subsequent incubation buffer (65, 80). Further, potassium uptake into such cells can be used to raise pH, in a controlled way, since the uptake of this cation occurs by net exchange for H⁺ (see below). This has allowed some aspects of pH homeostasis to be investigated.

The problem with each of these methods is that the precise events taking place to initiate the perturbation of pH are not clear. For example, in the pH shift experiments the proton-conducting pathways responsible for the change in pH are not known. This ignorance reflects the complexity of whole cells as an experimental system and ultimately limits the type of information gained. As an alternative, investigators have turned their attention to the analysis of transport components which could play a role in pH homeostasis (81; B. P. Rosen, Methods Enzymol., in press).

### Components of the pH Homeostatic System

As discussed above, it is likely that the intracellular pH of a bacterial cell will be subject to perturbations in both the acid and the alkaline direction. Thus it is evident that a truly homeostatic system must be capable of coping with both types of deviations from the regulated state. The same conclusion can be reached for *E. coli* from the reported measurements of pH as a function of external pH (84, 101). Since pH can be either more alkaline or more acidic than pH₀, it follows that there must be systems both to raise and to lower pH in a controlled manner (84). It is the identity of these systems which has occupied many workers for the last 10 years.

Possible mechanisms for cytoplasmic pH regulation include preexisting cytoplasmic buffers, biochemical production of H⁺ or OH⁻, and active transport of H⁺ or OH⁻ (88).

**Cytoplasmic buffers.** Cytoplasmic buffers can offset a limited amount of acidification or alkalization. There is a remarkable similarity in the buffering capacity of organisms despite different types of metabolism and different pH ranges for growth (Table 1). This is not very surprising, since the principal components of the cytoplasmic buffer are the amino acid side chains of proteins (97). An unusually high buffering capacity has been observed in *Bacillus subtilis*, but there is no evidence that this increases the pH range for growth of the organism (65b).

**Production of acids and bases.** The production of H⁺ or OH⁻ from metabolism is a very neglected area of pH regulation studies. There is little evidence for metabolic acid or base production as a specific component of a pH homeostatic system. The synthesis of decarboxylases and deaminases is affected by the pH of the culture medium (30), but this has not been shown to be directly involved in pH control. In plant cells the pH sensitivity of phosphoenolpyruvate carboxylase and malic enzyme is such that their activities increase in response to shifts of pH₂O to alkaline and acidic values. The respective catalysis by interconverting phosphoenolpyruvate, malate, and pyruvate. Essentially a strong acid (malic acid) is synthesized from neutral materials (ultimately glucose and CO₂) when pH₂O is alkaline. When pH₂O is too acidic, malate is decarboxylated to pyruvate which has a higher pK than malate. This mechanism operates in vivo in plants (88). Evidence for its operation in bacteria is lacking. However, it should be relatively easy to test for its involvement since mutants of *E. coli* lacking phosphoenolpyruvate carboxylase are available.

Further, such a mechanism involves fixation of CO₂ and should be sensitive to experimental conditions in which CO₂ is limited. Carbon dioxide as bicarbonate has been reported not to be essential for pH homeostasis in *Bacillus alcalophilus* (T. A. Kruilich, personal communication), suggesting that the phosphoenolpyruvate carboxylase reaction is not of great significance in this organism.

Active transport of H⁺ (or its equivalent OH⁻). The controlled transport of H⁺ has long been considered to be the dominant mechanism of control of cytoplasmic pH. However, although it has been argued that primary proton pumps evolved with regulatable properties that would enable them to fulfill a role in pH homeostasis (89), it must be clearly recognized that their activity is constrained by the generation of proton motive force, especially its membrane potential component (78). Assuming a spherical microorganism of 1 μm diameter, a volume 5 x 10⁻¹⁰ μl, and a capacitance of 3 x 10⁻¹⁴ F, it can be calculated that approximately 4 x 10⁸ protons must be expelled from the cell to generate a membrane potential of 200 mV (36). Given a cytoplasmic buffering capacity of 50 nmol of H⁺ per pH unit per mg (dry weight) and assuming 5 x 10⁸ cells per mg (dry weight), this proton extrusion would raise pH by 0.001 pH units. Clearly, extrusion of large quantities of protons is required to generate a pH gradient relative to that needed to generate ΔΨ. The dissipation of excessive membrane potential by either cation influx or anion efflux is essential for the generation of the pH gradient. Thus in *N. crassa* lowering of the cytoplasmic pH led to a stimulation of the proton-pumping ATPase (96), but the extent of this stimulation was constrained by the resulting increased membrane potential. An increase in the permeability of the cell membrane to an ionic species other than H⁺ was necessary to allow high rates of the ATPase to counter the acidification of the cytoplasm (96).

In bacteria, net potassium uptake is the dominant means of compensating for the charge on the proton (7, 29, 42, 63, 80, 81). Since potassium is the major cellular cation, uptake of this ion during growth may well be a major factor acting to increase pH. This alkalization may be used to offset a fall in pH due to acid production, but may act adversely to perturb pH if the load of pH is causing the cytoplasmic pH to rise. Similarly it might be expected that factors which stimulate potassium transport, e.g., an increase in osmotic pressure (28, 52), might transiently perturb pH. There is at present no evidence that potassium uptake is regulated by the demands of pH homeostasis (see below, Alkalophiles).

The recognition that bulk proton extrusion in response to cation uptake is a major cause of elevation of pNa has led to most discussion and experimentation being focused upon mechanisms of acidification of the cytoplasm. Cation-proton antiports have been suggested to play the dominant role in acidification (8, 18, 81, 84, 85, 122-125; Rosen, in press). The basic principles of this hypothesis are illustrated in Fig. 2A. The antiport exchanges internal cations (either Na⁺ or K⁺)
for external protons, with the resulting acidification of the cytoplasm. This simple system is theoretically plausible. However, in a cell there may come a point when the internal concentration of the cation is so low that it limits antiport activity and must be replenished to allow acidification to continue (Fig. 2B). Thus, one must think in terms of cycling of the cation rather than simple extrusion (15). The problems created by this requirement for cation uptake are discussed below (site of pH control).

Although most research workers have primarily considered cation transport, there is no reason why anions should not play a role in pH homeostasis, possibly via anion-hydroxyl exchange (Fig. 2C). As yet there is no evidence favoring their involvement in pH regulation, but their role in animal cells is well documented (79).

Sites of pH Control

In addition to determining which systems are involved in pH homeostasis, an important aim must be to ascertain that the flux of ions through these systems is controlled (15). If these systems are not regulated then rather than regulating pH, they would simply facilitate a shift of pH in one direction or the other. The distinction between these two possibilities is that a regulatory system exhibits maximum activity under conditions where there is a need to reestablish homeostasis through controlled acidification or alkalinization. If a system does not possess this property, then its activity will be dictated by factors other than pH. Thus, on some occasions it will contribute to homeostasis, but on others its activity will be part of the load acting to perturb pH. In general primary proton pumps are not regulated, but compensatory proton fluxes are controlled. This theme will be expanded upon by consideration of the three groups of organisms, acidophiles, alkalophiles, and neutrophiles.

ACIDOPHILES

In the last 3 years there have been four reviews dealing with major aspects of growth and pH homeostasis in acidophiles (20, 47, 67, 73), and so no attempt at a comprehensive discussion will be attempted here. It is now accepted that the acidophiles maintain their cytoplasmic pH between pH 6.0 and 7.0, with the precise value being species dependent. In common with many other bacteria, acidophiles exhibit significant variations in their capacity to achieve a truly constant pH. Thus the intracellular pH of Coxiella burnetii varied from pH 5.2 to 6.95 over the range pH1 2 to 7, whereas in Bacillus acidocaldarius the pH was relatively constant at pH 6.5 to 6.9 over the range pH1 2.5 to 5 (41, 77). However, both studies were carried out with nongrowing cell suspensions; it may be that C. burnetii achieves a higher degree of regulation under growth conditions.

The metabolic diversity of acidophiles means that they do not all face the same metabolic load acting to displace pH away from the optimum value, but they do face the common problem of generating and maintaining a large pH gradient; several lines of work suggest that the lower limit of pH, at which cells remain viable is pH 5 (39), and that below this pH value denaturation of proteins occurs. The central debate has been how acidophiles generate and maintain pH close to neutrality when the external pH is very low.

Initial studies on a range of acidophiles suggested that uncouplers and inhibitors of respiration did not cause the complete dissipation of the pH gradient (67, 73). However, transport of solutes via H^-linked systems was inhibited (39). The maintenance of ΔpH in the presence of uncouplers has been attributed to either limited cation permeability across the membranes of acidophiles or high cytoplasmic buffering capacity in the cells (126, 127). However, after careful and critical examination of the bioenergetic properties of B. acidocaldarius and Thermoplasma acidophilum, it has been suggested that many aspects of the problem are methodological rather than deriving from unusual physiological properties of the cells (77). In particular the use of pH probes below their pK values, the use of flow dialysis leading to anaerobiosis, and the binding of probes have been suggested to be the primary methodological causes for the apparent insensitivity of ΔpH to protonophores (77). In T. acidophilum the membrane may also confer an increased resistance to protonophores (77).

The use of pH probes below their pK values to determine ΔpH is very sensitive to small errors, particularly when ΔpH is itself small (2). These errors can arise from the estimation of the cell volume, small errors in quench correction in liquid scintillation counting, and estimation of cell mass. In the case of the acidophiles, these problems are exacerbated by the binding of at least one routinely used probe, acetylsaliclyc acid, to cell constituents (77). In E. coli there is also a transport system for this probe (Hammond and Booth, unpublished data). It is not clear whether such a system is present in acidophiles, but this possibility must be considered. Thus the determination of ΔpH in the acidophiles requires not only careful quantitation but also correction for probe binding; in consequence, one cannot be too dogmatic about the magnitude of the residual pH gradient in the presence of protonophores.

It has been proposed that either a high cytoplasmic buffering capacity or a reduced membrane permeability...
might play a role in pH homeostasis when the cells are energetically compromised (26, 127). In the case of the former it is true that the cytoplasmic pH is poised just above the pH range in which the biological buffering capacity is maximal (Table 1). Thus, a much larger influx of protons is necessary to reduce pH from the regulated value than if pHs were higher. However, the buffering capacity of acidophiles is not significantly higher than that of nonacidophiles (Table 1). In the acid pH range, buffering capacity derives principally from acidic amino acids, both free and attached to proteins. These amino acids are usually charged compensated by cations, which are almost wholly free in the cytoplasm. If these cations were to be exchanged for H+, this would result in the buffering capacity being outstripped. Indeed, there is evidence that, when K+ -H+ exchange is stimulated by the addition of nigericin at acid pH, acidification of the cytoplasm occurs to an extent that causes denaturation of the proteins (39). Thus the intrinsic cation impermeability of the membrane of acidophiles may be important in protection against excessive acidification.

The evidence favoring limited permeability to cations in acidophiles is equivocal. Work by Zychlinsky and Matin on Thiobacillus acidophilus has established that the cells are permeable to cations (or anions), but that this permeability may be limited. When respiration of cells was inhibited, rapid uptake of protons was observed (127), which was thought to be mechanistically equivalent to that observed at the end of an oxygen pulse in other bacterial cells (21) or mitochondria. However, for this proton uptake to be evident and measurable it had to be charge compensated by movement of other cations or anions, since the proton movement would otherwise be limited by the increase in ΔΨ (positive inside). In the absence of anions other than sulfate in the buffer employed in this experimental system the only possible flux was that of cations out of the cell. On the other hand Zychlinsky and Matin have shown that the capacity for charge-compensating flux is limited (127). When a protonophore (carbonyl cyanide m-chlorophenylhydrazone) was added to T. acidophilus, the rate of proton influx was accelerated severalfold by either valinomycin-induced K+ efflux or thiocyanate influx (127). This clearly suggests a kinetic limitation on carbonyl cyanide m-chlorophenylhydrazone-induced H+ influx which can be relieved by systems allowing rapid charge-compensating fluxes. However it is questionable just how much less permeable to cations is the membrane of an acidophile than that of any other bacterium. Certainly the stimulation of H+ entry by valinomycin is a common observation and is not limited to acidophiles.

Two further observations are pertinent to this question of cation permeability. First, it has been observed in B. acidocaldarius that transport via H+ symports is more sensitive to ion-exchange ionophores (nigericin) and channel-forming antibiotics (gramicidin) than to protonophores (66). This again suggests that an increase in cation conductance of the membrane is essential for good uncoupling. Similarly, when 2,4-dinitrophenol was added to cells of B. acidocaldarius incubated in a nonpermeant buffer (β-alanine sulfate), an increase in ΔΨ (interior positive) was observed which could be prevented by NaCl (77). This was suggested to indicate that in the absence of a permeant cation (or anion) proton influx was not readily compensated for by movement of other charged species. However, the rate of dissipation of ΔpH was very similar in the presence and absence of NaCl (77); thus, charge compensation per se did not appear to be rate limiting for dissipation of the pH gradient. The involvement of Cl− flux in this system was not discussed, but in other systems Cl− uptake has been suggested to occur when ΔΨ is poised positive inside (90). Thus, the Cl− permeability of acidophiles may deserve further study.

To summarize, it is no longer disputed that acidophiles possess a pH gradient, the generation of which is almost wholly dependent upon proton extrusion across a proton-impermeable membrane. The buffering capacity and the intrinsic permeability of the membrane to cations are obviously important factors in stabilizing pH, but it is likely that buffering capacity per se plays only a passive role in pH homeostasis. However, the possibility remains that regulation of the cation permeability of the membrane limits proton flux down its gradient. In particular, it is feasible that channels which mediate potassium efflux may be sensitive to pH, becoming inactive below the optimum value of pH, for growth.

Generation of the pH Gradient in Acidophiles

The capacity to invert ΔΨ at acid pH is essential to the generation of a large pH gradient. Thus, B. acidocaldarius has a membrane potential of 20 to 30 mV, positive inside, at pH 2.5 to 3 (66, 77). However, with the primary proton pump directed outward, the initial generation of a membrane potential, inside negative, will occur. Generation of a large ΔpH sufficient to allow growth at acid pH requires dissipation and inversion of ΔΨ. It has been proposed that the inversion of ΔΨ might be the result of influx of protons down their concentration gradient (74). However, although such a mechanism may be involved in the fine balancing of the contributions of ΔΨ and ΔpH to the proton motive force, it cannot be the mechanism that operates to return charge during the generation of the pH gradient, since it would be self-defeating. In neutrophilic bacteria, potassium transport appears to be the major means of depolarizing the membrane, allowing the generation of a pH gradient, inside alkaline (see below). In E. coli, potassium uptake via the Kdp system at acid pH can cause complete dissipation of ΔΨ and, under some circumstances, may cause ΔΨ to invert (R. G. Kroll and I. R. Booth, unpublished data). In acidophiles, the relative contributions of ΔΨ and ΔpH will be dictated by external pH in a manner similar to that of any other organism. Thus, an increase in pH will reduce ΔpH, and ΔΨ will become less positive as a result of net proton extrusion. Conversely, a decrease in external pH will increase ΔpH and cause a transient increase in the proton motive force, the consequent increased proton influx rendering ΔΨ more positive. On a larger time scale, net potassium uptake, necessary during cell growth, may play an essential role in maintaining the membrane potential positive inside.

The properties of the potassium transport system(s) in acidophiles have not been described. Two outstanding questions have been recognized. First, what is the mechanism of energy coupling to potassium uptake (77), and, second, which systems contribute to pH homeostasis? It is clear that the potassium transport cannot simply be driven by ΔΨ alone, since this would lead to exclusion of potassium from the cytoplasm, not uptake. Thus, an alternative energy source is required. Indirect evidence suggests that in E. coli and S. faecalis this problem may be overcome by K+ -H+ symport (5, 6). This solution seems unlikely in acidophiles, since it would exacerbate the situation; once ΔΨ, inside positive, is established further, potassium uptake would be prevented. However, ATP-coupled systems have been char-
acterized in other organisms (27, 57), and this alternative may be employed by the acidophiles.

Recent studies with membrane vesicles from *B. acidocaldarius* have shown that the systems necessary to invert ΔH⁺ were absent (40). Thus, even at pH₆, 3 the initiation of respiration led to the development of a membrane potential inside negative, and although a large pH gradient was generated, true pH homeostasis was not achieved. An equally important observation was that at pH₆, 6 little or no pH gradient was generated, even though a membrane potential of the correct orientation (negative inside) was generated (40). The failure to generate a pH gradient at pH₆, 6 suggests either that systems which generate ∆pH at pH₆, 3 are inactive or that systems which prevent overalkalinization of the intravesicular space are active. This implies that some aspects of pH homeostasis may operate normally in vesicles, and it can be inferred that the failure to achieve pH homeostasis at pH₆, 3 is due to the inability of the vesicles to depolarize ∆H⁺.

In conclusion, the recent studies with vesicles represent a significant advance in the study of acidophiles. The data suggest that this group of organisms is unlikely to be significantly different in their solution to the problem of generating and maintaining an optimum value of pH for growth. Thus they require systems both to raise and to lower pH₆. These systems operate to interconvert the components of the proton motive force generated by the primary proton pumps. The major problem, then, is simply one of scale.

**ALKALOPHILES**

Alkalophilic bacteria maintain their intracellular pH up to 1.5 units more acidic than the external pH (67). It is widely recognized that this creates a number of problems, the solutions to which are of interest both to physiologists and to bioenergetics. How is the inverted pH gradient generated, and what are the problems arising from the generation of a large pH gradient, interior acid? As a result of the inverted pH gradient, the total proton motive force of alkalophiles is much smaller than that of other bacteria (37, 54, 67). Such cells should have difficulties in synthesising ATP by H⁺ uptake through the membrane-bound ATP synthase (67). Although the solution to this problem is of considerable importance, it will not be considered further here, and interested readers are directed to a recent review of this topic (67).

It has been suggested that sodium flux plays a major role in acidifying the cytoplasm of alkalophiles, and unequivocal evidence for this has now been presented (66a, 68, 75).

**Sodium and the Growth of Alkalophiles**

A requirement for Na⁺ for the growth of bacteria at alkaline pH has not been easy to establish. In *B. alkalophilus* it has not proved possible to demonstrate sodium dependence during growth on either organic acids or glucose (68). Growth of *B. firmus* on malate exhibited a stringent Na⁺ requirement. This sodium requirement probably reflects the involvement of this cation in transport of the carbon source as much as an Na⁺ requirement for pH homeostasis (68). Growth with glucose as the carbon source only required sodium when the medium pH was above pH₆, 9.7 (68). The concentration dependence of the sodium requirement for growth was not reported, but recent reports suggest that as little as 0.5 mM Na⁺ is sufficient for pH homeostasis at this value of external pH (106).

One of the limitations of the above studies was the failure to prevent changes in the external pH during growth. This complicates the analysis of the Na⁺ requirement for growth, since this parameter has been observed to be pH₆, 3 dependent (68; Mc Laggan, Ph.D. thesis). Thus, in *E. aurantiacum* under conditions of controlled pH₆, 3 (Mc Laggan, Ph.D. thesis), growth on glucose required added sodium only when the pH of the medium was above pH₆, 8.5. Sodium stimulation of growth was maximal at 0.9 mM, but above pH₆, 10 the sodium requirement rose steeply to 10 mM (Mc Laggan, Ph.D. thesis).

The presence of sodium in the incubation medium assists in the maintenance of viability of *B. firmus* (54). At pH₆, 10.5, the rate of loss of viability was decreased sevenfold by the presence of sodium. At lower pH values (pH₆, 7), intrinsic viability was somewhat greater, but Na⁺ still exerted some protection of the cells, slowing the loss of viability threefold. The effect of Na⁺ was transient, lasting only two hours, after which loss of viability occurred at rates approaching those in the absence of Na⁺ (54). At alkaline pH, the protective effect of Na⁺ is thought to be due to facilitation of the maintenance of a low cytoplasmic pH (54).

The moderate halophile *V. alginolyticus* grows at alkaline pH and has been reported to have evolved a novel mode of growth. It has been proposed that Na⁺ expulsion is directly coupled to electron flow through the NADH-quinone oxidoreductase (111, 113–115, 117, 118). Consequently, growth, Na⁺ extrusion, and transport of α-aminobutyric (Alb) acid are insensitive to protonophores (115). These properties are only expressed at alkaline pH and thus represent an adaptation to this environmental parameter. However, Na⁺ is not thought to be directly involved in pH homeostasis in this organism (81). Indeed, the insensitivity of growth at alkaline pH to protonophores appears to rely as much on the transport of K⁺ to maintain pH₆ as on an Na⁺ circulation for biochemical purposes. Thus, in the presence of carbonyl cyanide m-chlorophenylhydrazone pH₆, 5 was 8.05 and 6.29 in the presence and absence of potassium, respectively (115).

**Sodium and the Generation of the pH Gradient**

Good evidence for a role for Na⁺ in the development of a pH gradient, acid inside, has been obtained in three alkalophiles. In membrane vesicles of both *B. alkalophilus* and *B. firmus*, the reversal of the pH gradient in respiring vesicles was dependent on Na⁺ (68). This was achieved with relatively low concentrations of Na⁺ in *B. alkalophilus*, consistent with a high affinity of the Na⁺ cycle components for this cation. This observation may partially explain the inability to demonstrate an Na⁺ requirement for growth in this species (68).

Suspension of cells of *B. firmus* in the absence of sodium caused failure of pH homeostasis (54). This was observed most significantly at pH₆, 10.5, with lesser effects at pH₆, 9; this correlates well with the observed pH₆, 3 dependence of the Na⁺ requirement for growth discussed above. At pH₆, 7, where ∆pH is alkaline inside the presence or absence of Na⁺, prolonged incubation with Na⁺ caused the collapse of the pH gradient (54).

The development of a pH gradient, inside acid, in *E. aurantiacum* is also dependent upon Na⁺ (75). Thus, when cells incubated in Na⁺-free medium were subjected to a rapid shift of the external pH to 9.65, a partial failure of pH homeostasis occurred (Fig. 3): the pH₆, 3 was about 0.5 unit higher than normal. Such cells were shown to exhibit a controlled acidification their cytoplasm upon the addition of Na⁺ (Fig. 3). The rate and the initial extent of the acidifica-
tion were shown to be dependent upon the external concentration of Na\(^+\), but the final steady-state pH\(_i\) value was the same in all cases (75; Fig. 3). A similar phenomenon has recently been reported in *B. firmus* (66a), although in this case the endpoint pH\(_i\) value was dependent on the route of Na\(^+\) entry (Fig. 4). Thus, when AIB a substrate for a sodium symport, was present in the incubation medium, a lower value of pH\(_i\) resulted than when the cells were incubated with Na\(^+\) alone (66a).

**Mechanism of pH Homeostasis in Alkalophiles**

It is clear from the foregoing that Na\(^+\) plays a major role in the development of the pH gradient, inside acid, in alkalophiles. It has been proposed that acidification of the cytoplasm is achieved by the exchange of internal Na\(^+\) for external H\(^+\) via the sodium-proton antiport. The presence of such antiport activity in alkalophiles has been confirmed, and its activity has been shown to be dependent upon both Δψ and pH\(_i\) (31).

The antiport of *B. alcalophilus* was assayed by measuring the efflux of Na\(^+\) from cells which had been starved of energy. Artificial pH gradients of different sizes and orientations were imposed on the cells in the presence of a membrane potential, and the rate of Na\(^+\) efflux was then measured (31). The response of Na\(^+\) efflux to an imposed membrane potential was tested at pH\(_i\) 9 in the absence of a pH gradient (31). Under these conditions a linear relationship between antiport activity and Δψ was observed (31). At pH\(_i\) 9, with an imposed membrane potential of 200 mV, the rate of Na\(^+\) efflux was essentially independent of further increases in the proton motive force brought about by lowering the external pH. However, a reduction of the proton motive force by imposition of a pH gradient, acid inside, led to a fourfold reduction of antiport activity. The antiport did show increased activity as a function of pH\(_i\), increasing 10-fold from pH\(_i\) 7 to 10 with a constant driving force of 200 mV (31). This property is consistent with a role of the antiport in pH homeostasis.

At neutral pH the antiport could not be driven by Δψ. This may reflect control of the stoichiometry of the antiport by internal pH as has been reported for the Na\(^+\)-H\(^+\) antiport of *E. coli* (11, 12). However, in none of the experiments was it possible to determine whether the properties observed were those of a single Na\(^+\) efflux system. Moreover, mutants of *B. alcalophilus* which have lost the ability to grow at alkaline pH and which have been reported to have lost the major Δψ-dependent Na\(^+\)–H\(^+\) antiport (69) still possess a Δψ-independent antiport at neutral pH (31). Thus, the evidence regarding the possession of the Δψ-independent antiport by cells growing at alkaline pH is equivocal. However, the isolation of these mutants does indicate the importance of the electrogenic Na\(^+\)–H\(^+\) antiport for growth at alkaline pH.

It is now well established that Na\(^+\) plays a role in pH...
homeostasis of alkalophiles. The areas of uncertainty and thus the topics for discussion and speculation are as follows: first, the mechanism of Na⁺ entry into cells (15); second, the site of pH control; third, the identity of other systems involved in pH homeostasis; and finally, the identity of the genetic loci which are essential for growth at alkaline pH (69). Our views on these four points are moulded principally by data obtained with just three organisms, B. alcalophilus, B. firmus, and E. aurantiacum.

Mechanism of Na⁺ Entry into Cells

For Na⁺ to participate effectively in the regulation of cytoplasmic pH, there must be a route of Na⁺ entry (15). It is well established that cells can extrude Na⁺ and thus maintain an Na⁺ gradient directed inward, which is a component of the driving force for solute uptake. The activity of the antiport is dictated by several parameters; the concentration of Na⁺ and H⁺ at the two sides of the membrane, the magnitude of ∆Na and ∆H, and the kinetic parameters of the antiport protein itself. If the sodium-proton antiport is involved in regulation of pH, then its activity should be enhanced by a sudden increase in pH. Initially this could lead to an increase in the Na⁺ gradient due to net Na⁺ extrusion in exchange for protons. If the perturbation of pH is quite small, then the preexisting Na⁺ pool may be sufficient to allow the proton influx required to restore the pH to the regulated value. However, if the perturbation of pH is large, then the internal Na⁺ pool may be insufficient for the required acidification. Thus, Na⁺ entry must occur to allow the continued exchange of internal Na⁺ for external H⁺ (Fig. 2B).

Two specific proposals have been made to account for Na⁺ entry. It has been proposed that Na⁺ entry could be mediated by the symports involved in solute accumulation (67). Regulation of the symport activity by pH has been suggested such that, when pH is high Na⁺ influx is stimulated, potentiating acidification of the cytoplasm (66a). The alternative hypothesis is that there is an Na⁺-specific channel which is regulated such that Na⁺ entry only occurs when the pH is too high and acidification is required (15, 75). The rationale for this proposal was that it allowed the separation of the pH-regulating Na⁺ circuit from that involved in solute transport (15). In this way pH homeostasis would not be dependent on the translocation of solutes other than Na⁺. However, it is necessary to control the influx of Na⁺ so that the Na⁺ gradient is maintained and excessive acidification of the cytoplasm is avoided. The separate Na⁺ entry pathway would enable the regulation of Na⁺ flux for pH homeostasis (15). At the present time evidence has been obtained which suggests that both proposals have some validity.

In E. aurantiacum pH homeostasis was achieved with low concentrations of Na⁺ similar to those required for growth. After the pH was raised to 9.4, the pH stabilized at 8.8 (75). The addition of Na⁺ resulted in a rapid Na⁺ entry; it was evident that the rate of Na⁺ entry initially outstripped the antiport activity (Fig. 5). Indeed, the internal Na⁺ concentration only began to decrease once pH fell below 8.6. This would be consistent with a reduced rate of Na⁺ entry upon attainment of a more acidic value of pH. However, direct determination of Na⁺ entry rates after pH homeostasis has been achieved (i.e., pH 8.4) did not wholly substantiate the view that Na⁺ influx was regulated (McLaggan, Ph.D. thesis). Thus, the Na⁺ entry observed was quantitatively smaller than when pH was very alkaline, but the entry of Na⁺ was still substantial. Although the route of Na⁺ entry has not been elucidated, there was no evidence for a symport of Na⁺ with any solute. Thus, the rate of Na⁺ entry was independent of either glucose or any ion which was a constituent of the buffer system. It was concluded that Na⁺ entry was via a uniport possibly simultaneous with an efflux of potassium (McLaggan, Ph.D. thesis).

The generation of a pH gradient in B. firmus at pH 10.5 requires the presence of high Na⁺ concentrations (68). Under these conditions there was no evidence for Na⁺-solute symport as the mechanism of Na⁺ entry. However, at suboptimal Na⁺ concentrations acidification required the presence of AIB, a substrate for Na⁺ symport (66a; Fig. 4). Thus, in this situation the presence of a symported solute accelerated the rate of acidification of the cytoplasm. Moreover, in each of the experimental combinations of Na⁺ and AIB, the value of pH was different; this may reflect the Na⁺ flux occurring. Thus previous studies have shown that AIB transport continues for several minutes, whereas pH was constant after 30 s (37). The Na⁺ flux consequent upon the uptake of AIB may result in overacidification of the cytoplasm, since in such experiments pH increases gradually with time (66a). In other studies of B. firmus it has been reported that at pH 9.6 as little as 0.5 mM Na⁺ is sufficient to regulate pH (106). The intracellular pH was 8.6 and was attained in the absence of transportable solutes other than glucose (106).

We can conclude that, at very alkaline pH, high Na⁺ concentrations are needed to regulate pH (68; McLaggan, Ph.D. thesis). In this instance the presence of a solute for Na⁺ symport accelerates the acidification of the cytoplasm if the sodium concentration is low, but may delay the attainment of homeostasis due to overacidification of the cytoplasm if the Na⁺ concentration is high (66a). At lower values of pH, there appears to be no absolute requirement for solutes to allow Na⁺ entry (106).
Site of pH Control

The above experiments are inconclusive on the issue of the presence of a specific Na⁺ entry pathway. However, they do raise two questions, namely, how does Na⁺ entry lead to acidification, and what are the sites of pH₃ control?

The mechanism by which Na⁺ entry leads to acidification of the cytoplasm is not simple. Consider again Fig. 2B, in which the cell possesses a proton circuit, consisting of a pump and several sinks, which are in balance leading to constant values of pH and Δψ. Coupled to the proton circuit is the sodium circuit. Passage of a single sodium ion through the cell will result in the entry of \( n \times x \) protons, where \( n \) is the proton stoichiometry of the antiport and \( x (\sim n) \) is the Na⁺ stoichiometry of the antiport (Fig. 2B). However, the entry of protons should cause Δψ to fail, leading to either an increased rate of proton expulsion by respiration, or reduced rate of proton entry via the proton sinks, or both. In this way proton extrusion will occur to offset entry via the antiport, and a change in pH₃ should be prevented. However, in the alkalophiles the Na⁺ cycle does result in net acidification of the cytoplasm (66a; McLaggan, Ph.D. thesis); thus, the problem of proton entry stimulating respiration must be overcome. This could be achieved in three ways. First, Na⁺ entry could be overall an electroneutral flux, i.e., either Na⁺ enters with an anion or its entry is balanced by the efflux of another cation (15). These fluxes need not be obligatorily coupled, and the effect would be to provide charge compensation on the protons entering via the antiport. Thus each proton would bear an effective (1 - \( x/n \)) positive charge where \( x/n < 1 \). This still leaves the fate of the ion accumulated with Na⁺ or expelled in exchange for Na⁺ to be resolved. It is possible this is a temporary problem which can be solved by the cell when the load on pH₃ has diminished. Second, the respiratory chain could be constrained from expelling further protons by high values of pH₃. This seems unlikely, since Δψ is maintained during the Na⁺ cycle (McLaggan, Ph.D. thesis). Third, the influx of Na⁺ could be so massive as to overwhelm the capacity for net H⁺ extrusion. A similar situation to the latter is the influx of protons with lactose in E. coli, which can lead to a breakdown of pH homeostasis (3).

It is probably significant that the capacity of the cell to adjust the contributions of Δψ and ΔpH to the proton motive force diminishes as the pH₃ is raised (37, 54, McLaggan, Ph.D. thesis). Thus, at acidic and neutral pH₃ values there is a reasonable 1:1 exchange of Δψ for ΔpH. However, as one approaches alkaline pH₃, the capacity of bacteria to maintain high values of Δψ while acidifying the cytoplasm does not appear to be great. The failure to maintain the proton motive force is unlikely to reflect limitations placed on the magnitude of Δψ by the dielectric breakdown point of the membrane, since it is observed even in neutrophilic bacteria where the values of Δψ are relatively modest (3, 50, 81). At higher values of pH₃, this limitation on the magnitude of Δψ may become a significant factor in the maintenance of the proton motive force. However, an equally important factor in the failure to maintain the proton motive force at alkaline pH may be the energy cost of cation cycling to maintain the pH₃ more acidic than the pH₄.

The discovery in alkalophiles that systems exist to raise pH₃, in addition to those for lowering pH₃, opens up the debate on the site of control of pH₃ and removes the requirement for the Na⁺ cycle to be regulated. In experiments with both E. aurantiacum and B. firmus there was evidence of a second component of the pH regulatory system which operates to counter too great a fall in pH₃ (66a, 75). Thus, in E. aurantiacum the addition of further Na⁺ after pH homeostasis had been achieved led to measurably Na⁺ cycling, which did not cause perturbation of pH₃ from its regulated value (McLaggan, Ph.D. thesis). The possible presence of a system to raise pH₃ was evident in earlier work, when excessive acidification caused by rapid Na⁺ cycling was counteracted by another unidentified system (75; Fig. 3). Further, Na⁺ extrusion does not always coincide with acidification of the cytoplasm (Fig. 5). Thus, when glucose was added to cells preequilibrated with Na⁺, further expulsion of Na⁺ occurred concomitantly with an increase in pH₃, (Fig. 5). Similarly, the continued cycling of Na⁺ during AIB transport in B. firmus did not cause a further decline in pH₃ (Fig. 4). Although less convincing than the data for E. aurantiacum this result is consistent with the existence of a mechanism to raise pH₃.

Thus, one envisages that the control of pH₃ in the alkalophiles occurs via the counteractive action of at least two different systems. The Na⁺ cycle is one of the systems, but the identity of the other is at present unknown. In the absence of control of Na⁺ entry, the Na⁺ cycle would simply be an acidification mechanism operating to lower pH₃. Regulation of pH₃ would then be exerted by modulation of the properties of systems which cause pH₃ to rise. This is not to suggest that control of Na⁺ cycling and of mechanisms which raise pH₃ are mutually exclusive. Similarly, by focusing on regulation of Na⁺ entry, one is not excluding control of the antiport. Rather, if each of the individual components contributing to pH homeostasis is regulated, then overall a finer degree of control can be exerted over pH₃. However, the balance of the evidence suggests that control of pH₃ may be primarily by prevention of overacidification of the cytoplasm.

There remains a considerable amount of work to be done before the ion movements involved in pH homeostasis can be understood. The studies discussed above provide a valuable framework within which to expand our understanding of these organisms. One of the means of doing this is via a genetic analysis of the components essential for growth at alkaline pH.

Genetics of Alkalophilic Growth

Mutants have been isolated recently from B. alcalophilus and B. firmus which fail to grow at alkaline pH (38, 69). These mutants have a pleiotropic phenotype which is caused by mutation at a single locus (70, 71). Thus, the mutants lack the major Na⁺H⁺ antiport and Na⁺-coupled transport systems, possess different cytochromes, and fail to synthesize a chromophore (38, 70, 71). These properties suggest either that the primary mutation affects a regulatory locus or that the gene for one essential system is affected (the Na⁺H⁺ antiport) and the other aspects of the phenotype are physiological adaptations. There is a precedent for the latter in that unc mutants of E. coli have a pleiotropic phenotype caused by a single lesion affecting the ATPase operon (25; Booth, unpublished data).

Similarly, it has been reported that B. subtilis can acquire the ability to grow to alkaline pH by transformation with DNA from an alkalophilic Bacillus species (107). With genetically marked strains of B. subtilis, transformants which could grow at pH₁₀ were isolated as double transformants to alkalophilic growth and prototrophy with respect to either arginine or leucine. These auxotrophic markers were used to show that the organisms able to grow at
alkaline pH were *B. subtilis* cells which had acquired DNA from the alkalophile. In this way the authors excluded the possibility that they were simply either isolating spontaneous mutants able to grow at high pH or reisolating their original alkalophile. Such transformants arose at much lower frequencies than prototrophs able to grow only at pH 7.5, indicating either that they where the result of double transformation events or that large pieces of DNA were required for acquisition of both characters (107). The transformants exhibited growth properties of both *B. subtilis* and the alkalophile.

These observations suggest that growth at alkaline pH is dependent upon the acquisition of a single gene or at most a limited number of genes. Evolution of organisms capable of growth at alkaline pH may require the acquisition of both enzymes capable of operation at new higher values of pH and externally located enzymes which are active in the range of pH, to which the organism is adapting. Expression of the genes for such an adaptation may have been controlled to allow the organism maximum flexibility in the growth environment. As such they might have become organized into a regulon (35) under the control of a regulatory gene. The pleiotropic phenotype of the *B. alcalophilus* mutants suggests a regulatory mutation affecting the transcription of a specific subset of genes under common control. This would be analogous to the onset of differentiation in bacilli (72) or the regulation of nitrogen metabolism in enteric bacteria (24). In both cases regulation is by the action of a positive regulator, the loss of which by mutation leads to a pleiotropic phenotype. Mutations affecting RNA polymerase itself can also give rise to very pleiotropic phenotypes due to selective transcription defects (see below; 32a, 94, 94a). Thus, the basis of the pH sensitivity of the alkalophile mutants is of considerable interest.

In concluding this section one might consider the question, what makes an organism into an alkalophile? The genetic evidence favors a single locus controlling a number of attributes which enable an organism to grow at alkaline pH. Clearly these represent solutions to bioenergetic problems associated with pH homeostasis, but one suspects that other less immediately obvious activities such as those involved in cell wall and membrane synthesis are of equal importance. Further, given the relative intolerance of *E. coli* to shifts in pH (122), one might expect that alternative essential enzymes might be needed in the cell growing at pH 10.5 (pH 8.5 to 9.0) compared with that growing at neutral pH (pH 7.5 to 8.0).

The same question posed in reverse should also occupy us; namely, what prevents an alkalophile from growing at neutral pH? This is a very variable property, with some alkalophiles being able to grow at both neutral and alkaline pH (67; McLaggan, Ph.D. thesis). The proposal that failure of alkaliphiles to grow at neutral pH is due to an overactive Na’ cycle, causing excessive acidification of the cytoplasm, does not seem to be tenable (31, 48). A simple genetic solution in obligate alkaliphiles would be that the putative positive regulator of expression of genes for alkaline growth has a negative regulatory role on systems essential for growth at neutral pH (24). On the other hand, the pH range for growth may be dictated simply by the properties of the enzymes exposed to the external medium (67). This area is open to speculation, and the molecular basis of alkalophily is going to be of considerable interest.

**NEUTROPHILIC BACTERIA**

A great many organisms of diverse metabolism and habitat fall into the rather general category of neutrophilic bacteria. Thus, discussion of pH homeostasis must be in terms of specific organisms rather than a general grouping. Most of the work on pH homeostasis has been carried out with three organisms, namely, *S. faecalis*, *E. coli*, and *V. alginolyticus*. In many respects the work on the last two organisms is complementary; thus, they will be discussed together.

*Stereococcus faecalis*. *S. faecalis* was the first microorganism in which the generation of a pH gradient was investigated. It was established that glycolyzing cells maintained a pH gradient, interior alkaline, of 0.5 to 1.0 unit, depending on the pH of the medium (43). Generation and maintenance of the pH gradient were energy dependent and could be prevented by incubation of the cells with *N,N*-dicyclohexylcarbodimide, an inhibitor of the membrane-bound ATPase (43). This organism was also the first in which the relationship between potassium transport and the establishment of a pH gradient was established. Cells which were Na’ loaded and incubated in potassium-free medium possessed only a small pH gradient. The addition of potassium to the medium led to the generation of a pH gradient due to energy-dependent proton extrusion consequent upon potassium uptake down its electrochemical gradient.

The central importance of the proton-translocating ATPase in pH homeostasis in *S. faecalis* is underlined by both genetic and biochemical studies. Mutants have been isolated which fail to grow at pH 6, but which grow normally at pH 7.5 (61). The capacity of the mutants to generate a pH gradient was severely impaired (58, 61). The mutants had significantly reduced proton-translocating ATPase activities and a reduced capacity to transport potassium (61). Although the primary lesion in these mutants has not been elucidated, it has been proposed that the mutation affects the ATPase and that the potassium transport defect is a secondary consequence of the failure to generate a membrane potential (61).

The relationship between potassium uptake and the generation of the pH gradient has also been investigated with mutants. In the *S. faecalis* 687A, the rate of potassium uptake is reduced at least twofold, and this mutant has an impaired capacity to generate a pH gradient (59). However, the requirement for K’ transport is not absolute. Mutant strains deficient in Na’ extrusion accumulate Na’ via a leak pathway in response to the membrane potential (59). In such mutants Na’ accumulation can substitute for potassium in the generation of a pH gradient (59). Similarly, in the parent a lipolphic cation, dimethylbenzyl ammonium ion, can substitute for potassium, since it too will accumulate in the cell and dissipate the membrane potential (42). This is a very clear demonstration that cation uptake serves only indirectly in the generation of a pH gradient, i.e., its role is the dissipation of the membrane potential generated by proton extrusion.

Biochemical studies suggest that the synthesis of the ATPase of *S. faecalis* is regulated by the internal pH (1, 60). Membranes prepared from cells in which the capacity to generate or maintain a pH gradient has been impaired, by either limitation for potassium or addition of uncouplers, exhibit enhanced ATPase activity (1, 60). Similarly, the membranes of cells grown at acidic pH, were found to contain significantly higher ATPase activity than cells grown at alkaline pH (58, 60). This enhancement has been shown to reflect de novo protein synthesis, and it is assumed that this reflects control by internal pH of either transcription of the ATPase operon or translation of the mRNA (1).

The internal pH of cells grown at low pH, was always
approximately 0.2 unit higher than that of cells grown at high pHo (58). Although this is consistent with the higher ATPase activity, it must be borne in mind that the generation of the pH gradient depends as much on the activity of the potassium transport system as on the ATPase per se. Thus, the higher pH gradient in cells grown at low pHo may reflect other adaptations to the acidic environment than simply the increased ATPase activity.

Finally, it has been proposed that the intracellular pH is regulated by the intrinsic pH sensitivity of the ATPase itself (59). There is an intimate relationship between the rate of potassium accumulation and the rate of alkalization of the cytoplasm. This relationship was found to reflect the pH sensitivity of the ATPase enzyme (59). Further, the cells possess no capacity to invert the pH gradient at alkaline pH (58). Thus, it was proposed that as pHo rose the activity of the ATPase declined until it simply matched the activity of inwardly directed proton leaks. In current models of pH homeostasis, no role is envisaged for cation-proton antiports, although at least one such system is known to exist (45).

Thus, at present it is envisaged that S. faecalis generates a pH gradient by the combined action of the primary proton pump and the potassium uptake system(s). The critical experiments to demonstrate pH homeostasis as outlined above (Assessment of Homeostasis) have not been reported. However, when fermentation products are eliminated from the incubation medium, the intracellular pH of Clostridium thermoaceticum is held quite constant at pH 7.2 in the range pHo 5.5 to 7 (10). This suggests some capacity for homeostasis which is independent of the simple considerations of the pH activity profile of the ATPase. The generality of this observation for other fermentative organisms needs to be established.

Escherichia coli and Similar Organisms

Despite intensive efforts to understand pH homeostasis in E. coli, we are still a considerable way from achieving that understanding. The first experiment that bacteria can maintain a relatively constant pH was made in E. coli (82) by Rottenberg and his colleagues. The thrust of this work was directed at testing one aspect of the chemiosmotic hypothesis (78), namely, that cells maintain a transmembrane proton motive force constituted of a membrane potential and a pH gradient. It was found that the magnitude of the pH gradient was a function of the external pH and that this reflected a relatively constant internal pH (76). As the external pH was raised, the membrane potential also increased. It was proposed that the change in the pH gradient consequent upon increasing external pH is mostly electroneutral, possibly involving an electroneutral proton conductor across the membrane—either antiport with a cation or symport with an anion. The earlier demonstration of an electroneutral Na+-H+ antiport in E. coli (120) led to the proposal that Na+ flux could account for the decrease in the pH gradient as the external pH was increased (82). Despite many attempts to generate evidence in favor of this and other models, the mechanism of acidification of the cytoplasm in E. coli and similar organisms remains unclear.

The evidence presented above for pH homeostasis in E. coli has been amply substantiated by studies in growing cells (3, 49, 50) which exhibit approximately the same value of internal pH and the same invariance of pH with variation in external pH. To define the mechanisms responsible for this homeostasis in E. coli, we need to determine which components act to raise pH and which act to lower pH (15, 84). In addition, we need to identify points of control of intracellular pH.

Studies in a range of organisms have pinpointed potassium uptake as the major means by which a pH gradient, inside alkaline, might be generated (7, 29, 42, 59, 63, 64, 80). In potassium-depleted cells, it was demonstrated that the pH gradient was very small at all values of external pH (64, 65, 80). The addition of potassium resulted in the generation of a pH gradient, the magnitude of which depended upon the external pH (see below). Thus potassium-dependent alkalization of the cytoplasm resulted in cells with a pattern of ΔpH generation similar to that seen in growing cells (65). Further, it was shown that if potassium uptake was constrained either by the external concentration of this cation or by the use of appropriate mutants, then generation of the pH gradient was likewise limited (7, 64). Further, within certain limits there was a direct relationship between the potassium uptake and the pH gradient generated (64).

In E. coli, the relationship between net potassium transport and the pH gradient breaks down when the internal pH becomes too alkaline (Fig. 6); the addition of potassium results in a rapid increase in internal pH until pH 8.1. At this point a mechanism for acidification of the cytoplasm overcomes the tendency for pH0 to rise, and it falls back to a value close to pH7.6 (65). It should be noted that the final value of internal pH is independent of both the initial and its peak values, exemplifying the high degree of homeostasis of pH in E. coli cells.

The mechanism of the acidification of the cytoplasm remains unknown, but the rate of the process was shown to be stimulated by the magnitude of the preformed pH gradient (65). This effect of ΔpH on the rate of acidification of the cytoplasm is evident in Fig. 6. Thus, the rate of acidification is greatest when the peak value of ΔpH is 1.25 units (pH6.8) and becomes slower as ΔpH is reduced (i.e., pH6 is increased). These differences in rate, although small, were consistently observed (65). Furthermore, the overshoot of pH could be prevented when the magnitude of the preexisting pH gradient was large (i.e., pH4.5, initial pH 7.2; 65). This effect did not appear to reflect differences in the rate of potassium uptake at different values of pHo, but rather a stimulation of the rate of acidification of the cytoplasm by the acidic external pH (65). Thus, it was proposed that the mechanism of acidification was sensitive to both pHo and pH and that these effects might be mediated via the magnitude of ΔpH (65).

The attainment of pH homeostasis was not absolutely dependent on potassium transport, but did require pH to be raised to a value greater than 7.6. Thus, potassium-depleted cells washed at pH8 had an internal pH close to the value of the wash buffer. Subsequent incubation with an energy source led to a controlled acidification of the cytoplasm to pH7.6. Omission of an energy source led to uncontrolled acidification of the cytoplasm (65), and it has been generally observed in E. coli that pH homeostasis requires either a high respiratory rate or ATP (Kroll and Booth, unpublished data). Thus, cells respiring on endogenous reserves have a low respiratory rate and a limited capacity to synthesize ATP and cannot attain a high degree of pH homeostasis. Such cells, however, can generate a proton motive force and carry out H+-coupled active transport (2). Thus, the capacity for pH homeostasis may require more than the capacity to establish a proton motive force.

There is some evidence to suggest that potassium is involved in raising intracellular pH to a more alkaline value
Trk system is operating in the net uptake or exchange mode (9; Kroll and Booth, in preparation).

Thus, potassium transport does play a role in the generation of the pH gradient, interior alkaline, as described above (Perturbation of pH, S. faecalis). However, it is likely that its role in generation of the pH gradient is a passive one rather than under the control of pH$_i$.

**Acidification of the Cytoplasm**

It is generally recognized that there are two principal systems which have been identified which could account for the generation of a pH gradient, inside acid, in E. coli and similar organisms. These are the sodium-proton and potassium-proton antiports. The evidence for a role for either of these systems is very indirect, and it remains a possibility that there may be other systems which are equally important (see below).

**Potassium-proton antiport.** The discovery of the potassium-proton antiport was made by Rosen et al., who suggested that it might be a component of the pH regulating system (13, 18). The system was given the name KHA and was recognized by the reversal of quenching of 9-aminoacridine in inverted membrane vesicles (Rosen, in press). However, the use of 9-aminoacridine has been criticized on the basis that it is not quantitative; thus, the interpretation of the data obtained can be difficult (84). Certainly, the reversal of quenching of fluorescence mediated by K$^+$ is small compared with that caused by the addition of Na$^+$ (18). Further, it has been reported that inverted membrane vesicles do not exhibit exchange of $^{38}$K and that this is not consistent with the presence of a potassium-proton antiport (84).

Genetic evidence points to at least three separate systems involved in potassium exit in E. coli. Epstein and Kim isolated several classes of mutants which required high potassium concentrations in the medium (27). Two of these mutant classes, trkB and trkC, were affected in potassium retention rather than potassium uptake. Recently these two loci have been shown to encode components of separate potassium efflux systems (14a), which correspond to the N-ethylmaleimide-stimulated K$^+$ channels discovered by Kepes et al. (52, 76). The addition of N-ethylmaleimide to E. coli cells resulted in potassium efflux, which was found to be due to direct reaction of N-ethylmaleimide with a small chemical in the cytoplasm which may regulate the activity of the potassium channels (76). The efflux of potassium was found to be essentially electroneutral; thus, it is thought that the N-ethylmaleimide-stimulated systems may be antiports (8). Insertion of transposons or isolation of nonsense mutations in the trkB and trkC loci (14a) prevents the N-ethylmaleimide-stimulated K$^+$ efflux (W. Epstein, E. P. Bakker, and I. R. Booth, manuscript in preparation). Loss of the trkB- and trkC-encoded systems does not impair diethanolamine-stimulated potassium efflux at alkaline pH (81; see below), suggesting the presence of a third potassium efflux system. Preliminary results also suggest that neither of these potassium efflux systems is involved in the acidification of the cytoplasm in overshoot experiments (Fig. 6; Booth, unpublished data). Thus, at the present time there is no evidence to suggest that the trkB- and trkC-encoded systems are involved in pH homeostasis.

Finally, mutants of E. coli which are sensitive to growth at alkaline pH have been isolated, and vesicles prepared from these cells were found to lack the KHA system (85). These mutants have not been investigated at the genetic level, but...
again pH homeostasis was not affected in these mutants (101).

The strongest evidence for a potassium-proton antipart has been obtained with whole cells of *V. alginolyticus* (81). Potassium-replete cells of this strain were incubated at pH, 9, and diethanolamine was added to raise the pH. The weak base permeates the membrane in its dissociated form and binds a proton in the cytoplasm and thus causes pH, to increase. K⁺ efflux was stimulated and continued until pH, was close to the regular range, pH, 7.7 to 7.9 (81; Fig. 7A). One caveat to these elegant experiments is that pH homeostasis was only achieved by net exchange of K⁺ for another cation, DEAE⁺. However, in a further series of experiments it was demonstrated that net potassium efflux may be involved in the generation of a pH gradient, inside acid. Thus, incubation of potassium-replete cells at alkaline pH in the absence of a potassium gradient led to dissipation of the pH gradient (Fig. 7B). The generation of a pH gradient, inside acid, was dependent upon the presence of a potassium gradient, directed outward. Once pH, 7.7 to 7.9 was reached, further increases in the potassium gradient were without effect on the ΔpH (81; Fig. 7B). A potassium gradient in excess of 100, 40, and 30 mV was necessary for pH homeostasis to be achieved at pH, 9.6, 9, and 8.6, respectively (81; Fig. 7B). These data suggest that potassium exchange for protons, possibly via an antipart, is an intrinsic component of pH homeostasis in *V. alginolyticus*.

These experiments raise the question of how pH homeostasis is maintained when the potassium concentration of the medium is high, i.e., when the transmembrane potassium concentration gradient is very small (84). Analysis of Na⁺ flux suggests that this too may play a role in pH homeostasis in this organism (see below).

Finally, one of the arguments against the involvement of potassium extrusion in pH homeostasis is that the accumulation of this cation is regulated by the osmotic pressure of the medium (15, 28, 52). It appears that *V. alginolyticus* may have found a solution to this problem. Cells in their natural environment may not encounter such large perturbations of pH, thus, the extent of potassium efflux may be correspondingly smaller, or the cells may have evolved a system for compensating for the potassium efflux. It should be noted that *V. alginolyticus* is a marine organism and thus is usually found in an environment in which the ambient potassium concentration is low and the transmembrane gradient is high. Certainly there can be no doubting that these experiments clearly establish the potential of potassium extrusion as a mechanism for lowering pH.

**Sodium-proton antipart.** The sodium-proton antipart was first demonstrated in *E. coli* by West and Mitchell (120) and has subsequently been found to be ubiquitous in bacteria (reviewed by Krulwich [65a]). The antipart has been assayed by Na⁺-dependent proton flux in resting cells, ²²Na uptake in inverted vesicles, and fluorescence-based assays in both inverted and right-side-out vesicles (65a). In deenergized cells the antipart was reported to be electroneutral at pH, 7 (120). A recent nuclear magnetic resonance evaluation of the Na⁺ gradient in respiring cells at moderately acidic pH, values has similarly suggested that the Na⁺ gradient is close to equilibrium with the pH gradient, an observation which again is consistent with an electroneutral antipart (A. N. Castle and R. M. Macnab, personal communication). Recent studies on Na⁺ extrusion from *E. coli* cells have suggested that the antipart is electronegative, since at pH, 7.5, where there is no pH gradient to drive an electroneutral antipart, cells still generate a significant Na⁺ gradient (16).

In membrane vesicles the antipart has been reported to be electroneutral at acidic pH and electronegative (2 H⁺ per Na⁺) at alkaline pH like a number of sugar-acid symport systems (86, 98). It was subsequently proposed that an important aspect in the change of the stoichiometry could be the intracellular pH (62). Recently, this has been shown to be the case. In right-side-out vesicles it was shown that the kinetics of the antipart depended upon the internal pH of the vesicles.
(12). Thus, even at acidic pH the antipor was electrogenic when the intravesicular pH was poised above pH 6.6 (12). It has been suggested that at low values of pH, the release of the second proton at the inner face of the membrane is kinetically limited. The intracellular pH could therefore play a significant role in regulating the antipor. However, it is difficult to ascertain the value of this kinetic control of the antipor to the cell, since pH is usually poised to a much higher value.

Are there multiple routes for Na+ exit? This is an open question. It was suggested by Beck and Rosen that the KHA system had a modest affinity for Na+ (13), although it seems unlikely that this system can act as a sodium-proton antipor in cells due to the presence of high concentrations of potassium. Chemical modification studies in right-side-out membrane vesicles have suggested that there may be two routes of Na+ exit. This was determined by chemical modification of the antipor with diethylpyrocarbonate. Coupling of Na+ exit to energy was inhibited by diethylpyrocarbonate, whereas efflux of Na+ down its concentration gradient was unaffected (22). This may represent the type of uncoupling by diethylpyrocarbonate previously observed with the lactose transport system of E. coli (83), or it may reflect the presence of two Na+ exit pathways. Without genetic evidence on this point, one cannot be certain.

Is there a role for Na+ in acidification of the cytoplasm? By analogy with the alkalophilas, it might be expected that Na+ cycling could play a role in acidifying the cytoplasm. In V. alginolyticus cells incubated in the presence of respiratory inhibitors to inactivate the primary Na+ pump, Na+ extrusion occurred in response to alkalization of the cytoplasm by diethanolamine (81). When pH was restored to approximately 7.9, Na+ extrusion ceased. This clearly suggests that the Na+ extrusion system can respond to alkalization of the cytoplasm. However, the role of this system in an organism which normally pumps Na+ out via a primary pump must be complex. In the absence of such a pump, it is possible that Na+ exchange for protons is an alternative to potassium when the potassium gradient is low.

In an attempt to obtain unequivocal evidence on this point, E. coli mutants lacking the sodium-proton antipor were sought (125). Two transport systems in E. coli were known to be preferentially coupled to the Na+ gradient generated by the sodium-proton antipor, namely, melibiose and L-glutamate. Mutants which had simultaneously lost the ability to grow on melibiose and glutamate were isolated after UV mutagenesis (125). The mutants had a defect in their ability to couple the sodium gradient to transport, but were normal in the transport of L-proline (122). The activity of the sodium-proton antipor also appeared to be impaired (125). When the pH sensitivity of the mutants was examined, they exhibited a markedly decreased growth rate at alkaline pH (122–125). This pH sensitivity led to the mnemonic phs being applied to the affected locus (125).

It was subsequently shown that there was a failure of pH homeostasis in the mutant of alkaline pH (123, 125). When the pH of the growth medium was titrated to pH 8.6 to 8.7, the pH increased rapidly to the same value as the external pH. Generation of a pH gradient, inside acid (pH 7.8 to 8.0), occurred over a 10- to 15-min period. During this period essentially no growth occurred, but the restoration of pH to a more acidic value was not dependent upon new protein synthesis (122). In the phs mutant, pH did not recover to pH 7.8, and growth did not resume.

Subsequent studies have raised major doubts about the role of the sodium-proton antipor in generating the pheno-type of the phs mutant. The major L-proline transport system in E. coli and Salmonella typhimurium is an Na+ symport (19, 104), yet its activity is not affected by the phs mutation (122; Giffard et al., submitted for publication). Genetic mapping of the phs mutation showed it to be an allele of rpoA, the gene encoding the α-subunit of RNA polymerase (94; Rowland et al., submitted for publication). The mutation causes a relatively selective transcription defect which affects the genes for cysteine biosynthesis and the arabinose regulon (32a, 94, 94a; Giffard et al., submitted for publication; Rowland et al., submitted for publication), in addition to the melibiose operon and the glutamate transport system. No evidence could be found for a defect in the sodium extrusion capacity at either neutral or alkaline pH (Giffard et al., submitted for publication), and it was concluded that the metabolic defects caused by the phs mutation could not be mediated by a lesion in the antipor.

In a recent analysis of the behavior of the mutant at alkaline pH it became clear that the growth rate defect was not as clear cut as was originally thought (123). Incubation at alkaline pH led to the formation of chains of cells, and growth continued with no increase in colony-forming capacity (123). At first sight this does not appear consistent with a defect in pH homeostasis, since growth itself has been reported to be very sensitive to changes in internal pH (125). At the present time the basis of the pH sensitivity of this mutant is an open question.

If the phs mutant is truly pH sensitive, then what is the basis of this sensitivity? The finding that the phs mutation affects RNA polymerase suggests that transcription of some gene vital for growth at alkaline pH is affected by the mutation (32a). One can envisage two types of effects. The level of synthesis of a gene product may be depressed by the transcription defect. Sufficient gene product is made to allow growth at neutral pH, but not at alkaline pH. Obviously an antipor could fall into this category, as could any essential enzyme. In the latter case the formation of chains by the phs mutant is consistent with the reduced activity of enzymes involved in cross-wall synthesis. The second possibility is that there is a group of genes the transcription of which is controlled by external pH and the products of which are essential for growth at alkaline pH. There is at present no major evidence for this, but changes in transcription of the heat shock genes at alkaline pH have been observed in E. coli (A. Travers, personal communication). Further, perturbation of pH at alkaline pH results in induction of the SOS genes (S. Schuldiner, personal communication). It is clear that a considerable amount of work remains to be done in this area.

Are there other transport systems involved in pH homeostasis? The acidification of the cytoplasm of E. coli cells (Fig. 6) is thought not to be due to the activity of either potassium-proton or sodium-proton antipor (65). The rate of acidification was independent of the external Na+ concentration in the range of 38 μM to 10 mM. On this basis it was calculated that the Na+ would need to cycle through the cell 7,000 times per min to account for the acidification rate. In experiments where overshoot is prevented by the presence of a preformed pH gradient, the rate of Na+ cycling would need to rise by 1 order of magnitude! The permeability of cells to Na+ is thought to be very low. Recent determinations of the Na+ permeability of E. coli cells suggest an Na+ conductance of 0.3 μS, which is very much smaller the measured value of 20 μS for protons (Castle and Macnab, personal communication). The exclusion of the potassium-proton antipor from a role in acidification in these experi-
ments was less indirect. Potassium uptake was being used to raise pH, since the accumulation of this cation was shown to be independent of either pH or P_H+, it was concluded that any potassium extruded to lower pH must be taken up again (65). If this is the case, then the potassium cycle is entirely futile as far as changing pH is concerned. Thus, another system capable of acidification of the cytoplasm must be present in E. coli. The identity of this system is at present unknown.

**Site of pH Control in E. coli**

In view of the opinions expressed above, one may think it premature to discuss sites of control in pH homeostasis in E. coli. However, there have been several interesting reports in this area. First, E. coli exhibits chemotaxis away from both acidic pH and weak acids and toward weak bases (53, 91, 100). Careful analysis of these phenomena has shown that a decrease in internal pH causes the cells to tumble, which potentiates movement in a more favorable direction (53, 91, 100). This change in swimming behavior is achieved through the control of methylation of a transmembrane protein called MCPI. The protein was demethylated in response to acidification of the cytoplasm and became remethylated when pH homeostasis was restored (100). However, the methylation state of MCPI was also affected directly by external pH, and it was suggested that this protein could sense both the external and the internal pH and that averaging of the two signals takes place (100). Thus, although sensing of internal pH is important in pH taxis, the external pH is also sensed.

The second type of observation derives from studies on the generation of a pH gradient by inverted membrane vesicles (90). The pattern of formation of the pH gradient in reference to the periplasmic face of the membrane was very similar in both inverted and right-side-out vesicles (90). This result was taken to indicate that the external pH plays an important role in determining the magnitude of the pH gradient and that the external pH is sensed. This result is analogous to the observation of acceleration of the rate of acidification of the cytoplasm by low values of pH during overshoot produced by potassium uptake into potassium-depleted cells (65).

**CATION TRANSPORT AND pH HOMEOSTASIS: A SUMMARY**

It is clear that we are a long way from a satisfactory understanding of pH homeostasis and the role that cation flux plays in this phenomenon. However, it must be stated that the last 5 years have brought several advances. It is now clearly established that control of pH in alkalophiles does involve the circulation of Na⁺—this is one of the few facts which have become well established. In neutrophiles, potassium efflux at alkaline pH in exchange for protons exhibits the right properties to be involved in pH homeostasis if the problem of simultaneously achieving potassium homeostasis can be overcome. Potassium uptake systems may play a universal role in elevation of cytoplasmic pH, but the role of such transport systems is not completely defined, especially in the alkalophiles and the acidophiles.

Finally, in this section one should consider the biochemical mechanisms which are involved in regulation of components of the pH homeostatic system by pH. Two basic mechanisms operate in bacteria to effect control of enzyme activity; these are the binding of small-molecular-weight regulatory compounds to the enzyme and the covalent modification of proteins (119). Various examples of both phenomena in response to changes in pH and P_H+ have already been referred to above; thus, it is not speculating too far to suggest that such mechanisms may operate in regulation of ion flux in pH homeostasis. This is an area which will repay further study. In particular, the energy dependence of pH homeostasis observed in E. coli and E. aurantiacum (65; McLaggan, Ph. D. thesis) may reflect control by the chemical modification of transport functions (103).

**CONCLUDING REMARKS**

Since the last major review on pH homeostasis in bacteria (84), there have been several advances. The most important of these is the confirmation of the role of Na⁺ in the generation of a pH gradient, inside acid, in the alkalophiles (66a, 75) and the demonstration of the capacity of K⁺ excretion for regulating pH (81). An encouraging development has been the application of genetics to the problem of pH homeostasis; however, the dearth of work in this area and for caution in the interpretation of genetic data is also timely (32a, 94, 94a). Perhaps the most striking aspect of recent studies is that we still do not have a clear understanding of the mechanisms involved in pH homeostasis. Furthermore, the studies on pH taxis (53, 91, 100) should serve to warn us not to expect the cell to be unsophisticated in the mechanisms adopted to regulate components of the pH homeostatic system.

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