The Effect of Food Preservatives on pH Homeostasis in *Escherichia coli*

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The effects of cinnamic, propionic, benzoic and sorbic acids on the growth and intracellular pH of *Escherichia coli* were investigated. The data suggest that the potency of weak acids as food preservatives is related to their capacity to reduce specifically the intracellular pH. The data also suggest that although both the undissociated and dissociated forms of the acid cause the intracellular pH to fall, growth inhibition is due predominantly to the undissociated acid.

INTRODUCTION

It is generally recognized that bacteria maintain their cytoplasmic pH (pH_i) relatively constant despite variations in the environmental pH. The mechanisms by which this homeostasis is achieved are poorly understood, although controlled, cation-dependent proton flux appears to be central (Padan *et al.*, 1982; Booth & Kroll, 1983). The requirement for homeostasis has been demonstrated unequivocally in *Streptococcus faecalis* (Harold & van Brunt, 1977). Gramicidin-treated cells incubated in an enriched growth medium demonstrated a marked growth inhibition as the incubation pH (pH_o) was lowered. Inhibition was complete at pH_o 6.0 (Harold & van Brunt, 1977). Similar data are not available for respiring organisms in which the capacity to regulate cytoplasmic pH is somewhat greater (Booth & Kroll, 1983).

The constancy of cytoplasmic pH in growing cells leads to the presence of a progressively larger transmembrane pH gradient (ΔpH) as pH_o is lowered (Kashket, 1981; Ahmed & Booth, 1983). In consequence it is not surprising that growth inhibition by weak acids should be more potent at low pH. The weak acid traverses the membrane in its undissociated form and dissociates in accordance with the intracellular pH, liberating a proton in the cytoplasm. When ΔpH is large, the amount of acid dissociating in the cytoplasm is correspondingly larger. Consequently, the potential effect of the weak acid accumulation on intracellular pH increases as pH_o is lowered. In such a model, H^+ is viewed as the inhibitor. However, it has long been held that with weak acid food preservatives the undissociated acid is the inhibitor (Bosund, 1962). Recently, in reviewing the effects of sorbic acid on bacterial cells Eklund (1983) proposed that the undissociated acid was some 10–600 times more effective as an inhibitor than the dissociated acid.

Previously, as a result of studies on membrane vesicles, the antimicrobial activity of food preservatives had been attributed to inhibition of transport by reducing the ΔpH component of the protonmotive force (Freese *et al.*, 1973). However, Eklund (1980) concluded that while this was correct for the parabens it could not explain the inhibition by weak acids. We have investigated the effects of four weak acid food preservatives on growth and intracellular pH of *Escherichia coli* to determine the relationship between pH_i and growth inhibition. The data suggest that the weak acids which are potent inhibitors of growth have a greater effect on intracellular pH than other weak acids of similar pK. There was a significant correlation between

Abbreviations: pH_i , intracellular pH; pH_o , extracellular pH; HA, undissociated acid; A_i^- , intracellular anion; A_o^- , extracellular anion.

intracellular pH and growth inhibition. The data point to the involvement of the undissociated acid in inhibition of pH_i maintenance.

METHODS

Chemicals. All chemicals were purchased from BDH (Analar grade) except benzoic acid, acetylsalicylic acid, propionic acid, sorbic acid and Triton X-100, which were from Sigma. PPO was from Packard Instruments; POPOP was from Koch-Light; *trans*-cinnamic acid was from Aldrich. Radiochemicals were from Amersham, except [7-¹⁴C]benzoic acid, which was from New England Nuclear.

Bacterial strain and growth conditions. *Escherichia coli* strain 7 (Hayashi *et al.*, 1964) was kindly supplied by Dr B. P. Rosen, University of Maryland School of Medicine, Baltimore, Md., USA. Cell suspensions were grown aerobically at 30 °C overnight in 250 ml flasks containing 100 ml minimal medium which contained per litre: NaH₂PO₄ · 2H₂O, 5.3 g; K₂HPO₄, 11.15 g; (NH₄)₂SO₄, 2.64 g; MgSO₄, 74 mg; FeSO₄, 278 µg; CaCl₂, 1.47 µg; ZnSO₄, 161 µg; glucose, 2 g; adjusted to pH 7.0. The last five ingredients were autoclaved separately in concentrated form and added when the medium had cooled.

Effect of weak acids on bacterial growth. The pH range of the growth media was adjusted by making up medium without the phosphate compounds but containing either 0.1 M-citric acid or 0.2 M-K₂HPO₄. The two solutions were mixed to give the desired pH and KCl was added as required, to make the K⁺ concentration constant at 190 mM. The overnight cell suspensions were diluted into this medium at the required pH to give an OD₆₅₀ of approx. 0.1, using a Pye Unicam SP600 spectrophotometer. Cultures were incubated aerobically at 30 °C in a shaking water bath and the OD₆₅₀ of the culture determined. When OD₆₅₀ had reached approx. 0.2, weak acids were added and growth was followed for a further 4 h. Weak acids were dissolved in DMSO, which in control experiments did not interfere with growth at the concentrations used.

Measurement of rates of glucose consumption. Overnight cell suspensions were washed twice by centrifugation at 16000 g for 5 min and resuspended in minimal medium. Cell suspensions were then diluted into growth media and incubated as described above after addition of 4 mM-glucose. At intervals, two 1 ml samples of culture were transferred to plastic Eppendorf tubes (1.6 ml capacity) and centrifuged for 20 s at 12000 g in a microcentrifuge. A sample (200 µl) was removed and incubated with 1 ml of assay reagents (Sigma glucose oxidase kit) for 45 min at room temperature. The A₄₅₀ of the samples and a calibration series treated identically was read and thus the extracellular glucose concentration determined.

Respiration studies. The rate of oxygen consumption by cell suspensions prepared as above was measured using a Clark O₂ electrode, as described previously (Booth *et al.*, 1979).

Measurement of intracellular pH. The pH_i of growing and non-growing cell suspensions was measured by determination of the distribution of radiolabelled weak acids (Booth *et al.*, 1979). Cell suspensions were incubated (with weak acid preservative when desired) at 30 °C in stirred glass vessels to ensure continuous aeration. The weak acids [2-¹⁴C]dimethylxazolidinedione (10 µM final concn: sp. act. 14.3 Ci mol⁻¹; 529 GBq mol⁻¹) or, more usually, [7-¹⁴C]benzoic acid (2 µM final concn: sp. act. 24.4 Ci mol⁻¹; 903 GBq mol⁻¹) were added together with ³H₂O (approx. 1 µCi ml⁻¹ final concn) as a total water marker. At intervals, two 1 ml samples were centrifuged in Eppendorf tubes for 20 s, as described above. A portion (100 µl) of the supernatant was removed and transferred to a centrifuge tube containing a similarly treated cell pellet that had not been incubated with radiochemicals. This procedure ensured that the degree of quenching in the supernatant and pellet samples was equivalent. This enabled calculation of trapped and intracellular ¹⁴C label in the experimental pellet (Booth *et al.*, 1979). The remaining supernatant was aspirated with a vacuum line. All pellets were resuspended in minimal medium to 300 µl final volume. A portion (200 µl) was added to 2 ml of scintillation fluid (Triton X-100, 333 ml; toluene, 666 ml; PPO, 4 g; POPOP, 100 mg). The samples of supernatant and pellet were counted for radioactivity using a Packard TriCarb 300C scintillation counter with the following window settings; ³H, 0–12 keV; ¹⁴C, 12–156 keV. The spillover of ¹⁴C into the ³H channel was estimated by using identically prepared samples which had been incubated with ¹⁴C only. From a knowledge of pH_o, pH_i was calculated (Booth *et al.*, 1979).

Although benzoate is itself a food preservative it did not reduce pH_i when used in the concentration range 2–100 µM at pH_o 5.

RESULTS AND DISCUSSION

Growth inhibition by food preservatives

The concentration dependence of the growth inhibition produced by benzoate, cinnamate, propionate and sorbate was investigated at pH_o 5.0. Exponentially growing cultures of *E. coli* exposed to these weak acids exhibited inhibition which was dependent both on the identity of the weak acid (Fig. 1) and on its concentration (data not shown). Of the four acids, cinnamate was the most potent inhibitor and propionate the weakest. Thus despite their similar pK values

Fig. 1

Fig. 2

Fig. 1. Inhibition of growth of *E. coli* at pH₀ 5.0 by addition (arrow) of 0.5 mM propionic (■), benzoic (○), sorbic (●) and cinnamic (□) acids.

Fig. 2. Effect of addition (arrow) of 0.1 mM (●) and 0.5 mM (■) cinnamic acid on the consumption of glucose by *E. coli* at pH₀ 5.0. ○, Control.

the acids were differentially effective as growth inhibitors. This suggests that the acids did not exert their effects on growth simply as a function of their excessive accumulation causing acidification of the cytoplasm. If this were the case the pattern of inhibition would reflect the pK values of the acids. In support of this suggestion, it was observed that the predicted intracellular concentration of the dissociated weak acids did not correlate with either the pK or the severity of growth inhibition (data not shown).

At higher values of pH₀ the same pattern of inhibition emerged, although higher concentrations of the acids were necessary to cause complete inhibition (data not shown).

During growth inhibition, glucose consumption continued, although its utilization was uncoupled from growth (Fig. 2). Thus during exponential growth an approximately linear relationship was observed between the residual glucose in the culture and the cell density. This relationship held in the presence of low concentrations of the food preservative cinnamate when growth inhibition was slight (Fig. 2). However, at higher concentrations (0.5–2 mM) of cinnamate, glucose consumption continued, while growth was severely inhibited (Fig. 2).

Similar data were obtained for all four acids. It is apparent that unlike the situation in *Saccharomyces cerevisiae* (Krebs *et al.*, 1983), in *E. coli* glucose uptake is not the primary site of action of the food preservatives.

Effects of food preservatives on pH_i

All weak acids which are freely permeable across the cytoplasmic membrane will reduce pH_i in a concentration-dependent manner. In the absence of direct effects on metabolism the potency of any weak acid at a given external pH value will depend on the pK of the acid. Acids of a low pK are generally more effective at collapsing the pH gradient than those of higher pK. Thus for comparison of the effects of the acids on pH_i it is necessary to normalize the data by reference to the concentration of the anion in the incubation medium. Cells of *E. coli* in the exponential phase of growth were incubated with the four food preservatives at a range of concentrations and also with the weak acids acetate and acetylsalicylate, and the internal pH was

Fig. 3

Fig. 4

Fig. 3. Effect of weak acids on pH_i of cultures of *E. coli* growing at pH_o 5.5. ▲, Acetylsalicylic (pK_a 3.5); △, acetic (pK_a 4.7); ■, propionic; ●, sorbic; ○, benzoic; □, cinnamic.

Fig. 4. Relationship between growth rate and pH_i of *E. coli* at pH_o 5.0. Cinnamic (□), propionic (■), benzoic (○) and sorbic (●) acids were added to final concentrations of 0.1, 0.5, 1.0 and 2.0 mM. ▲, Control.

determined. Acetylsalicylate and acetate had identical effects on the internal pH (Fig. 3). At an external anion concentration of 1 mM the acids reduced pH_i by 0.38 units at pH_o 5.5. All the food preservatives reduced the internal pH by a greater extent than either acetate or acetylsalicylate (Fig. 3). At an external anion concentration of 1 mM the drop in pH_i was 0.53, 0.65, 0.8, and 0.84 for benzoate, propionate, cinnamate and sorbate, respectively. Thus when the acids were compared with each other on equal terms a different pattern of potency was observed from that seen when studying growth at a single pH value (Fig. 1). The fall in intracellular pH was complete within 20 s and no further significant change in pH_i was detected with prolonged incubation.

In view of the action of the food preservatives on the intracellular pH, a correlation was made between the degree of growth inhibition and pH_i (Fig. 4). Despite the apparent differences in their effectiveness as growth inhibitors, all the four acids fitted the same relationship between pH_i and growth. Thus at pH_o 5 growth was not inhibited until pH_i fell below pH_i 7.1, but became severe once pH_i was reduced below pH_i 6.8 (Fig. 4). It thus appears that food preservative weak acids may all inhibit the capacity to maintain cytoplasmic pH above pH_i 7.1.

Is inhibition due to undissociated or dissociated acid?

Using benzoate we investigated the efficacy of benzoate anion and benzoic acid as inhibitors by (a) varying the undissociated acid concentration at constant pH and (b) varying the undissociated acid concentration by changing pH_o at a constant total acid concentration. A low total acid concentration (2.5 mM) was chosen to minimize the effects of dissociated acid on pH_i . Growth inhibition correlated strongly with the concentration of the undissociated acid regardless of the method of varying this parameter (Fig. 5a). The correlation between pH_i and growth inhibition was again evident despite the two different methods of varying the concentration of the undissociated acid (Fig. 5b).

Fig. 5. (a) Effect of varying the concentration of undissociated benzoic acid on the growth rate of *E. coli* by varying pH_o (□) or by varying the concentration of total acid at constant pH_o 5.0 (●). (b) Relationship of pH_i and growth rate in cultures exposed to 2.5 mM-benzoate at different values of pH_o (pH_o 5.0-7.0; △) or to different concentrations of benzoate at pH_o 5.0 (▲). Values corresponding to 100% growth rate were, typically, 0.28 h⁻¹ at pH_o 5; 0.31 h⁻¹ at pH_o 5.5; 0.33 h⁻¹ from pH_o 6 to 7.5.

Model for growth inhibition

On the basis of the above data we propose that growth inhibition consists of two components. Specific inhibition of an unidentified metabolic function by the undissociated acid (HA), and a generalized inhibition caused by acidification of the cytoplasm. It is clear that although the latter is a significant cause of inhibition, the former specific effect is the more potent. Thus cells of *E. coli* can tolerate quite large changes in cytoplasmic pH, down to pH_i 6.8, without significant growth inhibition (Figs 4 and 5a). Below pH_i 6.8, growth inhibition becomes severe but this arises in all cases from an increase in both HA and A_o⁻, i.e. an increase in both specific and non-specific inhibition. Thus at this point the data cannot be simply interpreted in terms of the effect of the acids on pH_i. Clearly it is significant that pH_i is lowered to a greater extent by food preservatives than by weak acids of a similar pK (Fig. 3). This suggests that the inhibitory action of HA has a synergistic effect with accumulation of the acid (as A⁻) on pH_i. However, growth inhibition correlates most strongly with the concentration of HA (Fig. 5b). Thus the decline of pH_i is unlikely to be the primary cause of growth inhibition.

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