The Effect of Sorbic Acid and Esters of p-Hydroxybenzoic Acid on the Protonmotive Force in Escherichia coli Membrane Vesicles

By TRYGVE EKLUND
Norwegian Food Research Institute, POB 50, N-1432 Aas-NLH, Norway

(Received 9 May 1984; revised 5 July 1984)

The effect of three food preservatives, sorbic acid and methyl and butyl esters of p-hydroxybenzoic acid, on the protonmotive force in Escherichia coli membrane vesicles was investigated. Radioactive chemical probes were used to determine the two components of the protonmotive force: $\Delta p$H (pH difference) and $\Delta \psi$ (membrane potential). Both types of compound selectively eliminated $\Delta p$H across the membrane, while leaving $\Delta \psi$ much less disturbed indicating that transport inhibition by neutralization of the protonmotive force cannot be the only mechanism of action for the food preservatives tested.

INTRODUCTION

It has been repeatedly claimed that several food preservatives act by inhibiting the uptake of reducing substances into microbes (Sheu & Freese, 1972; Sheu et al., 1972, 1975; Freese et al., 1973). It has been suggested (Freese & Levin, 1978) that lipophilic acids (e.g. benzoic and sorbic acid) specifically eliminate the transport-driving pH difference across the cellular membrane, while permanently uncharged compounds (e.g. esters of p-hydroxybenzoic acid, 'parabens') destroy membranes.

Eklund (1980) confirmed several food preservatives as transport inhibitors, but questioned whether this inhibition could in all cases be sufficient to explain growth inhibition. According to the chemiosmotic hypothesis (Mitchell & Moyle, 1969), the protonmotive force is defined as $\Delta p = \Delta \psi - Z\Delta p$H, where $\Delta \psi$ and $\Delta p$H are the differences in electrical potential and pH across the membrane and $Z = 2.3RT/F$ where $R$, $T$ and $F$ are the gas constant, absolute temperature and Faraday constant, respectively. A thorough discussion of the various forms this equation takes in the literature was presented by Lowe & Jones (1984). Eklund (1980) suggested that elimination of $\Delta p$H alone might not be sufficient to stop uptake totally, since $\Delta \psi$ alone is sufficient to drive the uptake of several substances (Ramos & Kaback, 1977a).

The aim of the work described here was to determine the effect of various preservatives on the two components of the protonmotive force. As in the works cited, membrane vesicles were used to exclude the possibility of measuring secondary effects, which may occur when whole cells are used. The pH difference was measured by uptake of benzoic acid (Kashket & Barker, 1977), while the charge difference was measured by uptake of the lipophilic cation triphenylmethylphosphonium (TPMP$^+$) (Ramos et al., 1976).

METHODS

Vesicle production. Membrane vesicles from Escherichia coli ML 308-225 were made as described by Kaback (1971) to a concentration of 8 mg protein ml$^{-1}$.

Uptake of benzoic acid. The modified flow dialysis technique described by Ramos et al. (1976) was used with only minor modifications, except that membrane vesicles were pre-treated with phenazine methosulphate (PMS) rather than PMS being added to the reaction mixture (Boonstra & Konings, 1977). A double chamber divided by a

Abbreviations: PMS, phenazine methosulphate; TPMP$^+$, triphenylmethylphosphonium ion.
dialysis membrane (Colowick & Womack, 1969) was used. The reaction mixture (0·8 ml 0·05 M-potassium phosphate buffer pH 6·6; 0·01 M-magnesium sulphate; 0·02 M-sodium ascorbate; 3·2 mg membrane protein; various concentrations of preservative) was placed in the upper chamber, while buffer (0·05 M-potassium phosphate pH 6·6) was pumped through the lower at a constant rate, and collected in fractions. The experiments were initiated by addition of [14C]benzoic acid (final concentration 25 μM). Uptake in response to the ΔpH was indicated by a fall in the radioactivity in the dialysate. This interpretation was confirmed by eliminating the membrane gradient by the addition of the ionophores valinomycin and nigericin, causing the radioactivity to rise to the original level of the control series.

The fall in radioactivity was measured, and used to calculate an inhibition index for ΔpH. The inhibition index was calculated according to the formula 1 - U/Uc (Freese et al., 1973), where Uc is the uptake in the control and U, the uptake in the sample. No inhibition gives this index a value of zero, while total inhibition gives a value of one. The control value for ΔpH was calculated according to Ramos et al. (1976).

Uptake of TPMP+. The filter technique described by Kaback (1974) and Ramos et al. (1976) was used, with Gelman Metricel GA 6 filters, 0·45 μm pore size. The reaction took place in 50 μl aliquots containing 0·05 M-potassium phosphate buffer pH 6·6; 0·01 M-magnesium sulphate; 0·02 M-sodium ascorbate; 0·1 mM-PMS; 20 μM [14C]benzoic acid; 0·08 mg membrane protein; various concentrations of preservative. The inhibition index for Δψ was calculated similarly to the inhibition index for ΔpH. The control value for Δψ was calculated according to Ramos et al. (1976).

Chemicals. [methyl-3H]Triphenylmethylphosphonium bromide, specific activity 3·59 Ci mmol⁻¹ (132 GBq mmol⁻¹), was from New England Nuclear. Preliminary experiments were made with similarly labelled TPMP⁺, specific activity 4·38 Ci mmol⁻¹ (162 GBq mmol⁻¹), kindly donated by Hoffmann La Roche. [carboxyl-14C]Benzoic acid, specific activity 57·8 mCi mmol⁻¹ (2·14 GBq mmol⁻¹), was from Amersham. Valinomycin was from Sigma, while nigericin was a generous gift from Hoffmann La Roche and Eli Lilly.

RESULTS

Fig. 1(a, b and c) shows the inhibition of the two components of the protonmotive force as a function of preservative concentration. The effect of the preservatives was expressed as inhibition indices for ΔpH and Δψ. The standard error of the inhibition index was in general less than 0·1 units. The control value of ΔpH was 1·9 (interior alkaline) corresponding to -110 mV. For Δψ, the control value was -45 mV.

The effect of sorbic acid is shown in Fig. 1(a). The neutralization of ΔpH was complete at a concentration of 10 mM, while only one-third of Δψ was eliminated by this concentration. Even at a concentration of 100 mM, one-third of the initial Δψ still remained.

A similar pattern, but with steeper dose-response curves, was apparent for methyl and butyl paraben (Fig. 1 b and c). At the concentrations needed for elimination of ΔpH (3 and 0·3 mM, respectively), one-third of Δψ still remained. Increasing the concentration of the parabens to near the limit of solubility did not destroy Δψ completely.

DISCUSSION

The weak, lipophilic sorbic acid and the uncharged methyl and butyl esters of p-hydroxybenzoic acid appeared to affect the protonmotive force in E. coli membrane vesicles in a similar manner, eliminating ΔpH while leaving Δψ at levels sufficient to energize uptake of substances needed for growth maintenance in whole cells.

The selective action of sorbic acid on ΔpH is not unexpected since the basis of ΔpH measurements by uptake of weak acids is the supposition that these probes enter vesicles or cells as undissociated molecules. When used in high concentrations these dissociate in the interior until the pH difference across the membrane is eliminated (Ramos et al., 1976; Cramer & Prestegard, 1977). It was more unexpected that parabens, which supposedly act by increasing membrane permeability in general (Furr & Russell, 1972), preferentially neutralized ΔpH at concentrations which left Δψ almost intact. A somewhat similar phenomenon may have been observed however in the studies of Denyer et al. (1980) on the antibacterial action of 4-n-alkyl phenols, which appear to act by enhancing the proton translocation rate. The possible effect on Δψ was not investigated by these authors.

A comparison can be made between the present results and previous data on growth and uptake inhibition by the same inhibitors on the same organism (Eklund, 1980). Evidently the
Preservatives and protonmotive force

Fig. 1. Effect of three food preservatives, as a function of concentration, on ΔpH and Δψ in E. coli ML 308-225 membrane vesicles. Food preservatives used were: (a) sorbic acid; (b) methyl ester of p-hydroxybenzoic acid; (c) butyl ester of p-hydroxybenzoic acid. ○, inhibition index for ΔpH; ●, inhibition index for Δψ. The inhibition index is defined in Methods. No inhibition gives a value of 0 and total inhibition gives a value of 1.

inhibition indices for growth and ΔpH do not correspond; the concentration of sorbic acid needed to inhibit growth is much higher than that required for a complete elimination of ΔpH. Better correspondence is found between the inhibition indices for Δψ and growth, and better still between the inhibition indices for Δψ and for uptake of amino acids in whole cells and vesicles, but the correspondence is not complete. Since the amino acids tested are neutral (serine, alanine, phenylalanine) they are expected to depend both on ΔpH and Δψ for active uptake (Niven & Hamilton, 1974) and it is possible that the remaining Δψ is too small to support active uptake. However, the work of Ramos & Kaback (1977b) shows that there seems to be no threshold value for the ability of membrane gradients to drive uptake processes. The same relationships observed with sorbate can be seen for the two parabens tested. Thus the mechanism of action for food preservatives, exemplified by sorbic acid and methyl and butyl paraben, cannot be regarded as one single mechanism connected with the cell membrane. Specifically, the mechanisms claimed by Freese et al. (1973) and Freese & Levin (1978), although important, seem inadequate as the sole basis for growth inhibition by sorbic and other lipophilic acids (Eklund, 1980). The theoretical reasons suggested (Eklund, 1980) have been supported by the present results. As for parabens, the apparently selective inhibition of ΔpH, similar to that caused by sorbic acid, seems more difficult to explain. One must assume that parabens selectively increase the permeability of the membrane to protons, while leaving the membrane more or less impermeable to other charged molecules.

Thanks are due to Janina Magnussen for excellent assistance.

REFERENCES


